Taxonomy and Ecology of *Vibrionaceae* Associated With *Red-Spot* Disease of Queensland Aestuarine Fish.

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Indemnity Statement.

The work presented in this thesis is, to the best of my knowledge and belief, original, except as acknowledged in the text. The material has not been submitted, either in whole or in part, for a degree at this or any other university.

Dated this thirteenth day of July, Nineteen Hundred and Eighty Nine.

.....Steve Nearhos.....

Steven P. Nearhos

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IV ABSTRACT.

Red-Spot disease (RSD) is a chronic ulcerative condition of aestuarine fish attributed (Rodgers and Burke, 1981) principally to *V.anguillarum,* and which yearly costs dependent Queensland fisheries *ca* $100 000. An investigation of endemic *Vibrionaceae* and their host interactions in an environmentally relatively unperturbed ecosystem where RSD occurs was aimed at deriving a disease management strategy for wild fish stock. Samples from diseased and control fish, and of different components from the fishes aquatic environment were taken over *ca* 14 months, for *Vibrionaceae* identification and deduction of environmental cycles by observation of floral compositions.

These samples were inoculated to various bacteriological media with unknown selectivities for non-enteropathogenic *Vibrionaceae.* From pure cultures of 996 isolates 728 were classified as presumptive *Vibrionaceae.* Excluding replicates, 391 of these presumptive *Vibrionaceae* strains and 145 external type, reference or partially identified strains were phenotypically examined in 8 batches, for up to 190 characters. The data was used in a progression of numerical analyses. Batch one, with the highest proportion of reference OTU's (Operational Taxonomic Units), served as a diagnostic master -key or *Rosetta* to identify strains. Initial examination was by cluster and parsimony analyses and distributions were checked against Jaccard and Euclidean similarity matrices. Similar procedure for a batch of 96 OTU's in a comparative review of four cluster protocols sorting Euclidean and Jaccard matrices indicated Average linkage sorting of the Jaccard matrix produced results most consistent with current classification. This method was used on an IBM 3083 computer to test placement of 277 partial and complete replicates from different standardized batches, and then to cluster *Rosetta* OTU's with 240 OTU's from 3 other batches. Similar personal computer driven analysis clustered *ca* 240 OTU's from residual batches. OTU groups from two batches were compared using a graphic multivariate-procedure. Stable differential characters were abstracted into *Vibrionaceae* identification matrices and their diagnostic potency assessed relative to other schemes.

Most provincially isolated strains were placed into extant taxa while strains from Benalla and Tasmania with different growth requirements were ecologically grouped. The first substantiative Australian records for several species were recorded and it was suggested that two species *V.orientalis* and *V.tubiashii* should be combined, with *V.orientalis* as senior synonym. A new phenetically and genotypically distinct species *V.zobellii,* was described from calanoid copepods. New biogroups most prominently of the species *V.aestuarianus, V.orientalis,* and *V.fluvialis* were described and descriptions of other were taxa emended. The related or phenetically similar species, [*V.fluvialis* and *V.furnissii*] and [*V.carchariae* and *V.harveyi*], respectively were not resolved. The twelve most common taxa *V.alginolyticus, V.carchariae/-V.harveyi, V.fluvialis/-V.furnissii, V.parahaemolyticus, A.hydrophila, V.aestuarianus* I & II, *S.putrefaciens, V.carchariae, Photobacterium*-like, *V.fluvialis/-V.furnissii*-like, *V.zobellii* and *V.vulnificus* accounted for 85 % of provincial OTU's. The fish pathogen *V.anguillarum* was seldom found, and restricted to advanced fish lesions. Likewise other *Vibrionaceae* were found on diseased but not control fish and so it was suggested that epizootics might occur in waves not necessarily caused by the same taxa. The same halophilic taxa isolated from Moreton Bay fish with acute *vibriosis* were also recovered from fish with chronic RSD so it was implied that disease stasis in river fish was due to interruption of halophile pathogenesis as fish migrated to lower salinities. Invasive encystment by cestode larvae was suggested to mechanically vector non-specific gut flora to mesenteries of undiseased fish. Some taxa from diseased fish were also carried by their sedentary and transitory isopod parasites, indicating the potential for these also as mechanical vectors. The narrow ecological range of *V.zobellii,* indicated that copepods may be a specific biological host, so supporting the thesis that *Vibrionaceae* might have specific rather than general micro-ecologies. It was found that young RSD prone fish were resistant to *Vibrio* spp. inferring RSD as an old rather than recent condition. An *in situ* RSD management regimen was proposed.

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TAXONOMY AND ECOLOGY OF *VIBRIONACEAE* ASSOCIATED WITH *RED-SPOT* DISEASE OF QUEENSLAND AESTUARINE FISH.

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Disk File Trees:

Two 360 k. IBM format disks, (1 & 2), are in the inside back pocket of Volume 2. Sub-directories specified in the file trees below may be rapidly accessed by typing the name only of the corresponding directory changing batch files, (FileName.Bat), and keying Return.i.e. Expert <R>; A08 <R>; A11 <R> etc. These batch files are present in respective disk root-directories and will be shown by DOS upon a DIR command.

```
DISK_1
  Expert.Bat
  EXPERT
    SAMPLE.BAS
    COMP.EXE
    COMP87.EXE
    PRIOR.EXE
    PRIOR87.EXE

DISK_2
  A08.BAT
  A11.BAT
  A12.BAT
  A13.BAT
  APNDICES
    APNDX_08
      API2ONE.BEN
      SYSTEK1.BEN
    APNX_11
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      VIB05.DAT
      VIB05A.DAT
      VIB06.DAT
      VIB06A.DAT
      VIB07.DAT
      VIB07A.DAT
      VIB08.DAT
      VIB08A.DAT
    APNDX_13
      PRINCOMP.EUC
```

List of Relevant Publications.

Volume 2 : Inside front pocket.

Pham A.V., Nearhos S., & Davis G.H.G. (1985) Ecological significance of siderophore production in marine bacteria. *Microbios Letters* **30**, 79-83.

Nearhos S.P. and Fuerst J.A. (1987) A re-analysis of 5S rRNA sequence data for the *Vibrionaceae* using the CLUSTAN program suite. *Current Microbiology* **15**, 329-335.

(IV) GLOSSARY.

Amend: To alter the definitive criteria of a taxon. See **Emend.**

Assign: To place a name to an OTU on the basis of available tentative information. See Classify.

Attribute: A character state for any of the single or synonymous manifestations of a feature e.g. nitrogenase positive.

Band: A series of features which together form a specified function e.g. operation of a particular metabolic pathway cf. **facet** and **feature.**

Batch: Is used here in the context of quality assurance and recognizes the temporal separation of individual segments in the phenetic data and the associated unique qualities of each segment with regard to each's uniform potential to be subject to different sets of localized skewing variations within the same general protocol.

Bayes' Theorem: The mathematical basis for a branch of statistics which utilizes previous experience to the estimate probability of future occurrences.

Biogroup: A taxonomically informal, often interim unit for phenetically distinct OTU's.

Biovar: Sub-species taxonomic grouping recognizable by sensitive genotypic methods such as DNA homology.

Character: A collective term which does not specify state, e.g. nitrogen fixation.

Cladon, (Clada): Group(s) derived by analysis of primary or secondary semantophores.

Classify: A collective term which concurrently includes both identification and classification.

Cluster: An aggregate of OTU's resulting from numerical analysis which may contain one or more phena.

Cohort: An ecological grouping of species into the same age class.

Consensus Tree: A taxonomic tree whose branchings reflect the consensus of overlapping hierarchical structure amongst equally parsimonious solutions (trees). The term "Strict" consensus tree is a tautology necessarily invoked when the first (Adam's, 1972) consensus trees were found not to satisfy this criterion.

Ecchymosis: Sub-surface pool of extra-vascular blood.

Ecology: Study of individuals, populations and species, and their interactions with each other and the abiotic environment. **Micro ....:** Study of the interactions between species.

Ecotype: Category which groups taxa by ecological rather than phylogenetic similarities.

Effective Publication: The date of issue of the *International Journal of*

Systematic Bacteriology in which a new taxon is described, or a previous publication in another generally available scientific journal, is accessed by a validation list becomes the effective date of publication. See also, **Valid Publication.**

Emend: To supplement the definitive criteria of a taxon.
Facet: A mechanistic grouping of "similar" phenetic characters e.g. carbon assimilation tests. See **Band.**

Feature: A grouping of characters which acknowledges weighting error introduced by homologous measurement of activity in different ways e.g. a feature of the nitrogenase enzyme is its action also as an acetylene and azo-reductase. See **Character Attribute, Band,** and **Facet.**

Gestalt: A collectivized symbiosis formed by two or more strains of bacteria which allows at least one symbiont to grow in conditions which would previously have been limiting.

Glyph: An internally standardized multidimensioned plot of a single OTU analyzed by principle component analysis.

Homoplasy, Homoplastic: The expression of phenetic but not phylogenetic or teleologic homogeneity.

Lepidorthosis: Scale erection.

Local: In the same qualitative-statistical sphere of influence.

Maximum Likelihood: See Bayes' Theorem.

Mutualism: Describes an association which at some stage is beneficial to both species involved in a symbiosis.

Not Parsimoniously Complete, (NP-Complete):, Parsimony analyses can only be fully completed when all branch swappings have been undertaken. This is practical only for small datasets. NP-Complete analyses recognize the computational impracticality of investigating lengths all possible taxonomic branchings. These are arbitrarily truncated by program criteria before all branch permutations have been tested. Sometimes this results in a solution which is not the most parsimonious.

Operational Taxonomic Unit, (OTU): Individual population considered during numerical taxonomic process.

Parasitism: An association between different taxa which is random, or highly correlated i.e. a subset of the interactions which occur in a species micro-ecology. A parasitic bacterial species must remain reproductively viable as a result of the association and can be injurious or beneficial to the host. See also **Symbiosis and Mutualism.**

Parsimony: Transition of species from ancestral to the present form by the most conservative process i.e. by a minimum number of steps.

Petechium: Small surface scab or eruption of extra-vascular blood.

Phenon, (Phena): Group(s) derived by analysis of phenetic (phylogenetic significance unknown) data.

Rosetta: The original translatory purpose of the archaeologic *Rosetta Stone* has also been applied here, although here the term refers more to the use of a comparative reference library than a translating dictionary.
Semantides: Molecular documents of evolutionary history. **Primary s...,** DNA or mRNA; **Secondary s...,** Ribosomal RNA; **Tertiary s...,** most polypeptides,

all enzymes (Zuckerkandl and Pauling, 1965).

Significant: Statistical term denoting assigning relevance of result with specified probability of error i.e. 5 %.; **Highly S...** corresponds to 1 % probability of error, and **Very Highly S...** with 0.1 % or lesser probability of error.

Species: A contiguous population of phenetically and genotypically varied bacterial strains. Relative species age may be inferred from variation in 5S rRNA sequences amongst strains shown as related by DNA hybridization.

Streaking: A solid medium dilution process which through sucessive intersecting strokes of a sterile wire and subsequent incubation demonstrates bacterial culture purity.

Strain: Designation to individual cultures, of a status which acknowledges the specific teleological differences in origin or subsequent treatment of cultures. **Provincial s...** has been used here to include all isolates obtained within a 350 kilometer, *ca* 3°. radius of Brisbane. **Reference ...s**, are previously firmly or tentatively designated cultures which have been deposited in the UQM or another culture collection and so which allow comparative taxonomic review in the current or a later study. **Type ...s** are reference strains deposited in collections accredited by the International Committee for Systematic Bacteriology and which, except in the case of **neotype** designation, are descendants of the original accredited culture deposited by the describing author and accessed through **valid publication.**

Symbiosis: The subset of **parasitic** associations which is highly correlated. The association of one species with another may be mutually beneficial, indifferent or pathogenic. See also **Mutualism.**

Taxon, (Taxa): A mechanistic aggregation of information drawn from of available cladistic and or phenetic sources into stepped hierarchical units.

Valid Publication: The requirement of the International Microbiological Societies, that for general recognition of new taxa, descriptive publications of those new or redefined taxa must either be in the *International Journal of Systematic Bacteriology,* or be accessed from its *Validation Lists* to other generally available scientific journals where the description is contained. See also **Effective Publication.**

Vector: An agent which, as a result of its biology, transfers or amplifies bacterial parasite populations so that these are likely to act as viable infectious packets. **Passive ...s:** Accidentally transfer parasites from one place to another, or incidentally package or amplify "environmentally lost" strains to increase the chance of infection. **Mechanical ...s** additionally breach host primary defense mechanisms to allow secondary invasion by bacterial parasites. **Biological ...s** by non-random associative behavior between the parasite and or vector increase the probability of parasitic infection of a new host.

(V) Citations and Abbreviations.

Full authorship to referenced papers has been quoted only at the first citation. In taxonomic sections scientific and sometimes also authors names in brackets indicate that this author does not recognize the validity of the bracketed taxon. Descriptions of recognized taxa are followed on the first occurrence by a full and unbracketed listing of authors names in bold type.

In some tables species names of *Vibrionaceae* have been abbreviated to the initial distinguishing letters. Most other abbreviations are consistent with those found in the *International Journal of Systematic Bacteriology,* additional and less frequently used abbreviations follow:-

Key to Symbols and Abbreviations.

‰	Parts per Thousand.
0/129	2,4-Diamino-6,7-diiso-propylpteridine.
ADH	Arginine Dihydrolase (Thornley, 1960).
ALE	Average Linkage, Euclidean Matrix.
ALJ	Average Linkage, Jaccard Matrix.
ATCC	American Type Culture Collection.
ATW	Alkaline Tryptone Water.
BTB-Teepol	Bromthymol Blue Teepol.
Ba	Phenotypic Analysis Batch.
Bor	Borate.
cf.	Confer, (compared with).
CLE	Complete Linkage, Euclidean Matrix.
CLJ	Complete Linkage, Jaccard Matrix.
CP	Consensus Parsimony.
ca	Circa.
calc.	Calculated.
d.f.	Degrees of freedom.
DPM	Decompositions per minute.
EDDA	Ethylenediamine Dihydroxyphenylacetic acid.
ESS	Error Sum of Squares, Ward's (1963) Minimum Variance Method.
EtGl	Ethylene Glycol.
FS	Filter Sterilized (0.2μm).
GS	Glutamine synthetase.
HBSENA	Hydroxybutrate SENA.
ip.	Intraperitoneal.
i.u.	International Units.
LDC	Lysine Decarboxylase.
MIC	Minimum Inhibitory Concentration.
MLE	Median Linkage, Euclidean Matrix.
MLJ	Median Linkage, Jaccard Matrix.
MPBS	Marine Phosphate Buffered Saline.
MPBGS	Marine Phosphate Buffered Glucose Salts.
MRVP	Methyl Red, Voges Proskauer.
Molybdate	Ammonium Molybdate.
NA	Not Applicable.
ND	Not Determined.
Nb	Novobiocin.
Nf	Nitrofurazone.
Ns	Nifurstyrenate.
ODC	Ornithine Decarboxylase.
ONPG	o-Nitrophenyl-β-D-Galactopyranoside.
PBS	Phosphate Buffered Saline.
PEA	PhenylEthylAlcohol.
PHB	Poly-β-Hydroxybutyrate.

Key to Symbols and Abbreviations, (cont.).

PNPG	α-p-Nitrophenyl Glycerol.
PTA	Phosphotungstic acid.
Pc	Benzyl-penicillin.
Pm	Polymyxin.
Pt	0/129 phosphate.
RS,RSD	Red-Spot,Disease.
$S_{e.}$	Euclidean Similarity Coefficient (≡ Simple Matching Coefficient).
$S_{j.}$	Jaccard Similarity Coefficient.
SDS	Sodium-Dodecylsulphate.
SEDNA	Sodium-Enriched DNA Agar.
SEBHIA	Sodium-Enriched Brain Heart Infusion Agar.
SEBHIB	Sodium-Enriched Brain Heart Infusion Broth.
SEHIA	Sodium-Enriched Heart Infusion Agar.
SEHIB	Sodium-Enriched Heart Infusion Broth.
SENA	Sodium-Enriched Nutrient Agar.
SENB	Sodium-Enriched Nutrient Broth.
SEMHB	Sodium-Enriched Mueller Hinton Broth.
SETSB	Sodium-Enriched Tryptic Soy-Broth.
SLE	Single Linkage, Euclidean Matrix.
SLJ	Single Linkage, Jaccard Matrix.
SOD	Superoxide dismutase.
SR	Sodium Requiring.
SSC	Standard Saline Citrate (X 1).
Sm	Sulphamethoxazole.
Saline	0.9% aq. Sodium Chloride.
TCBS	Thiosulphate Citrate Bile Salts Sucrose.
TTC	Tri-Phenol Tetrazolium Chloride.
Tc	Tetracycline.
Tm	Trimethoprim.
tab.	Tabulated.
Tan	Tannate.
UPGM(A)	Unweighted Pair Group Method(Average), (Sokal and Michener, 1958) cf. **UPGM(S)**-Single; and **UPGM(C)**, Complete; **UPGM(M)**, Median.
UQM	University Queensland, Microbiology Department.
UAc	Uranyl Acetate.
X	Hybridized with.

(VI) Classification of Non-Bacterial Species.

Adapted from Barnes, (1974) and Grant, (1978).

A. Plant.

Sub-Phylum Chlorophyta: Green Algae *Gracilaria verrucosa.*

B. Macro-Invertebrates.

Phylum *Annelida,* **Class** *Polychaeta,*

Family *Nereidae:*	*Nereis diversicolor*
Family *Phyllodocidae:*	*Phyllodoce* sp.
Family *Terebellidae:*	Unidentified.

Phylum *Mollusca,* **Class** *Bivalvia:*

Oysters	*Crassostrea commercialis*
Mussels	*Modiolis* sp.

Phylum *Arthropoda,* **Class** *Crustacea,*

Sub-Class *Copepoda,*

Order *Calanoida:*

Acrocalanus gibber
A.gracilis
Paracalanus parvus
Pseudodiaptomus mertoni
Bestiola similis
Gladioferens pectinatus

Order *Cyclopoida:*

Oithona sp.

Order *Poecilostomatoidea:*

Bomolochid Copepod	*F.Bomolochidae*

Sub-Class *Malacostraca,*

Order *Isopoda:*

Cymothoid Isopod	*Cymothoa indica*
Cyralanid Isopod	*Pseudolana dactylosa*

Order *Decapoda:*

Soldier crab	*Mictris longicarpus*
Swimming crab	*Varruna littorata*
Banana prawn	*Penaeus merguiensis*
Southern tiger prawn	*P.esculentis*

C. Fish.

Phylum *Vertebrata,* **Class** *Teleostomi:* (Bony fish),

Bony Bream	*Nematolosa come*
Grey Mullet	*Mugil cephalus*
Silver Mullet	*Liza argentea*
Gold Fish	*Crassius auratus*
King Salmon	*Polydactylus sheridani*
Summer Whiting	*Sillago ciliata*
Golden Lined Whiting	*S.analis*

1.0 INTRODUCTION.

1.1 An Overview of *Red-Spot* Disease.

A remarkable aroma emanates from the putrefied muscle tissues of living fish with fully developed *Red-Spot* Disease, (RSD). Of course such affected fish are unmarketable and the recurrent losses to RSD disease have been estimated by Queensland government industry sources to be about $100 000 per annum.

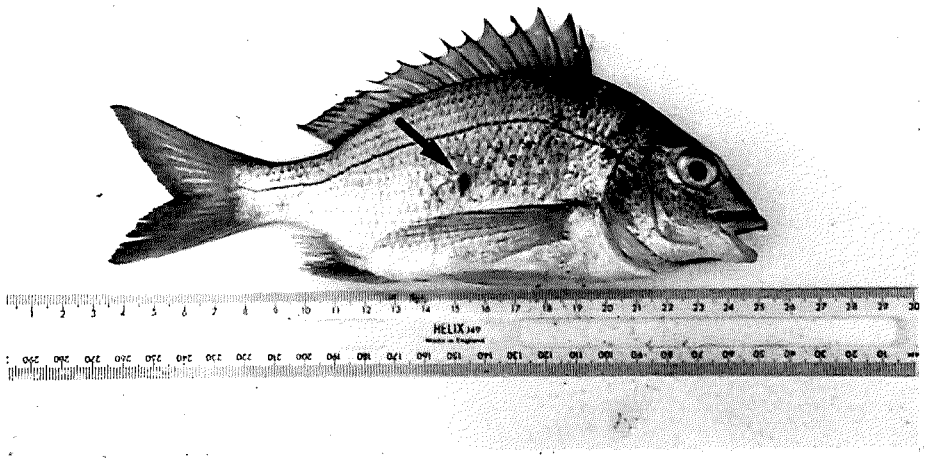
RSD in Queensland occurs principally in estuarine fisheries where the commercial species most commonly affected are mullet, *Mugil cephalus;* bream, *Acanthopagrus australis;* (Plate 1.1), and whiting, *Sillago ciliata*. The disease was attributed, after limited bacteriological examinations (Burke and Rodgers 1981, Rodgers and Burke 1981), principally to the bacteria *Vibrio anguillarum* and *Aeromonas hydrophila* and initiation related to stress induced through fluctuating temperature and salinity.

Anecdotal opinion variously attributes RSD to polluting effluents, from sugar refineries or cane farms, (as pesticides or fertilizers), and from sewage. Most recent conjecture attributes the pathogens as having recently arrived to Queensland in ships ballast tanks. Some fisherman however report that RSD is a condition which is perhaps more prevalent, but which occurred before 1950.

Now that susceptible commercial wild fish stocks are fully utilized and with potential for similar disease in the developing aquaculture industry, there is concern to at least maintain current yields. Such disease also can be financially catastrophic, and a major determinant of fish farm viability. Thus zoonoses which affect wild commercial fish species are increasingly economically relevant; and the need for disease control or management, obvious.

Plate 1.1: (a) Bream, *Acanthopagrus australis;* and (b-d) Mullet, *Mugil cephalus* with indicated early stage lesions from *Red-Spot* Disease.

a



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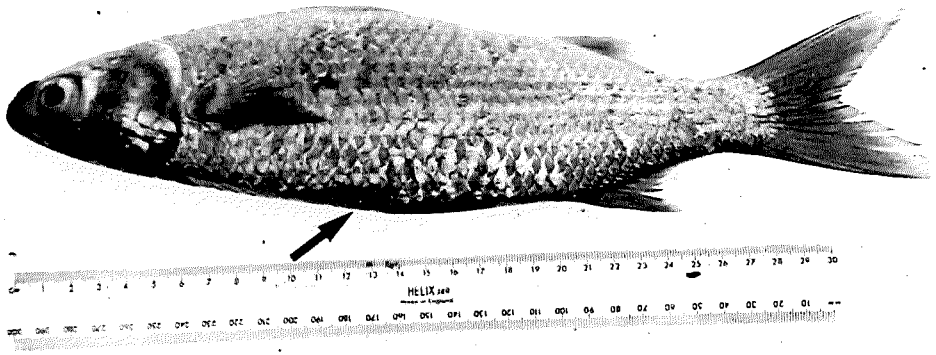

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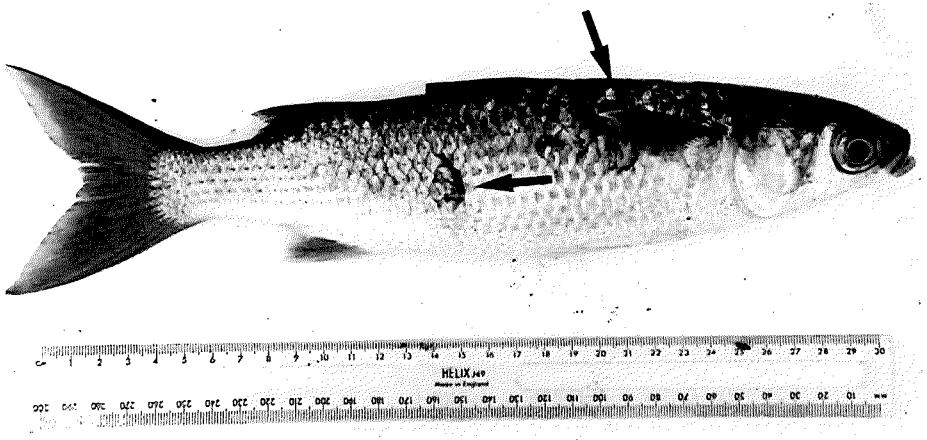

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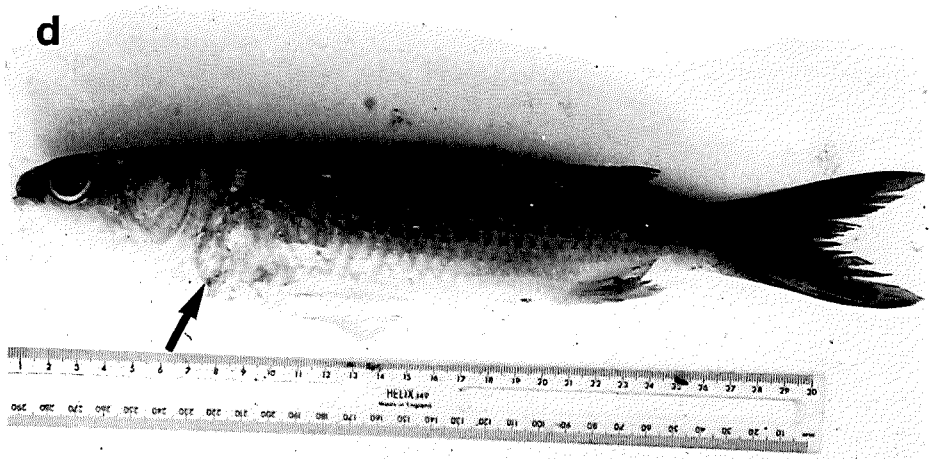


Traditionally ecological studies of infectious agents have focused upon terrestrial, rather than aquatic, host species. Although excursions into the realm of marine epidemiology introduce certain complications there are compelling economic reasons for work which could lead to the adoption of an *in situ* management regimen.

1.2 Study Aims.

This project addressed the following six objectives established for the deduction of some disease transmission mechanisms of RS associated *Vibrionaceae,* and potentially for development of a disease management strategy.

(i) To develop methods for the isolation, and classification of *Vibrionaceae*-like bacteria from fish and from niches in the aquatic milieu.

(ii) To classify the isolates using numerical taxonomic methods.

(iii) To compare and perhaps correlate isolates from different habitats.

(iv) To interpret the correlations in the context of their known and experimentally demonstrated micro-ecologies.

(v) To test the pathogenicity of bacteria, from selected phena, towards fish in controlled conditions and to examine some of the factors influencing virulence.

(vi) To investigate possible transmission modes of pathogenic or potentially pathogenic isolates between the environment and fish.

2.0 REVIEW OF THE LITERATURE.

2.1 *Vibrio* Disease in Fish: *An Introduction and Historical Perspective.*

According to Sindermann (1970) the first record of an infectious disease in fish was a zoonosis in migrating eels recorded in 1718 by Hoffer. Canestrini (1893) first implicated bacteria as causing this disease by isolation of the bacterium named as *Bacterium anguillarum* from diseased european eels, *Anguilla anguilla* (Moller and Anders 1986). Subsequently, *Vibrio anguillarum* Bergeman 1909, was described from diseased eels and was noted as the probable cause of the zoonosis known as "red disease" (Sindermann 1970). Later studies have shown that the symptoms of red disease of japanese eels, *Anguilla japonica*, caused by *V.anguillarum,* are also produced by at least two other species from the *Vibrionaceae, Vibrio vulnificus* Biogroup II, *(Pseudomonas anguilliseptica),* and *Aeromonas hydrophila* (Nishibuchi, Muroga and Jo, 1980). Similarly Rodgers and Burke (1981) reported three species of *Vibrionaceae, V.anguillarum, V.alginolyticus,* and *A.hydrophila*, from coastal Queensland fish with RSD. However no cultures from their work survived for third party evaluation.

Vibrio anguillarum has been isolated as a fish pathogen from many parts of the world (Austin and Allen-Austin, 1985) and is particularly widespread in Australia. Rodgers and Burke (1981) reported that the Queensland distribution of RSD extends northwards to the river systems of New Guinea and southwards to central New South Wales. Isolations of *V.anguillarum* and other fish pathogenic *Vibrio* spp. from farmed salmon in Tasmania (Cameron, Garland, Lewis and Machin, 1988) indicate the distribution of these species along the entire eastern seaboard i.e. in habitats where water temperatures range from 9-31°C. and in salinities between < 1 ‰ and 35 ‰. *Vibriosis* mostly occurs amongst schools of migratory fish (Anderson and Conroy, 1970) from a diverse range of teleost hosts extending from primitive groups such as eels and salmon (Nishibuchi *et al.,* 1980) to advanced fish such as cod (Larsen, 1982). *V.carchariae* was isolated from an elasmobranch (Grimes, Stemmler, Hada, May, Maneval, Hetrick, Jones, Stoskopf, and

Colwell, 1984). Reports of *Vibrio* disease from Northern Hemisphere wild fish stocks predominantly have been reported around the summer to late summer season (Sindermann, 1970).

Typically, the onset of *vibriosis* is indicated by inappetence, lethargy, darkening of the skin and eye opaqueness (Anderson and Conroy, 1970; Richards, 1980). Richards also found that turbot, *Scophthalmus maximus,* suffered peri-orbital and gut oedema. Anderson and Conroy observed that fins are initially congested with mucus, the vent reddened, and surface and sub-surface bleeding (petechia and ecchymoses) with some scale erection (lepidorthosis) also occur. Surface lesions later become ulcerative and damage underlying muscle tissue. The internal organs are typically oedematous with haemorrhagia and sometimes necrosis of the liver, spleen and kidney occurs. The gut and rectum are typically distended and filled with a clear viscous fluid by which stage anaemia is usually also apparent.

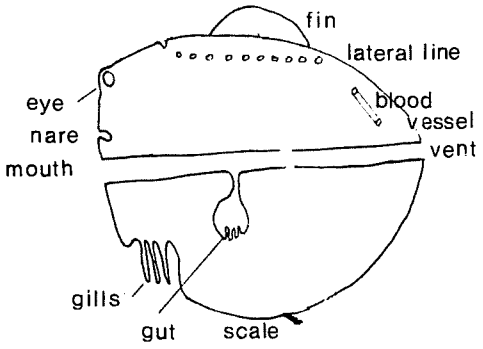
Because RSD infections were chronic rather than acute, Rodgers and Burke (1977-Unpublished) remarked on the low virulence of Queensland strains of *V.anguillarum.* Later (1981), they reported that RSD appeared to evolve from single inflamed scale pockets found colonized by *V.anguillarum* and secondarily invaded by saprophytes (See 2.6.1).

2.2 The Host Microcosm.

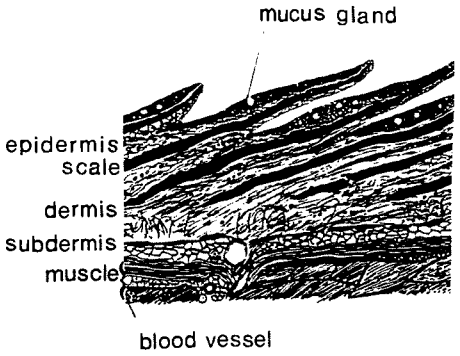
The fish host is covered by a slippery, layer of protective mucus (See 2.3.2) physically shielding against osmotic imbalance and against pathogens (Lagler, Bardach, Miller and Passino 1977, Hibiya, 1982). Under this mucus a diversity of specialized and consequently immunologically compromised sensory, absorptive, and locomotory tissues offer microcosm of different habitats to opportunistic or specialized parasitic invaders. Figure 2.1(a), adapted from Mims (1977) shows some potential sites for initiation of tissue invasion. An investigation of these sites may provide a key for isolating the routes of pathogenesis.

Figure 2.1: (a) Different Pathogen Niches Presented by the Fish Host (Modified from Mims, 1977); and from Lagler *et al.*, (1977) Structure of (b) Skin, (c) Nares, and (e) Lateral Line; and from Hibiya (1982), Structure of (d) Eye and (f) Gills.

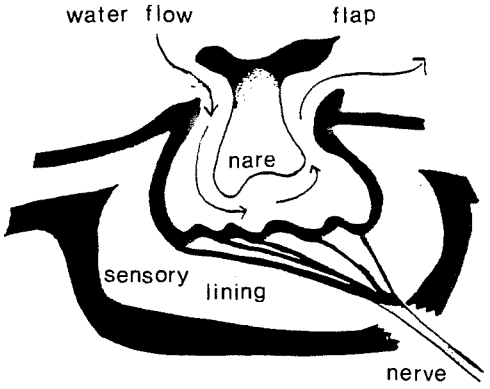
(a)


fin
lateral line
blood vessel
eye
nare
mouth
vent
gills
gut
scale

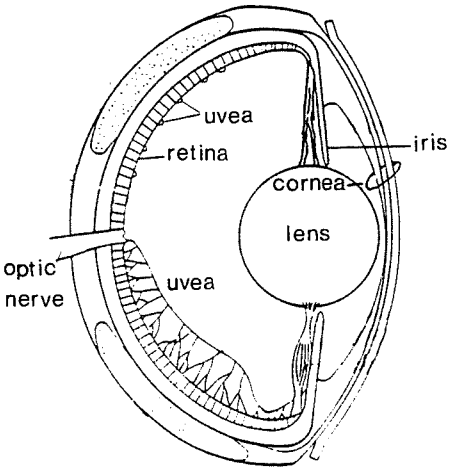
(b)


mucus gland
epidermis
scale
dermis
subdermis
muscle
blood vessel

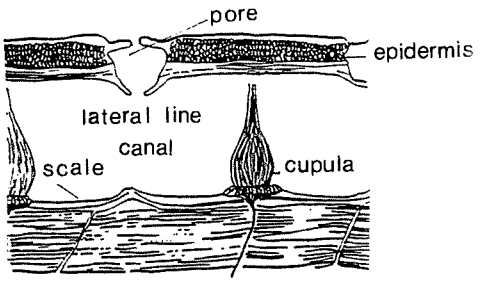
(c)


water flow
flap
nare
sensory lining
nerve

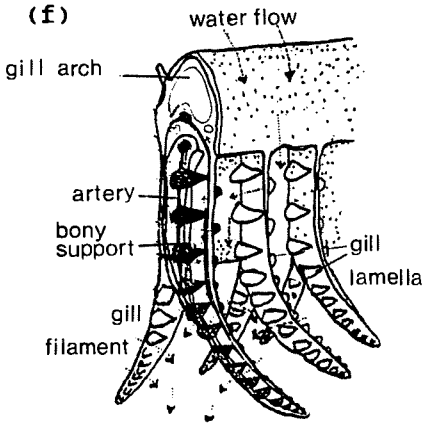
(d)


uvea
retina
iris
cornea
lens
optic nerve
uvea

(e)


pore
epidermis
lateral line canal
scale
cupula

(f)


water flow
gill arch
artery
bony support
gill lamella
gill filament

Fish skin, including mucus, (Figure 2.1(b)), generally forms a three layered, barrier between the host and its external environment, apart from the shedding properties of mucus against micro- organisms and debris, the skin protects against trauma by the imbricated arrangement of scales, but the scale pockets from which they arise are vulnerable to a specialized group of anadromous transversotremad fish flukes e.g. (Whitfield, Anderson and Moloney 1975). Feeding lesions to scale pockets left by *Prototransversotrema* spp. have been suggested by Rodgers and Burke (1977-Unpublished), as possible foci for infection of mullet *Mugil cephalus* by *Vibrio anguillarum* in RSD zoonoses.

Because of their membranous form and concomitant accessibility to blood, the fin tissues of fish are commonly breached by parasites, especially parasitic copepods. Secondary microbial infection of traumatized fins and tails of largemouth bass, *Micropterus salmoides* by saprophytes is mentioned in Section 2.3.3.2.

Fish nares (Figure 2.1.(c)) are blind pits where water is channeled over sensory nerves. Traxler and Li (1972) isolated *Vibrio anguillarum* from the nare of a laboratory cod, *Gadus morhua,* with necrosis to perinaral connective and muscle tissue.

Fish eyes, Figure 2.1 (d), are poorly structured to combat infections because for transparency, they lack extensive vascularization to the cornea and lens, and therefore are inaccessible to blood-borne immune processes. Eyes are both staging points to later systemic infection and susceptible to secondary infection from the uvea. Typically eye infections by bacteria, viruses, fungi, protozoans, digenea, nematodes, crustacea and probably also cestodes; result in opacity of the cornea or lens (Dukes 1975).

Horne, Richards, Roberts and Smith (1977) noted that in hatchery-raised turbot, *Scophthalmus maximus,* infected by *Vibrio anguillarum,* the most prominent early symptom was oedema, of the eye, (exopthalma), then the abdomen, (seen as lepidorthosis). Milleman and Knap (1970) cited in Dukes

(1975) suggested that oedema may be induced through upset fluid regulation from kidney damage. Because Horne *et al.* (1977) only examined moribund fish the initial site of infection remained unknown.

The lateral line, Figure 2.1 (e), is a sensory canal system underlying the epidermis of some fish with numerous pores opening to the epidermis. Moller and Anders (1986) reported that cod, *G.morhua,* had been found suffering an ulcerative condition to the lateral line with unknown aetiology. No infections via this route have been experimentally demonstrated but its feasibility has been indicated by the work of Amend and Fender (1976) who postulated that the perfusion of immunogen in hyperosmotic infiltration was via the lateral line system, then by cross-diffusion into neighbouring lymph ducts and so into the venous system. Later work using labeled bacteria as described below causes some moderation of this conclusion.

Gardner (1975), in Hibiya (1982), reported cellular necrosis in the lateral line of killifish, *Fundulus* sp., exposed to the heavy metals mercury and silver, indicating the lateral line as potentially susceptible to secondary bacterial invasion.

The fish mouth and digestive tract are lined with a soft mucus membrane (Lagler *et al.,* 1977) which coat food and protect underlying tissues from abrasions. Despite the protective effects of mucus, food was reported as one of the primary routes of infection by *Vibrio anguillarum* in farmed ayu, *Plecoglossus altivelis,* (Kawai, Kusuda and Itami, 1981).

Filaments and secondary lamellae of gill, Figure 2.1(f), tissues increase the surface-area-to-volume ratio for gas exchange and offer a readily accessible supply of blood to a diversity of pathogens. As for other external organs including the gut the primary cleansing mechanism is by shedding mucus. No reports exist of the gills being the focus of infections by fish pathogenic *Vibrio* species. However Cusack and Cone (1985) reported the occurrence of bacterial epiflora upon gill parasitic monogenoids. Such epiflora, if invasive, may colonize primary parasitic lesions. Additional

indirect evidence for permeation of gills by bacteria is work by Tatner and Horne (1983) following the route of perfusion of hyperosmotic infiltration of bacterial immunogen.

Killed ^{14}C-labeled cells of *V.anguillarum* perfused into rainbow trout, *Salmo gairdneri,* were found to absorb twice as much immunogen into the head region than the trunk region, (lateral line), but the workers were unable to say if this absorption was through the gills, lateral line or the mouth, or a combination of the three.

Reports from the literature do not indicate the primary sites of infection and pathogenesis however available evidence indicates that initial *Vibrio* infection is opportunistic rather than site specific. Comparative investigations (See 2.3.2) of different infectious routes indicate that tissues differ in their susceptibility to infection.

2.3.0 Infection and Pathogenesis.

The successful infection of any host is dependent upon three interacting sets of parameters, pathogen virulence, host susceptibility and environmental stress.

2.3.1 Pathogen Virulence.

A diversity of virulence/pathogenesis mechanisms are employed by invasive parasitic bacteria; these have been extensively reviewed e.g. in Brubaker (1985). The most important virulence mechanisms of *Vibrio* species seem to relate to their chemotaxis and tissue adherence; and production of siderophores, and cytolytic, mucinolytic, and proteolytic enzymes, and other toxic compounds.

From the evolutionary perspective whether these "virulence mechanisms" are serendipitous artifacts or highly evolved secondary functions of products with primary cellular maintenance functions is unknown, e.g. the neurotoxin, tetrodotoxin, produced by many *Vibrionaceae* also serves for sodium iron transport within bacterial cells (See 2.4.4.7). Another feature

which influences bacterial virulence is the capacity for both vertical and horizontal evolution, so permitting pathogenic populations to make quantum shifts to new, more successful (virulent? or symbiotic?) population phenotypes.

Crosa, Schiewe and Falkow (1977), found a correlation between the incidence of a 50 megadalton plasmid and the virulence of *V.anguillarum*. Subsequent experiments (Crosa 1980; Crosa, Hodges and Schiewe, 1980), with labeled plasmid demonstrated a correspondence between the loss of label, (and plasmid), and pathogen virulence. This plasmid was found to enhance virulence by coding for iron chelation, and allowed *V.anguillarum* to successfully compete with the host's siderophores (transferrin and lactoferrin) (See 2.3.2). Crosa and Hodges (1981) found virulent strains of *V.anguillarum*, with and without iron chelation plasmids, but which both produced siderophores in limiting iron conditions.

Trust, Courtice, Khouri, Crosa and Schiewe (1981) proposed that virulence of fish pathogenic *Vibrios* may also be related to bacterial adhesion to target cells on host gill and gut epithelia. They found that neither adhesive nor haemagglutination functions were plasmid coded. *In vitro* work has shown that *V.anguillarum* cells preferentially adhere to excised sections of mid and upper fish-gut in preference to lower gut regions (Horne and Blaxendale, 1983). Chemotactic movement of motile bacteria may also be a determinant of virulence as in the fish pathogen *Aeromonas hydrophila* which is attracted towards fish mucus (Hazen, Esch, Dimock and Mansfield, 1982). Haemolysin has been suggested as a further virulence factor of *V.anguillarum* behaving as a typical bacterial cytolytic toxin (Munn 1978; 1980).

Umbreit and Tripp (1975) showed that heat-stable toxin secreted from the cell wall of *V.anguillarum* i.e. lipopolysaccharide, (LPS), was toxic to laboratory gold fish. Rasmussen (1987) detected a heat stable polysaccharide, secreted by strains of *V.anguillarum*, which was antigenically distinct from LPS. Toranzo, Barja, Potter, Colwell, Hetrick

and Crosa (1983) found that pathogenic strains of *V.anguillarum* could be separated from non-pathogenic *Vibrio* spp. by their resistance to fish-serum lysis, indicating different responses to complement-mediated lysis and other serum components.

2.3.2 Host Susceptibility.

Gjedrem and Aulstad (1974) found a low but statistically significant hereditability of genetic resistance to *vibriosis*. Later, Winter, Schreck, and McIntyre (1980) correlated disease resistance of coho salmon, *Onchorhynchus kisutch,* and rainbow trout, *Salmo gairdneri*, with specific transferrin genotypes; but no difference in resistance to *vibriosis* of fish with different transferrin genotypes was experimentally demonstrated.

Mims (1977) reported properties of human mucus which prevent pathogenesis i.e. by behaving as a mechanical barrier, and sloughing surface, (already shown for fish), and by the presence of immune complexes which are resistant to proteolytic enzymes. The principal acid mucopolysaccharide in mucus of japanese eels, *A.japonica,* was shown (Hibiya 1982) to be neuraminic acid and so is susceptible to neuraminidases such as reported from *V.cholerae* e.g. Huang, Dietsch and Rott (1985),(See 2.3.1). Fletcher and Grant (1969); Bradshaw, Richard and Sigel (1971) and Harell, Etlinger and Hodgins (1976) found IgM antibodies in fish mucus towards bacterial pathogens. Harell, *et al.* (1976) found complement in fish mucus and serum but were unable to determine if non-immunized fish produced mucus which normally contained a non-specific low level antimicrobial system. An unspecified form of serum lysis was reported by Toranzo *et al.,* (1983) as a non-specific protection against *Vibrio* spp. (See 2.3.1).

Ellis (1982) found the development of a specific immune responses in fish was dependent upon the environmental temperature. Trust (1986) reported that these specific immune responses of cold and warm water fish were active over different temperature ranges.

Levin, Wolke and Cabelli (1972) and Watkins, Wolke and Cabelli (1981) found doses of *V.anguillarum* required to cause infection in *Pseudopleuronectes americanus* were less from intradermal than intraperitoneal (ip.), and oral routes. Muroga (1975), in a similar study of japanese eels, *A.japonica*, found the greatest susceptibility via intradermal routes, i.e. susceptibility differs between tissues consistently between species. Muroga also found large eels were slightly more resistant to infection than small.

Median lethal doses, (LD_{50}'s), of *Vibrio* spp. have been reported directly as the number of cells per fish e.g. LD_{50} of *V.anguillarum* to *P.americanus* of 640 cells by intradermal cf. 6 400 by ip. route (Levin *et al.,* 1972), or; [as by Muroga (1975) and others and irrespective of size dependant resistance], as mg cells/100g fish, where 1 mg wet weight is equivalent to about 8 X 10^8 bacteria, e.g. for *V.anguillarum* towards *A.japonica* the LD_{50} was 8.9 mg cells /100g. Jo and Muroga (1977) found the LD_{50} by intramuscular inoculation of *V.anguillarum* was equivalent to 8.75 mg cells/100g ayu, *P.altivelis.* This is consistent with later work (Kawai *et al.,* 1981) in which the median lethal dose (LD_{50}) for *P.altivelis* by intradermal injection was found to be 3 mg *V.anguillarum* per 20-40 g fish. An ip. Log_{10} LD_{50} of *V.vulnificus* Biogroup II towards *A.japonica* of 8.4 was found by Tison, Nishibuchi, Greenwood and Seidler (1982). The ip. Log_{10} LD_{50}'s of both *V.carchariae* and *V.damsela* towards spiny dogfish, *Squalis acanthias,* was found by Grimes *et al.,* 1984 as less than 6.6.

Watkins *et al.,* 1981 found minimal LD_{50} doses were achieved with 24-30 hour late log phase cultures. Lester and Budd, (1979) found older cultures (48 h) of a *Vibrio* sp. inoculated ip. into coho salmon *O.kisutch* in 10 000 and 100 000 cell doses killed fish mainly in the first week at the high dose, and in the first and second week at the lower dose. These findings were contrary to those of Muroga (1975) upon eels, *A.japonica*, which either succumbed to infection within 3 d of inoculation or not at all.

2.3.3.0 Environmental Stress.

The influence of stress in determining susceptibility has been stated by several authors; these are reviewed by Wedemeyer (1970) and Snieszko (1974). Snieszko reported a normally non-pathogenic *Aeromonas* species which opportunistically caused a zoonosis in fish in a polluted environment indicating that the environment can influence susceptibility to *vibriosis* by inducing stress conditions in fish so reducing their resistance; or such opportunistic pathogenic bacteria may be environmentally enriched to the level of an infectious dose. Brett (1958) cited in Wedemeyer (1970) defined stress as ... *"a state produced by any environmental or other factor which extends the adaptive responses of an animal beyond the normal range, or which disturbs the normal functioning to such an extent that, in either case, the chances of survival are significantly reduced."* Wedemeyer (1970) collated alterations to fish physiology which occur as a result of stress:

(i) Decrease in plasma albumen and transferrin.
(ii) Increase in plasma fibrinogen, haptoglobin and ceruloplasmin.
(iii) Decreased number of circulating lymphocytes.
(iv) Decreased inflammatory response.
(v) Impaired gamma-globulin formation.
(vi) Depressed interferon production.
(vii) Increased plasma proteolytic activity.

The induction of environmental stress may occur by a shock change of a normally stable parameter, or as a result of the prolonged alteration of a parameter. The major stress-mediating parameters can be sub-divided into abiotic and biotic components. The most important abiotic stress mediators are probably temperature, salinity, pH, dissolved oxygen, and exogenous pollution. The biotic stresses likely to be encountered are starvation, endogenous pollution, crowding and spawning cycles. Stress factors may interact synergistically with host and bacteria to facilitate infection.

2.3.3.1 Abiotic (Extrinsic) Stress Factors.

2.3.3.1.1 Temperature.

The role of temperature in determining the prevalence of *vibriosis* is indicated by reports of the disease off the Scandinavian coast when water temperatures rise above 10°C. (Gjedrem and Aulstad, 1974). Temperature has been instanced on a number of other occasions as a catalyst to disease. Sindermann (1970) reported that *vibriosis* in wild fish was most common for the summer to late summer period. Snieszko (1974) cited an investigation of interactions between winter water temperatures and depressed dissolved oxygen (Schaperclaus 1959) and their effects upon seasonally fasting wild carp. Despite depleted tissue reserves fish were found to compensate to lowering dissolved oxygen concentration by increasing haematopoesis; but as the dissolved oxygen continued to fall the ability of the carp to compensate was overtaken, and fish effectively became anaemic and prone to disease. In areas with cold winters the greatest losses of carp and other fish occur in spring from infections caused by *Aeromonas hydrophila (A.liquefasciens)* and other (unspecified) bacteria. Similarly Watkins, *et al.,* (1981) found that in flounder *Pseudopleuronectes sapidus* the LD_{50} of *V.anguillarum* in winter fish was three logs less than for summer fish. Muroga (1975) found that *A.japonica* infected with the same initial dose of *V.anguillarum* were more likely to die and to do so more rapidly at 20°C. than at 10°C. These inter-species differences in susceptibility are explicable by Trust's (1986) observations of temperature-dependent immune-responses (See 2.3.2). Esch, Hazen, Dimock and Gibbons (1976) speculated that thermal loading from a cooling plant might influence the infection of the cool-water largemouth bass, *M.salmoides*, by *A.hydrophila* by induction of higher metabolic rates leading to reduced body condition and a lowered resistance. This was experimentally proven (Gibbons, Bennet, Esch and Hazen 1978) and it was further shown (Esch and Hazen 1980) that the thermal effluent was inducing physiological stress in fish and that infection was a likely consequence of that stress however the contribution of increased temperature to decreased generation times of *A.hydrophila* was not addressed. It was suggested

(McCarthy 1976) that a zoonosis in British eels by a *Vibrio* sp. was a result of stress from high water temperatures and low freshwater flows in affected rivers at the time.

2.3.3.1.2 **Osmoregulatory Stress.**

Osmoregulatory stress to fish resulting from alterations of salinity have been suggested as the catalyst to zoonoses of *vibriosis* in wild (McCarthy 1976; Rodgers and Burke, 1981), and farmed, (Egidius, Wiik and Anderson 1977) fish.

High or low pH have not been specifically shown to facilitate infection. Most reported instances of *vibriosis* are in diluted seawater containing greater than 10 ‰ sodium chloride and consequently sufficient carbonate and bi-carbonate ions would be present to buffer the effects of acid conditions. Mc Donald (1983) showed that the blood chemistry of rainbow trout, *S.gairdneri,* was prone to alteration by acidic freshwater. Gunn (1986) reported mortalities, but not disease, in other freshwater salmonids exposed to episodic acidic pH, induced by spring "thaws," i.e. from acid snow. Sub-lethal changes to fish induced by acid pH were inhibited spawning, hyper or hypo-activity, reduced swimming activity and interference with chemoreception. Acid pH might initiate these effects by dispersing the protective and osmoregulatory mucus blanket to make fish physically, as well as physiologically more susceptible to infection.

2.3.3.1.3 **Dissolved Oxygen and Organic Pollution.**

Pollution, high temperature, and concomitant oxygen depletion have all been shown in the field to increase susceptibility to disease. Depletion of dissolved oxygen is usually a result of bacterial respiration, algal blooms, temperature increases, chemical oxidation of metals or the effects of surfactants. The specific contributions of these parameters have not been isolated in the laboratory but their influence upon fish health was shown by Plumb, Grizzle and Defigueiredo (1976). *A.hydrophila* was found in lesions of catfish, *Ictalarus punctatus*, in water with dissolved oxygen depleted by organic pollution. It was subsequently observed that as water quality

improved so too did fish health. Larsen (1982) and Larsen and Jensen (1982) correlated the incidence in Denmark of "Ulcus Syndrome" to induced background flora of *Vibrio anguillarum*-like organisms grown upon sewage and cellulosic effluents (See 2.6.1). The possibility here that suspended solids provided a pathogen staging platform (See 2.4.1) was not evaluated.

2.3.3.1.4 Pesticides and Heavy Metal Pollution.

Rodsaether, Olafsen, Raa, Myhre and Steen, (1977) suggested that the presence of copper in water, stressed eels sufficiently to allow infection by *V.anguillarum.* Sugatt (1980) similarly reported lowered resistance of fish to infection by *V.anguillarum* as a result of exposure to other heavy metals e.g. zinc and chromium (See 2.2 and 2.6.3). No reports are available for chronic toxic effects to fish by mercury compounds such as were used as fungicides in the Queensland coastal sugar industry before 1977. Little information is available for stress from chronic exposure of fish to pesticides such as might result in secondary *Vibrio* invasion. Snieszko (1974) reported that marine fauna exposed to the herbicides 2,4-D and diquat did not cause secondary bacterial infections, however DDT fed to trout and guppies increased susceptibility to bacterial infection.

2.3.3.2 Biotic (Intrinsic) Stress Factors.

Hille (1982) reported that Wedemeyer (1976) showed stress from crowding caused hyperglycaemia and moderate hyperchloraemia in rainbow trout. With crowding there is also the increased possibility of infection transmission through physical closeness of infected fish. The relevance of this to aquaculture and the occurrence of zoonoses amongst gregarious fish is obvious.

Stress from male and female reproductive behavior has been shown to induce bite and abrasion lesions lesions in largemouth bass, *M.salmoides*. Secondary infection of these lesions by saprophytes such as the myxobacteria has been reported (Gaines and Rodgers 1975).

Fouling of the water by excreta, mainly organic material and ammonia, introduces stress through enhanced growth of saprophytic bacteria involved

with degradation of organics, and nitrification of ammonia. Larmoyeux and Piper (1973) observed that ammonia at concentrations less than 1 mg/l caused a swelling and hyperplasia of gill lamellae and secondary involvement of filamentous bacteria. Smith and Piper (1975) found rainbow trout, *S.gairdneri*, chronically exposed to 1.6 p.p.m. ammonia have lowered growth rates and pathological changes to both the liver and gills.

2.4 Ecology of the *Vibrionaceae.*

Numerous regional studies of marine microbial flora have reported *Vibrio* sp. as major components, (Simidu, Ashino and Kaneko, 1971; Cook and Goldman, 1976; Kaneko, Krichevsky and Atlas, 1979; and Hauxhust, Krichevsky and Atlas, 1980). There is evidence that some species from the *Vibrionaceae,* e.g. *Photobacterium leiognathi* and *Vibrio diazotrophicus,* are ecologically restricted to symbiotic sites (See 2.4.4.3,5,6). The possibility that other ecologically less well studied, fish pathogenic, species might also occupy specific niches, at which specific disease control measures might be targeted is a possibility worthy of investigation.

2.4.1 Incidence and Seasonality.

Simidu and Tsukamoto (1985) found stratification of different species of *Vibrio* within the water column. Kaneko and Colwell, (1973, 1974, 1975a, 1978); Colwell, Lovelace, Wan, Kaneko, Staley, Chen and Tubiash, (1973); Shin, Horie, Okuzumi and Kobayashi (1976); Kaneko (1973) and Gjerde and Boe (1981), studied the seasonal distribution of different *Vibrio* spp. in temperate marine coastal regions. High counts were obtained in the water column in summer months, and during winter numbers fell below detectable levels (See 2.3.3.1.1); Kaneko and Colwell (1973, 1978) however found small numbers of *V.parahaemolyticus* persisting in marine sediments. A similar investigation on *V.cholerae* from the tropical Bay of Bengal (Nair, Abraham and Natarajan, 1980) and in freshwater around Calcutta Sarkar, Balakrish Nair, Banerjee and Pal (1985) also demonstrated an abundance of *V.parahaemolyticus* during summer, though the relative diminution during the winter period was not as severe as in temperate waters. This seasonality is

consistent with findings showing an increased incidence of *vibriosis* in summer despite increased fish resistance at higher temperature as reported by Watkins *et al.*, (1981), (See 2.3.3.1.1 and 2.6.2).

2.4.2 Starvation and the Gram Negative Spore State.

Amy, Pauling and Morita (1983) investigated a marine psychrophilic *Vibrio* capable of surviving for up to 1 year in starvation conditions. Despite cellular metabolite reordering and shrinkage, after six weeks 45 to 60 % of cells remained viable. These survival rates are substantially higher than found for *V.parahaemolyticus* by Kaneko and Colwell (1973) but probably reflect variability between species or resuscitation technique. MacDonell and Hood (1982) reported that < 0.3 μm "ultra-micro" starved dormant cells of *Vibrio* species grew to normal size in nutrient media. Subsequent work by Guckert, Hood, and White (1986) and Hood, Guckert, White and Deck (1986) showed that these starvation states resulted from a rearrangement of the relative proportions of macro-molecules and cell shape. It was suggested that recognition of this as a Gram-negative "spore state" was not inappropriate despite the absence of the outer membranes characteristic of terrestrial bacterial endospores. This concept is consistent with the definition by Mason, Hamer and Bryers (1986) of spores as morphologically differentiated structures.

2.4.3 Predation.

Apart from predation upon *Vibrionaceae* by filter feeders and other non-specific predators, some studies have been undertaken on loss of *Vibrio* spp. from the water column through bacterial predation. Miyamoto and Kuroda (1975) isolated a halophilic *Bdellovibrio* sp. parasitic for *V.anguillarum*. It was further suggested that over-wintering of *Vibrionaceae* only in the sediments was a result of removal from the water column by predation.

Baross, Liston and Morita (1978) isolated *Vibrio* bacteriophages from abiotic and biotic origins and found an apparent ubiquity of *Vibrio* bacteriophage in marine bivalve molluscs. It was suggested that phage may arrive via infected bacteria and persist in oysters and as digestive symbionts and susceptible

phage-types. While phages were frequently isolated for *V.parahaemolyticus,* phage active towards some abundant *Vibrio* spp. were rare.

2.4.4.0 Micro-Ecology.

Apart from their isolation from the water column and sediments *Vibrionaceae* have been found repeatedly associated with animals and plants in a large number of specialized niches. These niches range from simple colonized surfaces and biofilm communities to complex histological alterations by hosts to accommodate symbionts. Ecto and endo-commensal and potentially mutualistic, parasitic associations have been found between *Vibrionaceae* spp. and species of invertebrates and vertebrates e.g. with planktonic copepods, with sea urchins, in light organs of fish and squid, and with marine species accumulating bacterial tetrodotoxin.

2.4.4.1 *Vibrionaceae* Associated with Inert Surfaces.

Association of bacteria with surfaces was first suggested by ZoBell (1943) and the possible adhesive function of lateral flagella produced by *Vibrio* spp. upon contact with solid surfaces by De Boer, Golten and Scheffers (1975). Belas and Colwell (1982) confirmed this in competitive adsorption assays of separately, ^{3}H- and ^{14}C-, labeled cultures of *V.parahaemolyticus* and *V.cholerae,* onto chitin particles.

Pham, Nearhos and Davis (1985) found greater production of siderophores (a virulence indicator, See 2.3.1) in marine *Vibrio* spp. isolated from abiotic than from biotic exposed surfaces. This was interpreted as indicating that bacterial iron chelates trapped in animal surface slimes, could be poached by deficient strains otherwise unable to survive in the absence of such associations.

2.4.4.2 *Vibrionaceae* Parasitic on Healthy Fish.

Gillespie and MacRae (1975) reported that the predominant normal skin flora of fish were from the genus *Micrococcus* and *Pseudomonas* with only a small proportion of their isolates being *Vibrio* spp. However their results were based upon isolations from media containing only 0.5% saline which is less

than the minimal requirement of most *Vibrio* spp. (See 2.10.1, Appendix 1). In media with 3 % saline, Schandevyl, Van Dyck and Piot (1984) isolated a diversity of *Vibrio* species from the skin flora of healthy fish from the tropical coast of Africa.

Other studies of the bacteria from fish have repeatedly shown that *Vibrio* spp. are also significant active components in the gut flora of apparently healthy as well as diseased fish (Newman, Cosenza and Buck 1972; Ugagin 1979; and Kakimoto and Mowlah, 1980), (See 2.2 and 2.3.1).

2.4.4.3 *Vibrionaceae* Parasitic on Molluscs.

Extensive literature exist for *Vibrio* spp. isolated from commercially important lamellibranch and gastropod molluscs, (Tubiash, Otto and Hugh 1973; Tubiash 1974; Grischkowsky and Liston 1974; Lipp, Brown, Liston and Chew 1975; Di Salvo, Blecka and Zebal, 1978; Elston and Lockwood, 1983; Garland and Carson, 1987). The species most commonly reported are *V.parahaemolyticus, V.alginolyticus,* and *V.tubiashii (V.anguillarum).* Except perhaps in the case of the oyster pathogen *V.tubiashii,* (Hada, West, Lee, Stemmler and Colwell 1984), most may be incidental food contaminants rather than symbionts.

2.4.4.4 *Vibrionaceae* Parasitic on Plankton.

Kaneko and Colwell (1973) and Sochard, Wilson, Austin and Colwell, (1979) found that virtually all of the surface bacteria from planktonic (Calanoid) copepods were *Vibrio* spp. Kaneko and Colwell (1973, 1975a, 1978) and Colwell *et al.,* (1973) showed that there was an association between *V.parahaemolyticus* and zooplankton during the summer months. It was shown (Kaneko and Colwell, 1975b) that *V.parahaemolyticus* adhered to live copepods over inert chitin particles. Similar work (Thompson, Vanderzant and Ray, 1976) showed a preferential adsorption of *V.parahaemolyticus* to zooplankton rather than diatomaceous phytoplankton. When the external and internal flora of *Vibrio* spp. from copepods was phenotypically characterized, (Sochard *et al.,* 1979), the internal flora was partitioned into one phenon. All

operational taxonomic units (OTU's) from this phenon were chitinolytic, but only 3 of the remaining 7 phena containing OTU's from the outside surface of copepods were chitinolytic. OTU's in only one phenon were predominantly swarming i.e. produced lateral flagella. These results indicate first that external association of zoo-plankton is not primarily for metabolism of chitin, and that *Vibrio* spp. with lateral flagella are not more likely to be found associated on the external surfaces of copepods than those with only polar flagella.

Nalin, Daya, Reid, Levine and Cisneros (1979) investigated other benefits that might be conferred to associated bacteria and found that a strain of *V.cholerae* adsorbed to chitin particles was better able to withstand acid conditions, (such as are encountered in the host stomach), than could non-adsorbed *Vibrionaceae*. Similarly, improved survival rates at elevated temperatures were found for *V.parahaemolyticus* in the presence of chitin particles than with unaccompanied cells (Karunasagar, Venugopal, Karunasagar and Segar, 1986). Huq, Small, West, Huq, Rahman and Colwell (1983) observed that most *V.cholerae* were attached to copepod egg-sacs and preferred living to dead copepods, and that *V.cholerae* populations attached to living copepods remained viable longer than those with dead copepods. It was suggested that if the sole purpose of attachment was to degrade chitin then absence of protective waxes on the cuticle of dead copepods would have made the converse more likely. These results suggest that either a commensal symbiosis exists between copepods and *V.cholerae* or that copepods were feeding on bacteria i.e. no bacterial adherence was occurring. Cahoon (1982), reported feeding mucus in another calanoid copepod *Euchirella venusti,* bacteria captured in mucus from like species might escape to colonize copepod surfaces.

2.4.4.5 Luminescent *Vibrionaceae.*

Luminescent *Vibrionaceae* are represented by all *Photobacteria* spp. [*P.angustum, P.leiognathi, P.phosphoreum, P.fischeri* and *P.logei*] and *Vibrio harveyi, V.orientalis,* and *V.splendidus.* Desmarchelier and Reichelt,

(1984) additionally found some luminescent strains of *V.cholerae (V.albensis)*, (Hada, Stemmler, Grossbard, West, Potrikus, Hastings & Colwell, 1985). Reichelt and Nealson (1977) investigated the bacteria from light organs of six species of leiognathid fish and noted the preponderance of parasitic strains of *Photobacterium leiognathi*. It was suggested that there was selection by the fish for this species and that other luminous species present were only transient i.e. this association is a mutualistic symbiosis.

2.4.4.6 Nitrogen Fixation by *Vibrionaceae.*

Guerinot and Patriquin (1981) isolated a nitrogen fixing species of *Vibrio,* parasitic in sea urchins, which could be harvested by the urchins to yield additional nitrogen, putatively to offset urchins' problems of seasonal availability, and low nutritional value, of the urchins' normal food-plants. *V.diazotrophicus* was described from this and saprophytic sources (Guerinot, West, Lee and Colwell, 1982 and West, Brayton, Twilley, Bryant, and Colwell, 1985). The species lacks enzymes such as proteases, and lipases, normally reported from *Vibrio* spp. and correlated with virulence, which infers that these species have formed a mutualistic symbiosis.

2.4.4.7 Tetrodotoxin Production by *Vibrionaceae.*

Simidu, Noguchi, Hwang, Shida and Hashimoto (1987) reported several species of marine and fresh water polar flagellate bacteria which produced the neurotoxin tetrodotoxin. It was suggested that, in bacteria, this compound functions to transfer sodium ions across membranes. Horseshoe crabs, *Carcino scorpiusrotundicauda;* blue-ringed octopus, *Octopus maculosis;* starfish, *Astropecton polyacanthus;* and many species of puffer-fish, *Tetrodontidae,* were reported to accumulate this neurotoxin, sometimes into specially adapted organs, and to excrete it upon distress. It was assumed, but not proven that the toxin from these species was from bacteria. If this is the case and we assume evolution mono-phyletic; and co-evolution non-convergent, then the development, by phylogenetically diverse species, of protective toxin blocking compounds and specialized storage structures implies a long evolutionary association between these multicellular species and bacteria.

2.4.4.8 Conclusion.

The *Vibrionaceae* have been reported from a diversity of geographical sites although within these distributions i.e. presence or absence in the water column, is dependant upon seasonality and other unknown factors. Recently observation of spore-like stages and examinations of the micro-ecologies of the *Vibrionaceae* involved in symbioses with multi-cellular species has indicated a high degree of adaptation and specialization amongst species from this family.

2.5.0 Transmission of *Vibrio* Infections between Fish.

Trust (1986) described horizontal transmission as the principal pathway for fish disease. Because most disease reports are from gregarious or farmed fish for the purposes of this study horizontal transmission has been subdivided into active and passive sub-categories. The proven mode of active transmission in *Vibrionaceae* is chemotaxis (See 2.3.3.1). Passive transmission, i.e. by jostling fish, is probably the major transmission mechanism of pathogens between gregarious fish. Other forms of transmission can probably occur also through the interactive micro-ecologies of pathogen and host such as via contaminated food, and by secondary bacterial invasions of fish lesions.

2.5.1 Vectored Transmission.

In terrestrial environments, a main reason for the evolutionary trend towards the use of vectors by infectious agents is to circumvent the threat of dehydration. This is not a problem in aquatic habitats e.g. Gram-negative "spore state" without testa (See 2.4.2). In aquatic situations several other benefits occur from parasitic or hyper-parasitic transmission. Not the least of these is the ability to contact a host in a three dimensional environment. Marine multicellular parasites have adapted to their hosts' biology or behavior to increase the probability of contact and infection. Vectoring by such parasites may provide a means of packaging large infectious doses and so enhance zoonosis initiation.

Cusack and Cone (1986) suggested that vector borne parasitic transmission may be most relevant to obligate pathogens such as pancreatic necrosis virus, viral haemorrhagic septicaemia virus, and the bacteria *Renibacterium salmonarium* and *Aeromonas salmonicida*. The type of vector most suited to the transmission of such infectious agents, is one which has numerous contacts with different individuals of the host species. Cusack and Cone (1985,1986) described such intermediate hosts as "platforms".

Ahne (1985) classified microbial disease vectors of pathogens into mechanical and biological depending upon whether replication as well as transportation occurred. He found that while *Argulus foliaceus* and *Piscicola geometra* were able to transmit a virus of carp there was no viral replication within either vector. Such classification of vectors is less practical for bacteria, because they are able to replicate both inside and outside most vectors. Replication upon vectors is obviously a useful pathogenesis mechanism, but because this is a general property, other behavioral qualities of bacteria and vectors have been used here for redefinition of vectors into the categories of passive, (2.5.3) and mechanical, which depend for pathogenesis only on bacterial pathogenic opportunism to host behavior, and biological when associative behavior is demonstrated by bacteria towards specific hosts.

Mechanical vectors can transport reproducing bacteria and cause primary lesions. The first record of a mechanical vector was by Shiply in 1909 who attributed bacterial inflammatory swim bladder infections in fish to inoculation by invading nematodes (Cusack and Cone 1986). Similarly the ciliate *Epistylis* which produces lesions in fish secondarily invaded by *Aeromonas hydrophila* (Hazen, Raker, Esch, and Fliemans 1978) is a mechanical vector.

Other associations of bacteria with surface tissue invasive ecto-parasites have been shown but consequent infection were not shown, or bacterial pathogenesis not suggested or demonstrated. For example the monogenean gill parasite, *Gyrodactylus avalonia*, with adherent bacterial microcolonies was

observed by scanning electron microscopy (Cusack and Cone, 1985). The sub-scale pocket sedentary fish fluke, (Rodgers and Burke, 1977-Unpublished), *Prototransversotrema,* was suggested as perhaps providing the focal point for initial lesions in *Red-Spot* disease presumably by water-borne rather than ecto-commensal symbiotic bacteria. The ergasilid copepod, *Lepeophtheirus salmonis,* which produces primary lesions in salmonids was suggested as the possible route of invasion for pathogenic *Vibrio* spp. (Hastein and Bergsjo, 1976) but these authors viewed the parasite as they would a pointed stick, i.e. as a traumatizing agent, rather than as a potential vector.

The transitory carp louse *Argulus,* and other copepod and isopod crustacean parasites have also been implicated in the transmission of fish disease. Because of this transitory quality these ecto-parasites; if they contact an otherwise diseased fish are suited to the transmission of disease to new hosts. Praniza larvae of gnathid marine isopods, occupy the same transitory ectoparasitic niche as the carp louse and fish leech, consequently they might also be expected to act as vectors for the transmission of bacterial agents; such larvae have been recovered from european eels, *A.anguilla,* the archetypal host of, *V.anguillarum,* (Mugridge and Stallybrass, 1983), but not associated with disease initiation. Lawler, Howse, and Cook, (1974) suggested the possible involvement of another isopod *Lironeca ovalis* in promoting infection by a virus disease (Lymphocystis).

Larsen and Jensen (See 2.6.1) investigated viral initiation of "Ulcus Syndrome" but suggested no means for establishment of initial viral infection.

2.5.2 Bacterial Persistence.

Passive vectors of fish pathogenic bacteria may provide a means by which persisting bacteria from abiotic and biotic environments, can be amplified to an infectious dose e.g. when taken into the gut of another species. Using a streptomycin resistant strain of *V.alginolyticus* Gauthier and Clement, (1979) investigated the transfer from sediments to the benthos. It was

observed that such resistant strains persisted in the digestive tract of scavenger organisms and that these remained viable and were able to colonize species occupying a higher trophic level. However, selection of streptomycin resistance as a marker trait by these authors is unfortunate; *Vibrio* spp. are generally streptomycin resistant, and so the validity of their experimental procedure is doubtful.

As passive vectors seabirds have been shown to disseminate pathogens outside the aquatic ecosystem by passage through their intestines e.g. West, Lee and Bryant, (1983) isolated *V.cholerae* from gull and swan faeces. Hastings and Nealson (1977) were more authoritative in their earlier suggestion that the distribution of "marine enterobacteria", i.e. marine digestive tract symbionts, would be encouraged if they could incubate in parasitic or symbiotic mode on host food material, such as is evident on small crustacea and fecal pellets e.g. as found by Sochard *et al.,* (1979), and where luminosity would serve as an attractant to predators i.e. biological vectors.

2.5.3 Conclusion.

The potential role of intermediate associations as biological staging points to fish infection by *Vibrionaceae* have been shown. Ecto-commensal associations have been shown between bacteria and fish ecto-parasites and instances where parasitic lesions were foci for bacterial pathogenesis noted. Mechanisms which restore bacteria from the sediments to higher trophic levels, and which can move bacteria from one aquatic environment to another were noted.

2.6.0 Ulcerative Fish Diseases - Case Studies.

Ulcerative zoonoses in fish have been reported from around the world but relatively few detailed investigations are available for comparative examination.

2.6.1 Ulcus Syndrome in Cod.

In the period 1975-1983 Jensen, Block and Larsen investigated a condition of Danish wild and sea-pen reared cod, *Gadus morhua,* known as "Ulcus Syndrome" associated with water pollution loads imposed by sewage and cellulose factories. A limited bacteriological survey was conducted on fish with "Ulcus Syndrome" (Larsen and Jensen, 1979; Larsen 1982). It was found that *V.anguillarum* and *Aeromonas hydrophila* were the principle species isolated from skin lesions and anterior kidneys at all disease stages. Other bacterial species encountered were *V.alginolyticus*, *Pseudomonas aeruginosa*, *Alcaligenes* sp. and *Acinetobacter* sp.

Larsen and Jensen (1982) and Larsen (1982) reported that the incidence of "Ulcus Syndrome" was correlated with stress due to effluent loads and with a ten-fold increase in numbers of environmental *Vibrio*-like organisms (i.e. bacteria which grew on TCBS medium). Lesions were similar to those of RSD (Burke and Rodgers, and Rodgers and Burke 1981) in their final form, but primary development of lesions (Jensen and Larsen, 1979), is different:-

Stage 1: (Papulo-vesicular Stage). Firm papules 2-8 mm in diameter and 1-3mm high are produced. A petechial form was also present which allowed blood to escape from the apex.

Stage 2: (Erosion.) Papules are lost to leave crater shaped holes. At their bottoms these holes are coloured grey to yellow with a "pinkish touch".

Stage 3: (Early Ulceration.) Ulcers develop from these "craters" with hyperplasia at their margins.

Stage 4: (Late Ulceration.) A single or a few ulcers are present usually 2-8 cm in diameter. These ulcers, in aquarium studies were found either to develop from single papules or by a confluence of multiple ulcers. Ulcers were both superficial and deep.

Stage 5: (Healing.) This stage is characterized by an absence of scales and pigment cells in affected areas to produce small white patches in the skin. Mouth and other non-papular lesions induced through trauma e.g. from trawling nets and lamprey wounds were also found to develop to ulcers.

Ulcerous infections can be induced in control fish after 9-11 d of contact with fish in the papulo-vesicular stage (Jensen and Larsen, 1982). Inoculation of fish with papule filtrate (0.45 μm) was found to induce ulcus syndrome in *ca* 35 % of cases if inoculated into a scarified lesion and in *ca* 10 % of cases when injected ip. This filtrate may have included "ultra-microvibrios" as described by MacDonell and Hood, (1982). Direct inoculation of up to 5 X 10^6 cells, *V.anguillarum* or maintenance of fish in aquarium water seeded with 10^5 cells/ml of *V.anguillarum* in an immunosuppressive solution of horse serum, or placing culture suspension onto scarified tail tissue failed to cause infections.

An iridovirus and a rhabdovirus were isolated from some papular lesions. While rhabdovirus did not produce ulcus syndrome, in 25 % of cases injection of iridovirus resulted in later disease (Jensen and Larsen, 1979, 1982), however experimental fish were not free from normal bacteria. The iridovirus differed from the one later reported from Australian fish (Langdon, Humphrey, Williams, Hyatt, and Wrestbury, 1986) principally by its insensitivity to ether.

2.6.2 *Red-Spot* Disease in Queensland.

Rodgers and Burke (1977-Unpublished) correlated the occurrence of RSD in the Noosa R. Queensland mainly in mullet, with prolonged and extensive rainfalls during late summer and autumn. Samples taken from affected estuarine fish with damage to only 1-3 scales were assigned on the basis of limited bacteriological tests to *V.anguillarum*. The data suggested that as these lesions developed *V.anguillarum* was displaced from lesions by *A.hydrophila* and other saprophytic species of bacteria and fungi.

Jaw lesions which occurred in predator "Long Toms," *Stenocaulis kreftii* and

Stylosaurus macleayanus, were thought to have resulted from predation upon infected food species. Bony bream, *Nematalosa come*, were described as reservoir hosts of *V.anguillarum.*

The occurrence of *Prototransversotrema* sp. was noted and the earlier mentioned possibility; that RSD might develop from feeding foci left by this species was suggested without proof. The healing process described by Rodgers and Burke (1977-Unpublished) differs from the description of healing cod (Larsen and Jensen, 1979) principally by melanization and encapsulization of lesions to leave a depressed dark scar. This may reflect different immuneresponses by different hosts. No examination was made in this work for viral involvement or of river catchment and water use practices.

2.6.3 Ulcerative Fish Disease in the Northern Territory.

Humphrey (1986-Unpublished) undertook a preliminary investigation of ulcerative fish disease emanating from the Mary River and in other rivers in the Northern Territory. Eleven mainly freshwater and marine fish species were found to have been affected, including the commercial species mullet, *Liza diadema*, and barramundi, *Lates calcarifer.*

Mullet exhibited ulcers as described earlier (See 2.6.2). Pathogenesis was more similar to that found by Larsen and Jensen (1979) than by Rodgers and Burke, (1977-Unpublished). Metacercarial cysts were observed in the musculature of *L.calcarifer* and punctate holes produced by an encysted trematode were present in some fish both in the presence and absence of inflammation.

The bacterial species most commonly associated with lesions was *A.hydrophila.* This species was also recovered from an *Argulus* like parasite. Other bacterial species taken from lesions and identified by an unspecified testing regimen included *Plesiomonas shigelloides, Bacillus* sp., *Moraxella* sp. *Coryneform* spp. *Achromobacter* sp. and *Pseudomonas* sp. and *Aeromonas sobria.*

Cytopathic effects to fish cell cultures were observed in 20 of 42 tissue samples from several fish species and a passageable rhabdovirus found.

Ulcerative lesions found in this survey were said to have resulted from secondary invasion by bacterial and fungal saprophytes of initial viral lesions. Similarities were noted between this disease and the occurrence of ulcerative disease in south east Asia. Humphrey did not evaluate the possible influence of land use (mining) in these river systems to disease. East, Cull, Murray and Duggan (1988) reported that in 1985 radionucleides from a burst containment pond were lost into the Mary R. system. It was inferred that a high proportion of these heavy metals were adsorbed to silt and clay particles and carried to the sea. These, and copper mined also at nearby Rum Jungle strongly suggest that disease initiation could also have been due to heavy metal stress and "primary viral" infection, was by an opportunistic pathogen, enhanced by this stress (See 2.2 and 2.3.3.1.4.).

2.6.4 Ulcerative Disease in Thailand.

Tonguthai (1985) prefaced his highly speculative report on several ulcerative fish diseases in inland river systems in the rapidly developing country of Thailand with a listing of instances of ulcerative disease supposedly originating from Bundaberg in 1972.

The isolated bacteria identified by unspecified methods were primarily *A.hydrophila.* Other species incidentally present were; *Flavobacterium* sp., *Pseudomonas fluorescens, Pseudomonas* sp., *Edwardsiella tarda, Vibrio parahaemolyticus* and *Streptococcus* sp.

The occurrence of Reovirus and Picorna virus was cited. Later Frerichs, Millar and Roberts (1986) reported the isolation of a rhabdovirus with an extensive freshwater fish host range. No comparison has yet been made between the activities of these viruses or the viruses isolated from cod ulcus syndrome (See 2.6.1) or the rhabdovirus from the Northern Territory reported by Humphrey (1986-Unpublished) towards different fish-cell lines.

2.6.5 Conclusion.

The predominant occurrence of *A.hydrophila* amongst freshwater species in Thailand was consistent with isolations from fresh water sites in Australia, and identification of *V.anguillarum* from Denmark and Queensland indicate the common involvement of *Vibrionaceae* in fish ulcerative processes. While the bacteriological procedures used have been sufficient to diagnose the isolates as *Vibrionaceae,* the methods used and absence of reference cultures and recent taxonomic revisions to the *Vibrionaceae* (See 2.7) make it possible that the bacteria were misidentified, e.g. *V.fluvialis* as *V.anguillarum* and *V.furnissii* as *A.hydrophila.* A more comprehensive bacteriological analysis needs to be undertaken.

In all studies where examinations were made for viruses, they were found. However, in some fish, unlike the commonly isolated phenotypes from the *Vibrionaceae,* viruses were not found, and different viruses occurred between sites. The involvement of viruses may therefore be inconsequential to the pathogenesis of most cases of ulcerative disease, and viral involvement without bacteria does not generate all of the symptoms.

2.7 Current Taxonomy of the *Vibrionaceae* Veron 1965.

2.7.1 Phylogenetic Position.

The phylogeny of bacteria has recently been clarified by interpreting 16S rRNA catalogues (Fox, Peckman and Woese, 1977) in the context of neutral evolutionary theory as described by Kimura (1983).

Woese, Weisburg, Hahn, Paster, Zablen, Lewis, Macke, Ludwig, and Stackebrandt, (1985) catalogued sequences from the species *Aeromonas hydrophila,* a *Photobacterium* sp. and *Vibrio (Photobacterium) fischeri* from the *Vibrionaceae,* selected on the basis of groupings obtained from immunological distance with the superoxide dismutase (SOD) system (Baumann, Bang and Baumann, 1980), and placed them in the γ-sub-division of the purple bacteria.

A more extensive survey of taxa from this sub-division, but at a lesser resolution was undertaken by MacDonell and Colwell (1985) who used entire sequences from 5S rRNA molecules of 29 *Vibrionaceae* in different comparisons by cluster analyses to test the then current classification. The new taxonomic contributions of this work included an affirmation of most existing *Vibrio* species as valid. *V.marinus* was found as distinct and unrelated, to other *Vibrionaceae* but not reassigned. *Photobacterium angustum* and *P.leiognathi* had identical sequences and so were proposed as synonymous. The authors also erected the genera *Shewanella* (See 2.7.2.3) and *Listonella* (See Nearhos and Fuerst, 1987) and suggested that *Aeromonas* spp. were distinct and unrelated enough from other taxa to be in a separate Family and that *Plesiomonas* was a genus which had greater affinities to certain *Enterobacteriaceae* than *Vibrionaceae.*

Nearhos and Fuerst (1987) challenged the precepts of these earlier phylogenetic arrangements of the *Vibrionaceae.* MacDonell and Colwell (1985) were scrutinized particularly for the level at which taxonomic distinctions were drawn in the context of intra-specific variation and the potential for homoplasy in 5S rRNA molecules of closely related species and for the assertion of their results in isolation from other less extensive molecular

comparisons of relatedness as were in the literature. The work of Woese *et al.,* (1985) was seen a potentially an unreliable indicator of phylogenetic relationships because two luminescent species of the *Vibrionaceae* were used and one of these has had unresolved controversy associated with its genetic position. Baumann *et al.* (1980), in the presence of conflicting data from different molecular classifications ultimately made an arbitrary selection and assignment of the species first described by Beijerink in 1889 as *Photobacterium fischeri* to *Vibrio fischeri.* Because of this controversy *V.fischeri* can not be regarded as a representative species of *Vibrio* indeed it would seem that the most typical and representative species of any genus must be the type species.

Vibrio fischeri, is more closely related to *Photobacterium* spp. than *Vibrio* spp. by DNA/rRNA hybridization, (Baumann and Baumann 1976), by immunological distance with the glutamine synthetase (GS) system, (Baumann, *et al.,* 1980); and by 5S rRNA sequence comparisons (MacDonell and Colwell, 1985). Consequently, on available data, the phylogenetic placement of the genus *Vibrio* relative to the other representatives of the γ-sub-division of purple bacteria (Woese *et al.,* 1985) is better inferred from 5S, than 16S rRNA sequences e.g. MacDonell and Colwell, (1985) and MacDonell, Schwartz, Ortiz-Conde, Last and Colwell (1986), Nearhos *et al.,* (1987), and Appendix (10.).

A further range of 5S rRNA sequences from the γ-sub-division was analyzed using *Clustan* (Release 2.1) by Nearhos and Fuerst, (1987). The phylogenetic structure produced by their single linkage regimen upon *Vibrio* sp. was consistent with that of Woese *et al.,* (1985) and Baumann and Schubert (1984), and in accord with MacDonell and Colwell, (1985) in placing *V.fischeri* with *Photobacterium* spp. The closer affiliation of *Plesiomonas shigelloides, Aeromonas hydrophila* and *A.media* with species from the *Enterobacteriaceae,* rather than those from the *Vibrionaceae* was also consistent with results of MacDonell and Colwell. But under all procedures *A.salmonicida* was placed amongst *Vibrio* spp. (Nearhos and Fuerst, 1987).

Consensus parsimony analysis, (Appendix 10.) was used to test these conflicting results, and aggregated all *Aeromonas* spp.

The current composition of the genus *Listonella* was not supported by consensus parsimony analysis (Appendix 10.) or by any of the distance matrix analyses (Nearhos and Fuerst, 1987). The validity of designating new taxa using a new technique in the absence of verification by the best available recognized procedure, was contested e.g. no sequence data was presented for *V.ordalii* was reported in Baumann and Schubert (1984) to have a DNA relatedness high affinity (75 %) with that of *V.anguillarum*. It was suggested that in the absence of this, or other supporting evidence the use of the combinations *Listonella anguillara, L.damsela* and *L.pelagica* should be held in abeyance. It was also suggested, (Appendix 10.), because of the single linkage analysis, DNA homology, and other molecular and morphological characters that *V.damsela* was more like species in *Photobacterium* than from the genera *Vibrio* and *Listonella*.

A lesser similarity was indicated when *V.alginolyticus, V.parahaemolyticus* and *V.harveyi* were compared by 5S rRNA (MacDonell and Colwell, 1985) than by DNA (Reichelt, Baumann and Baumann, 1976) homology. These differences indicate the susceptibility of the much shorter rRNA molecule to influences from other factors such as from species age related variation. Consequently for greater confidence, or at least a knowledge of molecular resolving power such comparative analyses based on rRNA sequences should be conducted using more than one strain within a species or a larger molecule.

No analysis of 5S rRNA supported the existing placement of *V.marinus* in the *Vibrionaceae*. Consequently the combination *V.marinus* is tentatively assigned as a *nomen dubium;* and in accord with MacDonell and Colwell (1985) the species is not considered further, except for recommending its phylogenetic appraisal by other techniques, e.g. 16S rRNA cataloguing.

The elevation of the genus *Aeromonas* to **Family *Aeromonadaceae* Colwell, MacDonell and De Ley, 1986;** is supported by parsimony analysis (Appendix 10.) subject to the caveats in 2.7.2.1.

2.7.2 Description of the *Vibrionaceae*.

The general 5S rRNA base sequence for the *Vibrionaceae* in accord with Baumann and Schubert (1984), pooled from data from MacDonell and Colwell (1985) and incorporating sequences of available *Vibrio* species validly published since 1984, *Aeromonas* spp., *Plesiomonas shigelloides*, *Listonella* spp. *Photobacterium* spp. and *V.marinus* but excluding *Shewanella* spp., is:

--UG**UGG***CCAUAG*--****UGG**CCAC-CUGA****CC***CCGAAGUCAG---

--*AGUGAAAC*******C-G*C--GAU--GGU-AGUGUGG-G***U-**CCCAUGAGAGUA

-----GG*-*A**GCCAGGCA*---

** Denotes Variable Locus.*

The general base sequence after revisions to the Family *Vibrionaceae* proposed by MacDonell and Colwell (1985), and Colwell *et al.*, (1986) i.e. sequences from *Shewanella* spp. *Listonella anguillara*, and *L.pelagica* are included, but those of *Aeromonas* spp., *P.shigelloides*, and *V.marinus* are excluded, is:

--UGC*U*GC*A**AUAG*--**U*U*G**CCAC-CUGA****CCAU*CCGAACUCAG-----

*AGUGAAAC***A***C-G*C--GAU--GGU-AGUGUGC-GGU*U-CCCCAUGUGAGAGUA----GGA-

CAU***CAGGCAU---

** Denotes Variable Locus.*

The *Vibrionaceae* Veron, 1965 as defined by Baumann and Schubert (1984) include bacteria with G + C ratios of DNA in the range of 38-63 mole %. Constituent genera are Gram-negative rods which possess polar and sometimes lateral flagella. The group are chemoorganotrophs and facultative anaerobes with respiratory or fermentative metabolism. Most are oxidase-positive and all use glucose as a sole or principle source of carbon. Species have variable sensitivities to the "vibriostatic" compound 0/129 (2,4-*di*-amino-6,7-*diiso*-propyl pteridine [phosphate]) at concentrations of 10 and 150 µg/ml. Some species have since been found to have a characteristic morphology and physiology associated with starvation conditions, which has been characterized as a Gram-negative resting state (See 2.4.2). Most

species require, or growth is enhanced by sodium ions. Species are primarily marine but freshwater and terrestrial representatives occur. The type genus is *Vibrio* Pacini 1854. Other validly published genera historically included in this family are *Aeromonas*, *Beneckea*, *Plesiomonas*, *Listonella*, *Photobacterium* and *Shewanella* (Colwell *et al.*, 1986).

The type species of *Vibrio* is *V.cholerae* Pacini 1854, taxonomic revisions and subdivisions of species from this genus which lead to its current definition (2.7.2.5) follow.

2.7.2.1 Genus *Aeromonas* Kluvyer and van Niel 1936, Popoff 1984.

The general 5S rRNA sequence from *Aeromonas hydrophila, A.salmonicida,* and *A.media* (MacDonell and Colwell, 1985) is:

--UGCCUGGCGACCAUAGC--GCCGUGGAACCAC-CUGA**--CCAUGCCGAACUCAG-----

AAGUGAAACGCGGUAGC-GCC--GAU--GGU-AGUGUGC-GAUUU--GCCAUG*GAGAGUA----GGA-

CACUGCCAGGCA*---

The genus *Aeromonas* differs from other genera in the *Vibrionaceae* by the absence of a sodium requirement, sheathed polar flagellum, motility in some strains, and of reports of a "spore" state. Species of this genus usually produce gas from glucose and are insensitive to the vibriostatic compound 0/129 phosphate at concentrations up to 150 μg/ml. The G + C ratio of DNA for known species ranges from 57-63 mole percent. Most species are aquatic. Colwell *et al.*, (1986) erected the Family *Aeromonadaceae* on the basis of cited complementary results from different techniques i.e. DNA-rRNA competition experiments, 16S rRNA catalogs, 5S rRNA sequences, and rRNA cistron similarities. However the attributes used to describe this family go beyond the scope of parameters of previously described species and are sufficiently general to encompass all species from the *Vibrionaceae* i.e. currently described *Aeromonas* species have G + C values only in the range 57-63 Mole % DNA, and yet the range of the proposed Family *Aeromonadaceae* is 40 to 63 Mole %. Other descriptive characters for the *Aeromonadaceae* are: Gram-negative straight or curved rods, motile by polar flagella, no

endospores or endocysts formed, chemo-organotrophs and facultative anaerobes capable of respiratory and fermentative metabolism. Oxygen is a universal electron acceptor, and nitrate is reduced, but not to gas. Most species are oxidase-positive and use d-glucose as a sole or principle source of carbon and energy and ammonium salts as sources of nitrogen.

It would seem more appropriate, on the available evidence, that the families *Vibrionaceae* and *Aeromonadaceae* have ranges of G + C values which include only currently recognized species. These ranges can be adjusted and families progressively redefined as new and exceptional species are described. On this basis *Aeromonadaceae,* would have G + C ratios in the range 57-63 mole percent, and the *Vibrionaceae* G + C ratios in the range 38-51 mole percent.

Between the compilation of Baumann and Schubert 1984 and October 1988 descriptions for two new *Aeromonas* species were validly published, ***Aeromonas media*** **Allen Austin and Colwell, 1983 and *Aeromonas veronii* Hickman-Brenner, MacDonald Steigerwalt Fanning Brenner and Farmer 1987.** Respectively these species had G + C contents in the range 62 ± 0.2 and 58-60 mole % DNA $T_{m.}$. Both produced gas from glucose and neither was susceptible to 150 μg/ml 0/129.

A.media cells are non-motile, produce arginine dihydrolase, a diffusible brown pigment, reduce nitrates, grow in 0-3% sodium chloride, and at 37°C. but not 42°C., and do not produce hydrogen sulphide or phosphatase. Strains from this species were variable in their ablity to utilize many carbon sources but none was able to assimilate cellobiose, fructose, formate, sucrose or trehalose. All of these substrates except cellobiose could be used by the other non-motile species *A.salmonicida.*

A.veronii is a medically significant motile species with a decarboxylase pattern distinct from previously described species but common to many *Vibrio* spp. i.e. arginine - lysine + & ornithine +. Hickman-Brenner *et al.,* (1987) reported the species was similar by DNA homology to *Aeromonas* isolates which

were arginine and lysine decarboxylating. *A.veronii* may be otherwise distinguished from other *Aeromonas* species by tartarate hydrolysis by most strains. Larsen and Jensen (1977) reported an *Aeromonas* species from ulcerated fish associated with this decarboxylation pattern but the DNA base ratios (50.9 mole percent DNA) and the absence of gas production would suggest that these represented early isolations of *V.fluvialis.*

The taxon from the *Vibrionaceae* ***Allomonas enterica*** **Kalina, Antonov, Turova and Grafova, 1984** isolated enterically and environmentally, resembles *Aeromonas* spp. by its similar DNA base ratio (57.5) and by 16 % DNA homology with *A.hydrophila.* However the authors placed this new sodium-requiring, ornithine decarboxylating, anaerogenic species into a new genus.

Here, for consistency with the genus *Vibrio* where similarly low DNA relatedness has not in the past (Reichelt *et al.,* 1976) been used to fragment the genus, and pending further work e.g. by 5S rRNA sequencing to indicate discrete placement of this species, the more conservative but necessarily generically relaxed combination *Aeromonas enterica* will be used. Genus *Aeromonas* is so relaxed to include species which may or may not have a sodium requirement and which may or may not produce gas upon glucose fermentation.

2.7.2.2 Genus *Photobacterium* Beijerinck 1889.

Baumann and Baumann (1984) defined the genus *Photobacterium* according to the following criteria: Most species are motile by 1 to 3 unsheathed polar flagella, and do not utilize β-hydroxybutyrate. Cells accumulate poly-β-hydroxybutyrate in starvation conditions and all species require sodium for growth. Some species are luminescent although this feature is easily lost. The G + C ratio of DNA for this genus was listed as 40-44 mole percent.

Upon incorporation of the species *V.fischeri*, *V.logei* and *V.damsela* into the genus *Photobacterium* (2.7.1), the generic amendations required are only that a species may bear 3-12 polar flagella and that flagella may be sheathed or unsheathed. The species *Photobacterium damsela comb. nov.* has been reported

to produce poly-β-hydroxybutyrate (Grimes *et al.,* 1984), has an unsheathed polar flagellum (Appendix 10.), but has not been reported to utilize β-hydroxybutyrate. The range of G + C values for these species is 39-44. The general 5S rRNA sequence for this genus is:

--UGC*UGGCGACCAUAGC--*UU*UGGCCCCAC-CUGA****CCAUGCCGAACUCAG-----

*AGUGAAACG*A**AGC-GCC--GAU--GGU-AGUGUGC-GGU*U-CCCCAUGUGAGAGUA----GGA-

CAUCGCCAGGCAU---

The validly published fish pathogenic species *Vibrio salmonicida* Egidius, Wiik, Anderson, Hoff and Hjeltnes 1986, is not considered to have been adequately resolved from *Photobacterium fischeri* or *P.logei* as described by Bang, Baumann and Nealson (1978). No comparison was undertaken phenotypically or genotypically between these species which could endorse the erection of *V.salmonicida*. Instead, Wiik and Egidius (1986) performed DNA hybridization on other fish pathogenic species *(V.anguillarum, V.ordalii,* and *V.parahaemolyticus)* already demonstrated as phenotypically distinct. Specific similarities to *Photobacterium logei* and *P.fischeri* not addressed by this study were, the presence of polar tufts of sheathed flagella, and a G + C base ratio of DNA of 41-42 mole percent $T_{m.}$, an inability to hydrolyze chitin, and gelatin, and the ability to separately utilize glucose, maltose and ribose but not citrate, arabinose, cellobiose or sucrose.

2.7.2.3 Genus *Shewanella* MacDonell and Colwell 1985.

The genus *Shewanella* MacDonell and Colwell 1985, was described with *S.putrefaciens, (Pseudomonas putrefaciens,* Derby and Hammer, 1931), *(Alteromonas putrefaciens,* Lee, Gibson, and Shewan, 1977) as the type species. Species in this genus are described as oxidative and fermentative, but whether some *Shewanella* spp. are really oxidative or only weakly fermentative requires more precise evaluation. All species are Gram-negative, non-pigmented, and motile by polar flagella. Growth of *S.benthica,* is enhanced by 400-600 atmosphere pressure. The G + C ratio of DNA from

species in this genus is 44-47 mole percent. The general 5S rRNA sequence for *Shewanella* (MacDonell and Colwell, 1985) is:

--UGU*U*G*GA**AUAGC--**UGUGC**CCAC-CUGAUC--CCAU*CCGAACUCAG-----
*AGUGAAACGCAGUUGC-GCC--GAU--GGU-AGUGUGG-G***U-*CCCAUGUGAGAGUA----GG*-
CAU*G**A**CAU---

2.7.2.4 Genus *Plesiomonas* Habs and Schubert 1962.

Schubert (1984), described *Plesiomonas* as differing from other genera of the *Vibrionaceae* by possession of 2-5 lophotrichous flagella; the fermentation of carbohydrates without the production of gas, the production of lysine, ornithine and arginine decarboxylases, inability to produce lipases and proteases and a general susceptibility to 0/129. The only described species, *P.shigelloides,* has a G + C content of 51 mole percent DNA. The 5S rRNA sequence (MacDonell and Colwell, 1985) for this species is:

--UGCCUGGCGGCCAUACU--GCGGUGGUCCCAC-CUGACC--CCAUGCCCAACUCAC-----
AAGUGAAACGCUGUAGC-GCC--GAU--GGU-AGUGUGG-GGUCU-CCCCAUGUGAGAGUA----GGG-
AACUGCCAGCCAU---

2.7.2.5 Genus *Vibrio* Pacini 1854, including *(Beneckea*, Campbell 1957), and *(Listonella*, MacDonell and Colwell 1985).

The general 5S rRNA sequence (MacDonell and Colwell, 1985) for the genus *Vibrio* excluding *Photobacterium fischeri, P.logei* and *P.damsela* (Appendix 10.) and *V.marinus* (2.7.1) pooled from MacDonell and Colwell, (1985) is:

--UGC*UGGCGACCAUAG*--G*U*UGG*CCCAC-CUGA****CCAU*CCGAACUCAG-----
*AGUGAAAC**A**AGC-G*C--GAU--GGU-AGUGUGC-GG**U-CCCCAUGUGAGAGUA----GGA-
CAUCGCCAGGCA*---

The genus *Beneckea* (Campbell 1957, Baumann, Baumann and Mandel, 1971) was redescribed to accommodate species of *Vibrio*-like bacteria with sodium requirements greater than 20 mM. On the basis of evidence from immunological distance studies with SOD and GS, and from rRNA homologies, Baumann, Baumann, Bang, and Woolkalis (1980) abolished *Beneckea* and assigned all

constituent species to *Vibrio*. The following is a revision of the differential characters of the genus *Vibrio* as described by Baumann, Furniss, and Lee (1984) to incorporate refinements based principally upon 5S rRNA sequence analyses.

Vibrio cells are generally single straight or curved rods. When grown in liquid media they are motile by sheathed single polar flagella. Mixed flagellation as defined by De Boer *et al.*, (1975) is observed in some species, but the wavelength of the unsheathed peritrichous flagella is shorter than for sheathed polar flagella. Growth is improved on media containing 5-700 mM sodium (*ca* 0.05 - 6 % w/v as sodium chloride). Most species are oxidase-positive, and have fermentative rather than respiratory metabolism, and all are able to utilize d-glucose. Species are primarily aquatic but occur in a diversity of niches, some are free-living, opportunistic (flexible) animal pathogens, or form symbioses. Nitrogen fixation and luminescence are characters common to many species, (See 2.4.4.6).

Most species are sensitive to the vibriostat 0/129 (phosphate) at concentrations less than 150 μg/ml, (as phosphate); however, Muroga, Yoneyama and Jo (1979) showed that cross-resistance to 0/129 was conferred to strains that had previously been exposed to combinations of folic acid homonyms such as trimethoprim and sulphonamide. Resting or survival stages "ultra-microvibrios" have been reported for some species (See 2.4.2).

Working with over 60 strains of bacteria classified as *Beneckea* spp. Okada, Miyamoto and Yoneda (1977) described a genus-specific antigen, C2, from polar flagella. This confirmed earlier work by Shinoda, Kariyama, Ogawa, Takeda and Miwatani (1976) who had earlier shown that antigen from polar flagella of *V.parahaemolyticus* was common to all, i.e. many current, *Vibrio (Beneckea)* species tested except *V.campbellii (B.campbellii, B.neptuna).*

2.7.2.5.1 Genus *Vibrio* since Baumann and Schubert (1984).

Since the rationalization of *Vibrio* species in the "Approved Lists" (Skerman, McGowan, and Sneath, 1980), the number of validly published, new, amended and emended species has almost doubled from 20 to 35 by 1988. The probable taxonomic status of species validly and or effectively published as *Vibrio* species and not dealt with in Baumann and Schubert (1984), up to October 1988 are reviewed below.

2.7.2.5.2 Dubious Newly Described *Vibrio* Species.

The fish and human pathogenic species ***V.damsela* Love, Teebkin-Fisher, Hose, Farmer, Hickman and Fanning, 1981** was characterized as an arginine dihydrolase and gas producing species, with the mole % G + C of DNA of 43 $T_{m.}$ and may be better classified as *Photobacterium damsela* (Appendix 10.).

***Vibrio salmonicida* Egidius, Wiik, Anderson, Hoff and Hjeltnes, 1986** is not adequately resolved from the existing species of *Photobacterium* (See 2.7.2). The specific epithet used shows little foresight potentially allowing confusion in the *Vibrionaceae* with *Aeromonas salmonicida.* Analysis by Nearhos and Fuerst (1987) using 5S rRNA cataloging, implies the impossible situation where two species with the same specific epithet, could be reclassified into the same genus. The mole % G + C of DNA is 41-42 $T_{m.}$.

2.7.2.5.3 New Arginine Dihydrolase Positive *Vibrio* Species.

Most species of *Vibrio* described since 1980 have been arginine dihydrolase positive. Previously, these species would have been identified as either *V.anguillarum* or *A.hydrophila*.

The nitrogen fixing strains grouped as ***V.diazotrophicus* Guerinot, West, Lee and Colwell 1982**, most closely resembled *V.anguillarum*, differing primarily by lacking proteolytic enzymes and by utilization of xylose and salicin. No other species tested for DNA hybridization was closely related. The most similar 5S rRNA sequences (MacDonell and Colwell, 1985; Nearhos and Fuerst, 1987) are from other species producing lateral flagella, *V.carchariae* and *V.proteolyticus*. The mole % G + C of DNA is 45-47 T_{m} , B_{d} .

Given the widespread occurrence in bacteria of genes for nitrogen fixation the presence of this character alone is not sufficient reason for the description of *V.diazotrophicus* as a species separate from *V.anguillarum*. Later West *et al.*, (1985) and Urdaci, Stal and Marchand (1988) found that nitrogen fixation is a common feature of *Vibrio* spp.

V.aestuarianus **Tison and Seidler 1983,** differed in its published description from *V.anguillarum* only by the production of acid from lactose, however the species has sufficiently low homologies by DNA hybridization to warrant its elevation as a separate species. The mole % G + C of DNA is 43-44 $T_{m.}$.

V.orientalis **Yang, Yeh, Cao, Baumann, Baumann and Tang 1983** is a luminous species which is quite distinct from the other arginine dihydrolase positive species of *Vibrio*. This species phenotypically resembles *V.splendidus* Biovar I. It differs from the photobacteria by the presence of a single sheathed polar flagellum, and from other luminous species of *Vibrio* by its ability to accumulate poly-β-hydroxybutyrate. No hybridization studies were conducted but inter-species distances were assayed immunologically using the SOD rather than the empirically endorsed GS system (See 2.7.1). The mole % G + C of DNA is *ca* 45.5 $B_{d.}$.

V.tubiashii **Hada, West, Lee, Stemmler and Colwell 1984,** was also drawn from *V.anguillarum*. The species was defined by its genotypic distance, (< 30 % DNA homology), and biochemically distinct profile. Most notably the species utilized xanthine, and tyrosine and failed to produce acetoin. The mole % G + C of DNA is 43-45 $T_{m.}$.

V.furnissii **Brenner, Hickman-Brenner, Lee, Steigerwalt, Fanning, Hollis, Farmer, Weaver, Joseph and Seidler 1983** was originally described as an aerogenic biogroup of ***V.fluvialis*** **Lee, Donovan, and Furniss 1978.** Most strains of *V.furnissii* further differed from strains of *V.fluvialis* by the utilization of rhamnose and glycerol. By DNA hybridization *V.furnissii* was most closely related to *V.fluvialis*, with less than 25 % relatedness to other species of *Vibrio*. The mole % G + C of DNA is 50.8 $B_{d.}$.

2.7.2.5.4 New Decarboxylase Negative *Vibrio* Species.

The group which has come to be described as *V.ordalii* Schiewe, Trust and Crosa, 1981 was first resolved as *V.anguillarum* Biogroup II by Hastein and Smith (1977). In a comparison of phenotypic and genotypic aspects Biogroup II appears to be phenotypically distinct and sufficiently genotypically different from *V.anguillarum* Biogroup I (75 %) (Baumann and Schubert, 1984) to be classed as a separate species. The mole % G + C of DNA is 43-44 $T_{m.}$.

V.hollisae Hickman, Farmer, Hollis, Fanning, Steigerwalt, Weaver and Brenner, 1982 is a decarboxylase negative species most notably failing to ferment maltose, with a DNA base ratio of 49-51 $T_{m.}$ mole % and low DNA relatedness to 21 other species of *Vibrio* and *Photobacterium*.

2.7.2.5.5 New Lysine and/or Ornithine Decarboxylating *Vibrio* Species.

A fish pathogenic *Vibrio* from Japanese eels, *A.japonica, (Vibrio anguillicida)* (Nishibuchi, Muroga, Seidler and Fryer, 1979) was found by DNA hybridization to be distantly related to other species of *Vibrionaceae,* (12 to 23% similarity to *V.anguillarum* and 2 to 17% similarity with *P.fischeri).* This species was later re-described as *V.vulnificus* Biogroup II Tison, Nishibuchi, Greenwood and Seidler, 1982 because of the high DNA relatedness, (> 90%), to *V.vulnificus* Reichelt, Baumann and Baumann, 1976, and differed phenotypically from this species only by its failure to produce indole. The mole % G + C of DNA is 46-48 $T_{m.}$.

V.mimicus Davis, Fanning, Madden, Steigerwalt, Bradford, Smith and Brenner 1981 was separated from *V.cholerae* as a distinct DNA homology group (60-70 % relatedness with *V.cholerae).* Phenotypically it was characterized principally by its failure to produce acid from sucrose but Desmarchelier and Reichelt (1982) found that sucrose negative strains of *Vibrio cholerae,* verified by DNA hybridization, also existed. This supported the earlier contention of Davis *et al.,* (1981) that these species were best differentiated by genotypic rather than phenotypic methods. The mole % G +

C of DNA has not been reported but is assumed equivalent to *V.cholerae* i.e. 47-49 $T_{m.}$.

***V.carchariae* Grimes, Stemmler, Hada, May, Maneval, Hetrick, Jones, Stoskopf and Colwell 1984** was described from a single strain. This strain exhibited its highest DNA homology with *V.parahaemolyticus* (*ca* 40 %) from which it differed phenotypically by its fermentation of sucrose. The mole % G + C of DNA is 46 $T_{m.}$.

***V.cincinnatiensis* Brayton, Bode, Colwell, MacDonell, Hall, Grimes, West and Bryant 1986** was classified as a new species from a single isolate. In the absence of corresponding supporting data by other currently accepted molecular methods this was the first instance in which 5S rRNA was used as a differential criterion for such description. The species was found by the unweighted pair group method average (UPGMA) sorting protocol (Sokal and Michener 1958), to be most similar to *V.nereis* and *V.diazotrophicus*. However using parsimony analysis it was found that the closest association was with the oxidase-negative species *V.gazogenes*. The strain was found to be phenotypically distinct from all other species of *Vibrio*. The mole % G + C of DNA is 45 $T_{m.}$.

***V.mediterraniensis (V.mediterranei)* Pujalte and Garay 1986** was distinct from other lysine decarboxylase producing species; *V.parahaemolyticus, V.vulnificus, V.splendidus, V.nereis* and *V.natriegens*; as determined by DNA homology (<20 %) and by its failure to use d-gluconate. The mole % G + C of DNA is 42-43 $T_{m.}$.

2.7.3 Conclusion.

Eleven of fifteen species of *Vibrionaceae* validly described since Baumann and Schubert, 1984 seemed possibly individually valid, but closer comparative examination with other species from the *Vibrionaceae* is required. Some of these species have been described upon the basis of single, possibly atypical strains. The detailed comparative framework for diagnosis of species of the *Vibrionaceae*, composed and introduced by Baumann and Schubert, (1984) has been largely overlooked in recent years, and consequently further taxonomic revision is required.

2.8 Rapid Diagnosis of *Vibrionaceae.*

2.8.1 Diagnosis with Existing Systems.

Proprietary systems have been marketed which claim to identify species of the *Vibrionaceae.* The sodium and temperature requirements of *Vibrios* and lack of suitable testing regimens, are the main problems encountered with these systems. A comparative review of the *API 20E, API Rapid E,* and *API Rapid NFT* schemes was undertaken by Overman, Kessler and Seabolt (1985) using common medically encountered *Vibrio* spp. suspended in 0.85 % saline. The *API 20E* was found to give the best results, perhaps endorsing its choice as a base for another scheme by Dawson and Sneath (1985). Overman *et al.,* did not endorse the relevance of the system beyond the identification of other than commonly occurring (i.e. human clinical) species of *Vibrionaceae.* Kent (1982) found the *API 20E* alone failed to identify fish pathogenic *Vibrio* species and it was necessary to introduce supplementary tests to prevent API profile confusion between *Vibrio* and *Pasteurella* spp. Dawson and Sneath (1985) supplemented the *API 20E* scheme with further tests and a profile reference library of known *Vibrio* spp. to increase resolution, and used a probabilistic approach for identification of unknown *Vibrio* isolates. Their scheme suffers from the small library of reference OTU's, (172), and from the unbalanced choice of additional features assayed. These imposed biases by duplication e.g. tests for sucrose fermentation and yellow colonies on TCBS, growth in the absence of salt and on CLED, and by the proportionally high dependence *(ca* 15 %) upon cellular morphology.

2.8.1.1 Non-Proprietary Diagnostic Systems.

Lee and Donovan (1985) developed a two stage diagnostic scheme prefaced by screening for oxidase reaction, nitrate reduction, arginine (dihydrolase), decarboxylation of arginine, lysine and ornithine, sensitivity to 150 μg 0/129, and growth in 0, 3, and 6 % sodium chloride. Strains were assigned to eight groups and further characterized. This, like all keyed identification schemes, fails to acknowledge possible atypical, or incorrectly tested

strains, and diagnoses are slower than single battery arrays. West *et al.,* (1985) in a numerical taxonomic survey demonstrated affiliations of decarboxylase negative strains with decarboxylating type cultures i.e. these common strains would have been misidentified by the scheme of Lee *et al.,* (1985). It was consequently recommended that a polythetic approach is best for species diagnosis.

Based upon the diagnostic scheme of West, Lee and Bryant (1983) and West and Colwell (1984), Brayton *et al.* (1986) updated diagnostic characters for the identification of 21 *Vibrio* species on the basis of attitudes towards 39 characters. Comparative data does not exist for all described species from the *Vibrionaceae* and the diagnostic characters used will not resolve the recent species *V.aestuarianus* and *V.tubiashii* from *V.anguillarum.*

Bryant, Lee, West and Colwell (1986a,b) described a computer program which used a data matrix from a rendering of *ca* 1000 OTU's of *Vibrionaceae* over 81 characters into 38 taxa. For some species i.e. *V.cholerae, V.anguillarum, A.hydrophila, A.sobria V.parahaemolyticus, V.alginolyticus, V.campbellii,* and *V.vulnificus,* where greater than 30 OTU's were examined the frequencies of phenon attributes would reflect natural population frequencies i.e. follow a normal distribution rather than a *t*- distribution. Large analyses such as this also provide information as to the relative abundance of (common or rare) species and should also be a consideration in probabilistic identification.

2.8.1.2 Expert Systems.

Willcox, Lapage and Holmes (1980) described probabilistic identification based upon the implementation of Bayes' scheme of prior probabilities. Naylor (1987) described a computer program which has been compiled with and without acknowledgment of common and rare species from the data of Bryant *et al.,* (1986a,b) as follow: Data strings from phena first list the prior probability of occurrence of species and then list alternately two values for each character, the first value was the probability of a positive

reaction i.e. as published by Bryant *et al.* the second listed the probability of a negative reaction in the remaining phena. Four program shells (Disk_1) labeled as PRIOR, PRIOR87, COMP, and COMP87 were prepared. The programs labeled with a "COMP" prefix have had prior probabilities of species equalized i.e. they are equivalent to the program of Bryant *et al.* The "Prior" prefix makes distinction in identification between rare and common species. Programs with an "87" suffix have been compiled for faster operation with a maths co-processor chip.

The program has facility for graded individual responses and missing data, depending on confidence with a character state i.e. when operated the program produces a series of questions to which the operator may respond in the ranges -5 to -1 for negative reactions, 0 for missing data , and 1 to 5 for positive reactions. Species are listed as they are eliminated and ultimately a diagnosis is made.

2.9 Taxonomic Concepts and Methods.

2.9.1 The Validity of Phenetic Taxonomy For Diagnosis of *Vibrionaceae.*

Taxonomy has been divided into cladistic and phenetic schools. The preoccupation of the cladists is with the sequence of derivation of characters while pheneticists are concerned with the presence or absence of like characters. Janvier (1984) comparatively reviewed cladistic and phenetic taxonomies, his major concern being the implied monophyletic structure of a phenogram constructed from data "known" to be homoplastic. Sokal (1983) supported phenetic over cladistic taxonomy citing, like Sneath (1957) before him, stability as the major strength of phenetic over cladistic taxonomy. This stability was evident by addition of further characters and OTU's and consistency through changes of coding and/or similarity measures. None of the cladistic methods were considered sufficiently consistent. Cornish-Bowden (1983) showed that parsimony (cladistic) procedures do not make use of all available information and reinforced his argument by performing phenetic analysis using all information not used by parsimony procedures to produce an analysis similar to the one generated by phenetic methods with all available data. Cornish-Bowden concluded that both phenetic and cladistic methods ignored some of the data and that neither method was inherently better than the other.

Zuckerkandl and Pauling (1965) proposed the use of molecules as records of evolutionary history, particularly the "fossil" history in the degenerate code of DNA. Their work showed the validity of phenetic classifications by stratification of these informational molecules or semantides. The ultimate basis for cladistic analysis is in the molecules which code information for genes or their transcripts (DNA or mRNA), while phenetic analysis may be based on information derived from any of the higher strata.

Within the *Vibrionaceae,* phenetic classification has been evaluated from macromolecules (tertiary semantides) using micro-complement fixation

techniques (Baumann, Baumann, Woolkalis, and Bang 1983) to compare the enzyme systems, SOD, alkaline phosphatase (AP), and GS, Baumann, Baumann, Bang, and Woolkalis, 1980). Analyses of secondary semantides, (MacDonell and Colwell, 1985; Nearhos and Fuerst, 1987) using 5S rRNA data have demonstrated the superior ability of GS over SOD in indicating relatedness in the *Vibrionaceae.* The observations of these comparative analyses empirically justify the application of phenetic taxonomy as a primary tool for the segregation of species.

2.9.2 Bacterial Populations and the Species Concept.

The perception of species as populations derived by Sneath and Sokal (1973) from evaluation of the definitions of Mayr, Emerson, and Grant is of *a population which is a reproductively cross-fertilizing group of individuals, and which locates its variants around a median.* Sneath and Sokal further indicated that any such concept becomes non-operational if there is a lack of evidence concerning the extent of interchange of genetic material. Stanier, Adelberg and Ingraham (1977) made the observation that because prokaryotes are principally haploid, and reproduce asexually, the notion that bacterial species interbreed within a population like plants and animals is invalid. The best working definition of a bacterial species proposed by Stanier *et al.,* was: *"a group of strains that show a high degree of overall phenetic similarity and that differ from related strains groups with respect to many independent characters."*

This concept unofficially encourages authors to provide supplementary molecular information supporting the definition of new species from related bacteria e.g. Tison and Seidler (1983) provided a range of G + C base ratio values and demonstrated low DNA homology with other *Vibrio* species to support their establishment of *Vibrio aestuarianus.*

A further complication with the classification of bacteria into species stems from the potential for unrelated species to exchange genetic material i.e. for horizontal evolution. This possibility was evaluated by Baumann *et*

al., (1983) and regarded as a component of background noise which became insignificant when sufficient tests are used to demonstrate discontinuities of phenotype and genotype. From this, the empirical definition of a species in bacteriology has become: *a population comprising a cohesive, but not necessarily homogeneous group of phenetically and genotypically distinct strains.*

This appreciation of species as populations is fundamental to the valid application of multivariate statistical procedures formulated for the analysis of overlapping normally distributed populations.

2.9.3.0 Numerical Taxonomy.

2.9.3.1 General Concepts of Numerical Taxonomy.

The basic concept of numerical or Adansonian taxonomy is that taxonomic similarities between operational taxonomic units (OTU's) can be objectively determined through the comparison of large repertoires of equally weighted characters (Colwell and Austin, 1981).

The assignment of bacteria to categories was initially overwhelmed by the functionally heterogeneous but morphologically homogeneous nature of bacteria. Observations of secondary attributes e.g. presence of differing hydrolytic enzymes, produced much information for which the relative classificatory significance was unknown. While classical taxonomists were fundamentally correct (see 2.9.3.4) in their weighting of stable characters, e.g. Gram-stain reactions, the ability of one worker to evaluate the findings of another was, before the application of numerical methods, only possible within the same subjective framework. Sneath (1957) was the first to seriously apply numerical taxonomic technique to bacteria. The underlying principal provides an objective approach to classification, allowing the empirical selection of significant characters for population differentiation. Because such classifications are assemblages of many individually trivial characters (Sneath 1972), the skewing effects of error are minimal.

The decline of computing costs and the need to sort and interpret more information has allowed practical application of this concept and the evolution of more stringent statistical procedures now even accessible to desktop computers.

2.9.3.2 Weighting and Error in Numerical Taxonomy.

In practice, numerical taxonomic methods are applied to only small sub-sets from each OTU's total phenetic attribute profile. A typical analysis may include data from observations of morphology, assimilation ability and stress physiology and as such is as likely to produce an analysis which reflects ecological, as it is phylogenetic grouping. Lee, Shread, Furniss and Bryant (1981) studied environmental *Vibrionaceae* by a well structured testing regimen and produced a dendrogram which successfully resolved two new species of *Vibrio* but which grouped these more closely to *Aeromonas* than other *Vibrio* species indicating that phenetic classification can only approximate natural or phylogenetic relationships.

To force classifications into a more "natural" configuration *a priori* character weighting has been adopted e.g. by MacDonell, Ortiz-Conde, Last and Colwell (1986) for determination of frequencies of variation of all loci from 5S rRNA sequences and to downgrade the contribution of hypervariable regions in parsimony analysis (See 2.9.3.4).

A priori weighting is imposed simply by the selection of different similarity (or distance) coefficients (See 2.9.3.3). Sneath (1972) did not acknowledge this precept and objected to *a priori* character weighting because of difficulties in rationalization, but supported *a posteriori* weighting, i.e. to develop diagnostic classifications. Janvier (1984) listed five reasons for the assignment of weight to characters:

(i) When the character is conspicuous.
(ii) When the character was found by an "authority".
(iii) When the character was found by a specialist for that character.
(iv) When the character is widespread or restricted amongst taxa.
(v) When the character is adaptive or not adaptive i.e. vestigial.

Sneath (1970) justified the arbitrary selection of determinative criteria pragmatically; if the selection was good a stable and predictive classification was produced. To minimize the problems associated with improperly weighted features Colwell and Austin (1981) recommended that character profiles for 100 to 200 tests should be composed from a broad spectrum of phenetic facets, and include cellular and colony morphology, biochemical, nutritional and physiological attributes.

2.9.3.3 Similarity Coefficients.

By comparison of every OTU with itself and with all other OTU's, similarity and distance coefficients, compress multi-dimensional data into forms more reconcilable to analysis. There are many ways to assemble these coefficients consequently they have been designated into the categories of metrics and ultrametrics depending on the extent of their linearity (Williams and Dale, 1965).

Ultrametrics for OTU's (X), (Y) and (Z) with Similarity, (S), have the properties:

$$S(X,X) = 1 \quad \text{for all X}$$
$$S(X,Y) \geq 0 \quad \text{for all X, Y}$$
$$S(X,Y) = S(Y,X) \quad \text{for all X, Y}$$
$$S(X,Y) \geq \max(S(X,Z), S(Y,Z)) \quad \text{for all X, Y and Z.}$$

(Adapted from Milligan, and Isaac 1980).

Formulae for calculating similarity (or distance) coefficients are based upon the attributes sums for each of a series of 2 X 2 contingency tables comparing OTU's (X) and (Y) where:

a = Sum of attributes present in X and Y.
b = Sum of attributes present in X and absent in Y.
c = Sum of attributes absent in X and present in Y.
d = Sum of attributes absent in X and Y.

The methods used most commonly in microbiology have been Euclidean Similarity $S_{e.}$, (Simple Matching Coefficient) of Sneath (1957), and the Jaccard Coefficient $S_{j.}$ (Jaccard 1901). These are both ultrametrics and are expressed by the following formulae:

$$S_{e.}\ \% = \frac{(a + d).100}{(a + b + c + d)}$$

$$S_{j.}\ \% = \frac{(a).100}{(a + b + c)}$$

It can be seen that where the simple matching coefficient uses all of the data, the Jaccard coefficient selectively excludes negative matches and so imposes an *a priori* weight (See 2.9.3.2) to other attributes rather than matching absences. Sneath (1957) justified use of the Jaccard coefficient on the basis that in the absence of positive matches it was not possible to make logical selection of important data i.e. OTU's might be similar or they might be non-viable; and information about matching negative characters was present by inference. This is correct but nonetheless an *a priori* bias is imposed.

Sneath (1968) reported the partitioning of contingency tables into vigour and pattern components where vigour is an expression of comparative versatility and pattern a measure of the number of different attributes shared between OTU's. Non-metric coefficients based upon pattern similarities were suggested as being potentially useful for the differentiation of distantly related OTU's. Distance coefficients which have been adopted for modern clustering programs can be obtained by subtracting the percent similarity from 100.

2.9.3.4 Cluster Methods and Protocols.

Results of numerical analyses are obtained when analyses are compressed into similarity matrices and these are patterned into hierarchical structures by agglomerative sorting (clustering) procedures. MacDonell and Colwell (1985) observed that polythetic i.e. numerical taxonomy had produced a classification of *Vibrionaceae* which when tested by molecular methods proved essentially valid.

The clustering procedures which have been used in bacteriology are unweighted pair group method-complete (UPGMC), Sorrensen (1948); unweighted pair group method-single (UPGMS), Florek, Lukaszewicz, Perkal, Steinhaus and Zubrzycki (1951); and average linkage protocols (UPGMA), Sokal and Michener (1958); Error Sum of Squares (ESS) (Ward, 1963) and unweighted pair group method-median (UPGMM) Median (Gower, 1967). These algorithms are shown below for the similarity at fusion of clusters P and Q with any cluster R, all clusters containing N elements i.e. S(R,P+Q).

(i) Complete Linkage: (UPGMC)

$$S(R,P+Q)= 0.5S(R,P) + 0.5.S(R,Q) - .5\left|S(R,P)-S(R,Q)\right|$$

(ii) SingleLinkage: (UPGMS)

$$S(R,P+Q)=0.5S(R,P)+ 0.5S(R,Q) + .5\left|S(R,P)-S(R,Q)\right|$$

(iii) AverageLinkage: (UPGMA)

$$S(R,P+Q)=\frac{NP.S(R,P)+ NQ.S(R,Q)}{(NP + NQ)}$$

(iv) Error Sum of Squares (Ward's method): (ESS)

$$S(R,P+Q)=\frac{(NR+NP).S(R,P)+ (NR+NQ).S(R,Q)- NR.S(P,Q)}{(NR + NP + NQ)}$$

(v) MedianLinkage: (UPGMM)

$$S(R,P+Q)= 0.5.S(R,P)+ 0.5.S(R,Q)- 0.25.S(P,Q)$$

(Modified from Wishart, 1982).

The procedures were comparatively reviewed by Jardine and Sibson (1968) according to their conformity with following requirements:

(i) A unique hierarchic transformation result should be obtained from given data.
(ii) Small changes in data should not drastically alter dendrogram structure i.e. hierarchic transformations should be continuous.
(iii) If the similarity coefficient is already ultrametric (See 2.9.3.3) it should not be altered by hierarchic transformation.
(iv) The hierarchic transformation obtained should represent a minimum distortion subject to the requirements of all other criteria.
(v) The hierarchic transformation is independent of scale.
(vi) The hierarchic transformation should be independent of input permutations of the data set.
(vii) Excised clusters from hierarchic transformations should maintain the same relativity when analyzed separately.

The complete linkage method was discarded for being "ill defined," and the group average linkage method for its discontinuity. Only single linkage was able to satisfy all criteria. The chaining artifact of single link clustering was cited by Jardine and Sibson (1968) as a defect of all hierarchic classification schemes and it was suggested that multiple linkage (overlapping) cluster protocols were an appropriate alternative.

These protocol selection criteria have been seen as too stringent by Williams, Lance, Dale and Clifford (1971) and by Gower (1975). Everitt (1979) endorsed the pragmatic approach expressed initially by Williams *et al.,* (1971) and suggested from comparative examination between protocols, that the mathematically attractive single linkage method was least satisfactory while UPGMA and ESS methods generally derived groups consistent with prior classification. Because of the mathematical attraction and computational simplicity of the single link method modifications have been made which minimize the chaining effect. Wishart (1969) derived an algorithm which operates by restarting analysis when clusters reach a predetermined size, i.e. potentially when chaining occurs, but rather than linking new OTU's to the last cluster entrant, a modal type (=centrotype) is found from each cluster and these are used as attachment sites for remaining OTU's. Also a feature of Wishart's (1969) algorithm on the *Clustan* (Wishart 1982, 1986, 1987) and *SAS* (SAS Institute, 1984) programs are facilities which allow the comparison of new OTU's with a specified number, "k", nearest neighbours (k-linkage lists). These density dependent comparisons act to damp intrinsic noise and so reduce fragmentation of phena from variable or error prone data. By imposition of "trimming" to chaining clusters and through the use of these "k-linkage lists" increasingly large numbers of strains can be confidently compared.

Other more mathematically advanced procedures have been described. These are generally less available than matrix sorting procedures, but are typified by K-means (cited in Anderberg 1973 and Hogeweg's 1976) program. These operate by a feedback loop iteratively to develop and test phenon attribute

frequencies row by row as OTU's are added and removed on the basis of row averages or phenon stability. The latter procedure operates by weighting stable characters as analyses proceed.

2.9.3.5 Treatment of Missing Character Data.

Anderberg (1973) indicated two methods for treatment of missing attributes in data analysis. The first is for all characters which have missing data to be deleted from analysis. The second relies upon assignment of row or column averages to missing character loci before analysis. Wishart (1986) implemented a system where missing attributes were treated as wild so that within-cluster character averages could be separately assigned and reassigned to the same missing attribute during analysis. By this method only conflicting attributes are treated as non-equivalent.

Because of the stability of well balanced data similarity matrices derived from comparisons of more than 70 characters as advocated by Sokal and Sneath (1973) can also be expected to yield predictive similarities for separate comparisons of the same OTU's based upon different phenetic facets or other non-matching characters. Other treatments which compare OTU's only where matching character data is available rely on this premise.

2.9.3.6.0 Equating Phena with Species.

The ultimate separation of phena and then species from procedural artifacts requires consideration of possible errors introduced at all levels of analysis.

2.9.3.6.1 Procedural Errors.

Problems posed by phenetic analyses performed on small population subsets selected by specific, and possibly skewed, isolation and test procedures also need to be considered. For example if in a numerical taxonomic survey all isolations are performed in ways which differ from the original isolation of type cultures, the results may show skewing and resemble each other more than their respective type strains.

Another potential source of skewing particularly in separate carbon-source assimilation assays is from cross-feeding. In their study of *Pseudomonas* Stanier, Palleroni and Doudoroff (1966) observed that in taxonomic studies using multiply inoculated (23 strains) mineral salts agar plates, some problems of interpretation of growth arose from cross-feeding of some strains, and starvation of other slower growing strains. These problems were addressed by sorting, after primary evaluation, of like strains into a second testing regimen and by supplying a larger volume of more dilute substrate. Both of these solutions can be seen to be inadequate, first because heterogeneous populations of species will still cross-feed on at least some substrates, and then because of the short generation times of bacteria i.e. *ca* 20 min, species with prolonged lag phases or slower generation times would still be outgrown, and so unable to grow, except on the most refractile substrates. Further problems were suggested from incompetent strains within species which lacked the permeases necessary for uptake of substrates generally utilized by these species. Others, e.g. Franzmann (1983) have used multiwell plates but these, while preventing agar diffusion of substrates, cause new problems relating to condensation and subsequent cross-contamination of wells by motile species.

Baumann and Schubert (1984) recommended concurrent testing of unidentified *Vibrionaceae,* rather than their comparison with published results from reference cultures, presumably because of phenetic test errors or strain mutations. Sneath and Johnson (1972) divided procedural noise into test and sampling errors, where test errors represent incorrectly read results e.g. because of cross-feeding, and sampling errors; non-comparatively conducted tests i.e. tests read after different incubation conditions, or from strain mutations. Hada, Krichevsky and Sizemore (1983) additionally reported that *Vibrio* spp. had a high plasmid incidence, *ca* 30 %, and that these were gathered by numerical analysis into particular species groups and coded for commonly assayed phenotypic features i.e. to potentially skew results of numerical analysis (cf. 2.9.3.1, Baumann *et al.,* (1983)). It was proposed by

Sneath and Johnson (1972) that sampling error was reduced by increasing the number of comparative tests, and in Sneath (1974), that similarity coefficients were relatively stable to the effects of error; however Sneath and Johnson (1972) had earlier also recommended that such data was best treated by pattern coefficients (See 2.9.3.3).

2.9.3.6.2 **Analytical Errors.**

Possibly the worst abuse of numerical taxonomy has been the mechanistic assignment of arbitrary similarity values to differentiate between taxa e.g. genera (85 %) and species (75 %). Such practice based on phenetic criteria requires prior knowledge of differential characters within previously studied groups. If a study is exploratory then new species may be differentiated by two characters or twenty characters depending on the regimen used.

Sneath (1972) reported on procedures for objective definition of sub-populations from dendrograms. Normal distributions of OTU's in species sub-populations were assumed, and OTU's which were more than 2 standard deviations from the centrotypes were removed from "species". The problem with this approach is, that for valid use of the normal distribution more than 30 OTU's need to be present in each sub-population. Most often in small numerical analyses this is not possible.

Modern clustering programs such as SAS[r.] (1985) (Appendix 16.) offer objective facility to monitor cohesion of such populations formed at different similarity levels based upon specially devised small population probability *(Pseudo-F* and *T)* distributions.

Milligan and Isaac (1980) indicated that one of the potential problems of cluster analysis was the reproducible resolution of cluster partitions in a sample set of random data. Obviously the opportunity to objectively test and discard such artifactual classifications by methods other than varying cluster algorithms and similarity criteria is an attractive proposition. Multivariate procedures provide such an option and these are now widely

available on specific classification packages such as CLUSTAN (Wishart 1982,1986) and statistical packages such as SAS (SAS Institute, 1985).

2.9.3.7 Multivariate Methods.

Multivariate procedures presume that each OTU comes from a normally distributed population and that several such populations exist simultaneously in the data i.e. the data for (o) OTU's is composed initially by (n) characters in (n) dimensional space. Some of the characters are associated with others (dependent) others are unassociated (independent). Multivariate analysis operates by reducing the data from (n) dimensions to a specified lower number (m) finding independent characters and combining dependent characters, which offer no new information, then plotting (o) bits of information into (m) new vectors each containing successively less information i.e. an (o) by (m) dimensioned determinant matrix is produced.

Unlike the simple procedures of cluster analysis, multivariate procedures make more computationally rigorous analyses, which reduce the number of OTU's simultaneously examinable on the same computer system. One of the most conceptually attractive multivariate methods is factor analysis. The determinant matrix is rotated to permit observation from different perspectives and allow selection of the most agreeable perspective. The subjectivity of perspective selection prompted Chatfield and Collins (1980) to suggest that this technique was best avoided. Factor analysis without rotation of the matrix reduces it to principal component analysis. This procedure is reproducible and not operator sensitive, marginally less interpretable, and it perceivably produces the same result upon repeated analysis.

Chernoff (1973) addressed the problem of interpretation of multi-dimensional data by assigning scalar ranges to the features of cartoon faces and then fitting the data from multi-variate analyses to these faces. These glyphs allow easy aggregation of like subjects, but because the relative dimensions of faces are not equal they could be expected to bias interpretation.

Relative contributions of individual vectors from a principal component analysis may be assignable to increasingly trivial vectors but decision as to which facial feature is trivial is subjective. Other procedures for display and easy interpretation of multivariate vectors (Everitt, 1978) produce individual plots on equi-dimensional scales of "star symbol", or "sun-ray" glyphs.

2.10 Vibrio Physiology and its Relevance to Culture Media.

2.10.1 Effects of Media Ionic Composition on *Vibrionaceae* Growth.

The cell walls of Gram-negative bacteria are particularly sensitive to medium composition and stressed bacteria are less able to compensate for medium nutrient imbalances under poor osmotic conditions. Tamura, Kato, Iwata, Kotani and Kitaura, (1976) investigated the effect of hypotonicity on *V.parahaemolyticus* and suggested that sodium chloride deprivation may cause an increase in the porosity of the cell envelope allowing amino acid leakage. At lower pH, and with varying concentrations of lithium, sodium, potassium, magnesium and calcium chloride, lysis in hypotonic solutions could be prevented. Deneke and Colwell, (1973) suggested that excess cellular phosphate may be involved in the binding of cations necessary for marine salt requiring bacteria to form a stable envelope structure. Unemoto, Tsurouka and Hayashi (1973) suggested that $[Na^+]$ prevented cell lysis in *V.parahaemolyticus* and *V.alginolyticus* by providing the cell envelope with sufficient strength to resist mechanical stress, and $[K^+]$ by balancing the internal osmotic pressure of the cells. It was later shown that in *V.alginolyticus* (Tokuda, Nakamura and Unemoto, 1981) $[K^+]$ depletion renders cells insensitive to external pH and unable to maintain the optimal internal pH for metabolism.

For *V.parahaemolyticus, V.alginolyticus*, and *V.vulnificus* biogroup II some physical and biochemical properties have been demonstrated to vary depending on culture medium. *Vibrio alginolyticus* has been shown to have pH- and anion- dependent salt modifications to its alkaline phosphatase activity (Hayashi, Unemoto and Hayashi, 1973). The pH of the medium influences the

formation of lateral flagella in this species (Ulitzur, 1975a; Shinoda and Okamoto, 1977; and Kimura, Tateiri and Iida, 1979). De Boer, Golten and Scheffers (1975) reported that in *V.alginolyticus* heterogeneous flagellation was influenced by temperature, pH and salinity. Swarming in *V.parahaemolyticus* and *V.alginolyticus* is a result of negative chemotaxic response to metabolic by-products (Ulitzur, 1974, 1975b); (Reviewed Appendix 4.). Nishibuchi and Muroga, (1977) found that high salinity induced the formation of additional polar flagella in strains of *V.vulnificus* biogroup II *(Vibrio anguillicida).*

2.10.2 *Vibrionaceae* Isolation Media.

Appropriate isolation media are essential for the demonstration of marine bacteria. For example Gillespie and MacRae (1975) did not recover significant numbers of *Vibrio* spp. from fish skin using isolation media designed for terrestrial bacteria.

Most work on the development of isolation media for *Vibrio* spp. has been directed towards the common enteric pathogens *V.cholerae* and *V.parahaemolyticus*. Media for their isolation have traditionally included surfactants, (sodium cholate, bile salts, teepol), and alkaline conditions, a fermentable carbohydrate and a pH indicator or an oxidized heavy metal salt for growth detection. Additions of dyes such as ethyl violet served to restrict growth of Gram-positive species. Sucrose Teepol Tellurite agar was compared with Thiosulphate Citrate Bile-salts Sucrose (TCBS) agar by Chaterjee, De and Sen, (1977) and found to give a better recovery of typical isolates of *V.cholerae* than TCBS agar medium. Tests conducted by Nicholls, Lee and Donovan (1976) showed that only low recoveries (*ca* 35%) of *V.anguillarum* occurred on TCBS agar medium. Kusuda, Sako and Kawai (1979) investigated recoveries of 31 strains of *V.anguillarum* on selective media. The most promising of these was Bromthymol Blue teepol agar medium on which all but two strains grew strongly. A selective medium for *V.parahaemolyticus* utilizing copper sulphate, sodium cholate, high pH and 3% sodium chloride as selective agents (Watkins, Thomas and Cabelli 1976)

achieved 95% confirmation of typical colonies.

Beuchat (1974) and Ray, Hawkins and Hackney (1978) found that the minimum generation time for *V.parahaemolyticus* was 16.4 min at 36°C. in tryptic soy broth containing 2.9 % saline at pH 7.8. Larsen (1984) found optimal conditions for *V.anguillarum* were pH 7.0, salinity 1-2 % and temperature 25°C. This species was inhibited at temperatures of 30°C.

Beuchat (1977) demonstrated that 3 % sodium chloride in 0.1M potassium phosphate at pH 7.0 was the most suitable diluent for protection of cold- and heat- stressed *V.parahaemolyticus*. Ray *et al.*, (1978) recommended a low salt environment resuscitation stage for *V.parahaemolyticus* before selective enrichment. An earlier study by Beuchat (1976), evaluating enrichment media and broths for heat-stressed vibrios, showed that most showed better recoveries than the TCBS agar standard. The most satisfactory selective recoveries of *V.parahaemolyticus* were obtained with Water-blue Alizarin-yellow agar, Arabinose Ammonium Sulphate Cholate agar and Horie's Arabinose Ethyl Violet (HAE) broth. The recovery of *V.parahaemolyticus* in Glucose Salt Teepol broth improved when supplemented with magnesium and ferric ions but did not match the effectiveness of HAE broth (Ma-Lin and Beuchat, 1980). Alkaline Peptone Water (APW) was suggested as a suitable medium for the non-specific enrichment of *Vibrio* spp. (Furniss, Lee, and Donovan 1978).

Simidu and Tsukamoto (1980) developed a medium which addressed the possibility that *Vibrio* spp. are normally free-living environmental bacteria rather than enteric pathogens. The medium relied only on the fermentation of glucose at moderate alkaline pH in an anaerobic atmosphere.

3.0 EXPERIMENTAL STUDIES.

3.1 Introduction.

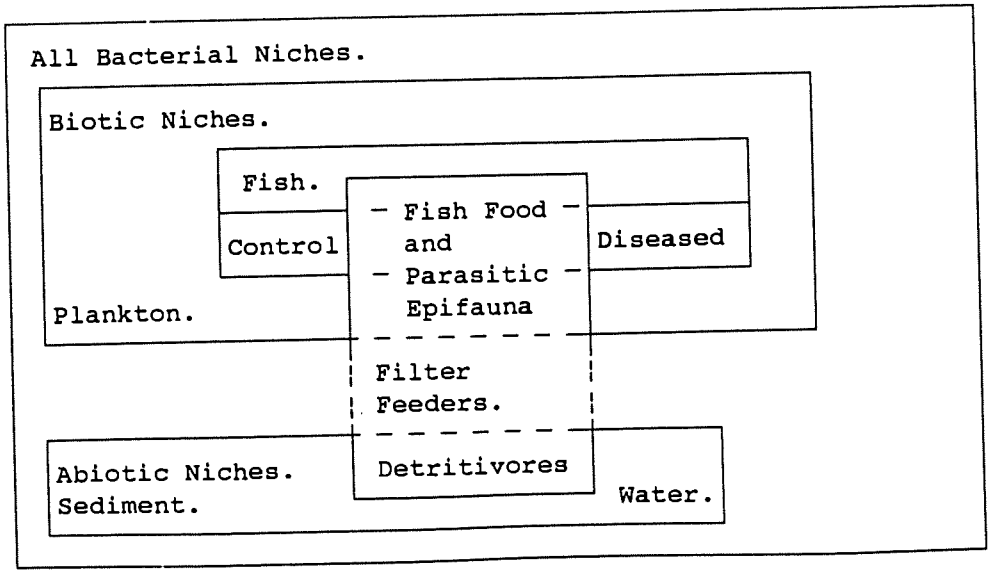
RSD is a typical ulcerative fish disease which seems stress related, but otherwise attributable to one or more previously imprecisely identified species from the *Vibrionaceae.* Similar taxonomic confusion is also present amongst other *Vibrionaceae* as a result of much recent uncoordinated revision by workers active in different microbiological disciplines, and based upon phenetic and molecular data. However, despite this lack of uniformity current classification has allowed the recognition of individual non-pathogenic environmental phenotypes of *Vibrionaceae* with micro-ecologies that can be opportunistic or highly specialized and occur as mutualistic symbioses.

3.2 General Experimental Design.

To most appropriately satisfy the study aims (1.2) an area of the Queensland coast (3.5) was selected where RSD was known to occur but where extrinsic factors were minimized.

Diseased and control fish and biotic and abiotic material from the fish's aquatic *milieu* (Figure 3.1) was sampled over a period of fourteen months.

Figure 3.1: Potential *Vibrionaceae* Environmental Distribution Within Aquatic *Milieu* Illustrating Basis for Strain Isolations.



These samples were inoculated to various bacteriological media with unknown selectivities for non-enteropathogenic *Vibrionaceae* and pure cultures of isolates were presumptively identified (3.7). Of almost 1000 isolates obtained, *ca* 40 % were phenotypically characterized, using a range of numerical and DNA based taxonomic systems (3.8.3-6, Appendix 9.).

The systematic and ecologic data was tabulated with a view to finding what components of *Vibrionaceae* flora from fish and biotic and abiotic components of its food web were restricted to, or overlapping between different trophic levels and so indicate the passage of bacteria, or their progeny through the aquatic milieu (3.10.1-5).

Results from these investigations were seen as potentially able to indicate environmental reservoirs of pathogenic species during quiescent periods of RSD and so also mechanisms for disease transmission (4.0).

3.3 Repetitive Methods.

3.3.1 Analytical Conventions.

All chemicals reagents used were analytical grade, or *Sigma* grade. Reagents and media except where qualified were prepared in aqueous solution on a weight/volume (w/v) or volume/volume (v/v) basis. Alkaline aqueous solutions were prepared by addition of 0.05 ml 5 N sodium hydroxide per 100 ml solution.

3.3.2.0 Sterilization.

3.3.2.1 Steam Sterilization. Except where otherwise specified sterilization was by autoclave (Silentia) at 121°C. for 15 min. Sterility was verified by pressure sensitive indicator tape (3M Australia) colouration.

3.3.2.2 Dry Heat Sterilization was conducted in a hot air oven at 160°C. with a holding time of 1h.

3.3.2.3 **Gamma Sterilization** was used to recycle expensive disposable polystyrene articles. These were decontaminated in sodium hypochlorite (*ca* 0.5% available chlorine), washed and rinsed extensively (detergent [**Diversy-***Pyroneg*], tap-water, distilled water), dried and sealed in plastic bags. They were sent to a commercial gamma irradiation plant operated by **Ansell International** and returned with an activated radio-sensitive indicator label attached. Gamma sterilization of small items was by exposure in a ^{60}Co cell **(Candu)** for at least 12 h. Concurrently exposed clear soda glass containers were discoloured to brown by this treatment.

3.3.2.4 **Filter Sterilization** was by passing solution through 0.2 μm sterile membrane filter cartridges (Flow-*Flopore D)* into a sterile reservoir.

3.3.3 Incubations except upon qualification were performed at 25 ± 0.1°C. in a refrigerated incubator **(Lindner and May).**

3.3.4.0 Culture Storage.

Reference, type or special interest cultures from the *Vibrionaceae* were deposited in the Microbiology Department culture collection, lyophilized in ampoules using horse serum as a cryoprotectant, and stored at 4°C.

Working cultures were stored in the dark at 22°C. on slopes of Sodium-Enriched Heart Infusion Agar medium (SEHIA), (3.4.1.03), overlaid with paraffin oil, (Faulding).

3.3.4.1 Culture Revival.

3.3.4.1.1 Revival from Ampoules.

Ampoules were opened and the cells suspended in Sodium-Enriched Nutrient broth (SENB) (3.4.1.01, Appendix 1.), and poured to plates of Sodium-Enriched Nutrient Agar medium (SENA) (3.4.1.02) and incubated 48h. For each culture a large loop-full of growth was streaked to a second plate of SENA and incubated. This growth, if pure was used as a source of inoculum in experiments. Primarily resuscitated cultures were examined also after 7d, to assure purity and the absence of slower growing contaminants.

3.3.4.1.2 **Revival from under Oil.**

Revival of stock cultures was by subculturing a loopful of growth into 2ml of SENB and incubating until turbid, or for 24 h. A loopful of this culture was streaked onto SENA and used as the source of inoculum in experiments, after observation for contaminants.

3.3.5 **Media Volumes.**

Tubed-media were dispensed in 3 ml volumes except ONPG/Tryptone where only 1 ml was used. Urease medium was gelled in slopes. Agar plugs were 2ml in volume. Most agar media were dispensed aseptically with a dispensing syringe (Becton Dickinson) to multiwell trays at 1.2 ml/well. Media used to support overlays were dispensed 1 ml/well and overlaid with 0.6ml of hydrolysis substrate medium.

3.4 Formulations Of Media, Buffers, And Reagents.

3.4.1 Nutrient Agars and Broths.

3.4.1.01 Sodium-Enriched Nutrient Broth, (SENB).

Lab Lemco	(Oxoid L 42)	5 g
Tryptone	(Oxoid L 29)	5 g
Yeast Extract	(Oxoid L 21)	3 g
Sodium Chloride	(Ajax)	20 g
Distilled Water		1 l

The pH was adjusted to 7.6 and the medium sterilized.

3.4.1.02 Sodium-Enriched Nutrient Agar, (SENA).

SENB	1 l
Agar (Oxoid L 11)	10 g

The pH was adjusted to 7.6 and the medium sterilized.

3.4.1.03 Sodium-Enriched Heart Infusion Agar, (SEHIA).

Heart Infusion Broth (Gibco M-23400)	25 g
Sodium Chloride (Ajax)	15 g
Agar (Oxoid L 11)	10 g
Distilled Water	1 l

3.4.1.04 Sodium-Enriched Brain Heart Infusion Broth, (SEBHIB).

Brain Heart Infusion Broth (Gibco M-06800)	38 g
Sodium Chloride (Ajax)	15 g
Distilled Water	1 l

The pH was adjusted to 7.6 and the medium sterilized.

3.4.1.05 Sodium-Enriched Brain Heart Infusion Agar, (SEBHIA).

SEBHIB	1 l
Agar (Oxoid L 11)	10 g

The pH was adjusted to 7.6 and the medium sterilized.

3.4.1.06 Sodium-Enriched Mueller Hinton Broth, (SEMHB).

Mueller Hinton Broth (Oxoid CM 405)	38 g
Sodium Chloride (Ajax)	15 g
Distilled Water	1 l

The pH was adjusted to 7.6 and the medium sterilized.

3.4.1.07 Sodium-Enriched Tryptic Soy Broth, (SETSB).

Tryptic Soy Broth (Gibco M-49800)	29.5 g
Sodium Chloride (Ajax)	15 g
Distilled Water	1 l

The pH was adjusted to 7.6 and the medium sterilized.

3.4.1.08 Sodium-Enriched Tryptic Soy Agar, (SETSA).

SETSB	1 l
Agar (Oxoid L 11)	10 g

The pH was adjusted to 7.6 and the medium sterilized.

3.4.1.09 Sodium-Enriched Tryptone Broth, (SETB).

Tryptone (Oxoid L 42)	10 g
Sodium Chloride (Ajax)	20 g
Distilled Water	1 l

The pH was adjusted to 7.6 and the medium sterilized.

3.4.2.0 Isolation and Enrichment Media.

3.4.2.1 Simidu medium (Adapted from Simidu and Tsukamoto, 1980).

Lab Lemco	(Oxoid L 29)	1 g
Microbiotone	(Oxoid L 72)	5 g
Yeast Extract	(Oxoid L 21)	1 g
Glucose	(Ajax)	5 g
TES	(Sigma)	2 g
Sodium Chloride	(Ajax)	20 g
Potassium Chloride	(BDH)	3 g
Magnesium Sulphate, Heptahydrate	(Ajax)	3 g
Calcium Chloride, Dihydrate	(Ajax)	0.1 g
Distilled Water		1 l
pH was adjusted to		8.4
Bromthymol Blue (1 % alkaline aqueous, Sigma)		4 ml
Agar	(Oxoid L 11)	10 g

The medium was sterilized.

3.4.2.2 Thiosulphate Citrate Bile Salt Sucrose Agar, Medium (TCBS).

TCBS Agar (Eiken)	89 g
Distilled Water	1 l

pH was adjusted to 8.6 and the medium melted at 108°C., 15 min.

3.4.2.3 EDDA Medium (1 μM).

SENA Medium	(Sterile Molten)	100 ml
EDDA (ICN Pharmaceuticals Inc.) alkaline aq. 360 μg/ml		0.1ml

EDDA deferrated according to Rogers (1973) was added to SENA medium and mixed.

3.4.2.4 Alkaline Tryptone Water, (ATW) (Adapted from Furniss *et al.*, 1978).

Tryptone (Oxoid L 42)	10 g
Sodium Chloride (Ajax)	20 g
Distilled Water	1 l

The pH was adjusted to 8.5 and the medium sterilized.

3.4.2.5 Bromthymol Blue Teepol Broth, (BTB-Teepol) (Adapted from Cowan, 1974).

Tryptone	(Oxoid L 42)	10 g
Lab Lemco	(Oxoid L 29)	3 g
Yeast Extract	(Oxoid L 21)	3 g
Sucrose	(Ajax)	20 g
Sodium Chloride	(Ajax)	20 g
Bromthymol Blue	(1 % alkaline aqueous, Sigma)	8 ml
Teepol 630	(Shell)	2 ml
Distilled Water		1 l

pH was adjusted to 8.5 and the medium sterilized.

3.4.3.0 Buffers.

3.4.3.1 Marine Phosphate Buffered Saline, (MPBS).

di-Potassium Hydrogen Phosphate	(Ajax)	1.21 g
Potassium di-Hydrogen Phosphate	(Ajax)	0.34 g
Sodium Chloride	(Ajax)	20 g
Distilled Water		1 l

pH was adjusted to 7.6 and the medium sterilized.

3.4.3.2 Saline-EDTA.

Sodium Chloride (Ajax)	0.15 M	8.77 g
di-Sodium -EDTA (BDH)	0.01 M	3.36 g
Distilled Water		1 l

pH was adjusted to 7.0

3.4.3.3 Standard Saline Citrate, (SSC).

Sodium Chloride	(Ajax) 0.15	8.77 g
Tri-Sodium Citrate	(Ajax) 0.015 M	3.87 g

pH was adjusted to 8.0

3.4.4 Reagents and Diagnostic Media.

3.4.4.01 Winkler Reagents (APHA, 1975).

(a) Manganous Sulphate Reagent.

Manganous Sulphate, Tetrahydrate (Ajax)	24 g
Distilled Water	50 ml

(b) Alkali-Iodide-Azide Reagent.

Sodium Azide (Ajax)	0.5 g
Sodium Hydroxide (Ajax)	24 g
Sodium Iodide (Ajax)	37.5 g
Distilled Water	50 ml

Sodium azide was dissolved in 50 ml distilled water, and sodium hydroxide and then sodium iodide were added as each dissolved.

(c) Sulphuric Acid (BDH)	10 ml

(d) Starch Indicator Solution.

Starch Soluble (Ajax)	1 g
Distilled Water	50 ml

Starch was dissolved in distilled water by heating.

(e) Sodium Thiosulphate Solution, 0.0250 N.

Sodium Thiosulphate Pentahydrate (Ajax)		3.1025 g
Sodium Hydroxide	(Ajax)	0.2 g
Distilled Water	to	500.0 ml

3.4.4.02 Gram Stain Reagents (Doetsch, 1981).

(a) Crystal Violet.

(i)	Crystal Violet (BDH)	20 g
	Ethanol (95 %) (Ajax)	200 ml
(ii)	Ammonium Oxalate (BDH)	8 g
	Distilled Water	800 ml

Solutions (i) and (ii) were combined and allowed to settle 24 h before filtering into a dark bottle.

(b) Lugol's Iodine.

Iodine (BDH)	20 g
Potassium Iodide (Ajax)	40 g
Distilled Water	2 l

Potassium iodide (40 g) was dissolved in 100 ml distilled water and 20 g Iodine added. The solution was diluted to 2 l and stored in a dark bottle.

(c) Safranin *O*, (2 %).

Safranin *O* (20 g) was dissolved in 1 l distilled water.

3.4.4.03 Catalase Reagent (Adapted from Collins and Lyne, 1970).

Hydrogen Peroxide 120 vol. (Ajax)	10 ml
Tween 80 (BDH)	0.6 g
Distilled Water	110 ml

Tween 80 (0.6 g) was dissolved in 110 ml distilled water and 10 ml 120 Vol. (36% w/v) Hydrogen peroxide was added. The solution was stored at 4°C. in a plastic stoppered bottle.

3.4.4.04 Oxidase Reagent (Steel, 1962).

Tetramethyl-p-Phenylenediamine Dihydrochloride (BDH)	0.4 g
Ascorbic Acid (Ajax)	0.2 g
Distilled Water	20 ml

N,N,N,N-tetra-methyl-p-phenylenediamine di-hydrochloride was dissolved in 20 ml distilled water and ascorbic acid. The solution was used to impregnate strips of filter paper (Whatmans No. 1), dried at 37°C., and stored in a dark bottle at 4°C.

3.4.4.05.1 Kovacs' Reagent (Kovacs 1928).

p-Dimethylaminobenzaldehyde (BDH)	5 g
Iso-Amyl Alcohol (BDH)	75 ml
Hydrochloric Acid (Ajax)	25 ml

p-Dimethylaminobenzaldehyde was dissolved in iso-amyl alcohol and hydrochloric acid was added. The solution was stored at 4°C. in an aluminium foil covered glass bottle.

3.4.4.05.2 Indole Medium.

SENB	1 l
l-Tryptophan	1 g

pH was adjusted to 7.6 and the medium sterilized.

3.4.4.06.1 Acetoin Reagent (Barritt, 1936).

(a) α-Napthol Reagent.

α-Napthol (Ajax)	25 g
Ethanol (Ajax)	500 ml

α-Napthol was steam distilled according to Fulton, Halkias, and Yarashus (1960) and 25g wet weight was dissolved in absolute ethanol as above. The reagent was stored at 4°C. in an aluminium foil covered glass bottle.

(b) Potassium Hydroxide Solution (20 %).

Potassium Hydroxide (Ajax)	40 g
Distilled Water	200 ml

Potassium hydroxide was added progressively to 200 ml distilled water and stirred until dissolved, and stored in a plastic bottle.

3.4.4.06.2 MRVP Medium.

SENB	1 l
di-Potassium Hydrogen Phosphate (Ajax)	5 g
Glucose	5 g

The pH was adjusted to 6.9 and the medium was sterilized.

3.4.4.07.1 Nitrite Reduction Reagents (Cowan, 1974).

(a) Sulphanilic Acid Solution.

Sulphanilic Acid (BDH)	4 g
Acetic Acid (Ajax)	150 ml
Distilled Water	350 ml

Sulphanilic acid was added to distilled water with glacial acetic acid and heated slowly until dissolved. The solution was stored in a bottle at 4°C.

(b) α-Napthylamine Solution.

α - Napthylamine (BDH)	2.5 g
Acetic Acid (Ajax)	150 ml
Distilled Water	350 ml

α-Napthylamine was added to distilled water with glacial acetic acid and heated slowly until dissolved. The solution was stored in a foil covered bottle at 4°C.

(c) Zinc Powder (Ajax) was dispensed to a 5ml plastic squeeze bottle.

3.4.4.07.2 Nitrate Reduction Medium.

SENB	1 l
Potassium Nitrate (BDH)	0.1 g

The pH was adjusted to 7.6 and the medium was sterilized.

3.4.4.07.3 Nitrite Reduction Medium.

SENB	1 l
Sodium Nitrite (BDH)	0.01 g

The pH was adjusted to 7.6 and the medium was sterilized.

3.4.4.08 Cholera-Red Reagent.

1 N Hydrochloric acid solution.

Hydrochloric Acid	50 ml
Distilled Water	450 ml

Hydrochloric acid, (50 ml) was added with stirring to 450 ml distilled water and the solution stored in a glass stoppered bottle.

3.4.4.09.1 Benedict's Reagent (Cowan, 1974).

Sodium Citrate (Ajax)	86.5 g
Sodium Carbonate [Anhydrous] (BDH)	50 g
Copper Sulphate [Pentahydrate] (BDH)	8.65 g
Distilled Water to	500 ml

Sodium citrate (Ajax) and carbonate (BDH) were dissolved in 300 ml distilled water and to this a solution of copper sulphate in 100 ml water was added. The combined solution was made to 500 ml and stored.

3.4.4.09.2 Gluconate Medium. (Adapted from Cowan, 1974).

Potassium gluconate (40 g, BDH) was dissolved in distilled water and made to 100 ml. The solution was clarified by filtration through activated charcoal (Oxoid) and filter sterilized. Fifteen ml was combined with 135 ml sterile SENB containing 1.5 g di-potassium hydrogen phosphate.

3.4.4.10 Arginine Dihydrolase (Adapted from Thornley, 1960).

(a) Support Medium.

Proteose Peptone (Oxoid L 46)	1 g
Sodium Chloride (Ajax)	20 g
di-Potassium Hydrogen Phosphate (Ajax)	0.3 g
Phenol Red (1 % alkaline aqueous, BDH)	1 ml
Agar (Oxoid L 11)	3 g
Distilled Water	900 ml
pH adjusted to	7.2

The medium was prepared as above, melted at 108°C. 15 min, and 450 ml aseptically dispensed to two bottles labeled test and control. The control bottle was supplemented with a further 50 ml distilled water and both bottles were sterilized.

(b) Arginine Solution.

l-Arginine Hydrochloride (Sigma)	12 g
Distilled Water	100 ml

The arginine was dissolved and filter sterilized. Medium in the "test" bottle from part (a) was aseptically combined with 50 ml sterile arginine from part (b). Indicator colours balanced by dropwise addition of 5 N sodium hydroxide.

(c) Agar Plug Medium.

Agar (Oxoid L 11)	20 g
Sodium Chloride (Ajax)	20 g
Distilled Water	1 l

The medium was sterilized in 200 ml portions.

3.4.4.11 Lysine and Ornithine Decarboxylase Media (Adapted from Moller, 1955; and Cowan, 1974).

(a) Support Medium.

Tryptone (Oxoid L42)	5 g
Lab Lemco (Oxoid L29)	5 g
Pyridoxal Hydrochloride (Sigma)	5 mg
d+Glucose (BDH)	0.5 g
Sodium Chloride (Ajax)	20 g
Distilled Water	900 ml
pH adjusted to	6.0
Bromocresol Purple (1.6 % alcoholic, BDH)	0.625 ml
Cresol Red (0.2 %, BDH)	2.5 ml

The medium was dispensed in 150 ml portions and sterilized.

(b) Amino Acid Solutions.

Aqueous solutions, (10 %), of amino-acid bases of l-Ornithine hydrochloride (Sigma) (13 g) and l-Lysine hydrochloride (Sigma) (10 g) were prepared and filter sterilized and volumes of each of these or an equivalent volume of sterile distilled water were added to produce a 1% amino-acid solution.

3.4.4.12 Glucose Fermentation Medium (Adapted from Hayward, 1964).

(a) Support Medium.

Tryptone (Oxoid L 42)	2 g
Yeast Extract (Oxoid L 21)	3 g
Ammonium di-Hydrogen Phosphate (Ajax)	1 g
Potassium Chloride (BDH)	0.2 g
Magnesium Sulphate, Heptahydrate (BDH)	0.2 g
Sodium Chloride	20 g
Agar (Oxoid L 11)	3 g
Distilled Water	900 ml
pH adjusted to 7.0	

(b) Glucose Solution.

d+Glucose (BDH)	10 g
Distilled Water	100 ml

Solutions (a) and (b) were sterilized separately and combined.

3.4.4.13 *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) Hydrolysis (Adapted from Cowan, 1974).

(a) ONPG Solution.

ONPG (Calbiochem)	3 g
Distilled Water	500 ml

Solution (a) was filter sterilized and stored at 4°C.

(b) Buffered Tryptone Water.

Tryptone (Oxoid L 42)	15 g
di-Potassium Hydrogen Phosphate (Ajax)	1.21 g
Potassium di-Hydrogen Phosphate (Ajax)	0.34 g
Sodium Chloride (Ajax)	30 g
Pyridoxal Hydrochloride (Sigma)	5 mg

The pH was adjusted to 7.6 and the medium was sterilized.

One volume of solution (a) was combined with three volumes of (b) and the medium was dispensed.

3.4.4.14 Christensen's Urea Medium (Adapted from Cowan, 1974).

(a) Christensen's Medium.

Tryptone (Oxoid L 42)	1 g
Sodium Chloride (Ajax)	20 g
Potassium di-Hydrogen Phosphate (Ajax)	2 g
d+Glucose (BDH)	1 g
Agar (Oxoid L 11)	10 g
Distilled Water	900 ml
pH was adjusted to	6.8
Phenol Red, (1 % alkaline aqueous, BDH)	1.2 ml

The medium was sterilized in 300 ml volumes and stored.

(b) Urea Solution.

Urea, 20 g, (Sigma) was dissolved in 100 ml distilled water, sterilized by filtration and stored. To make the complete medium Parts (a) and (b) were aseptically combined to produce a final urea concentration of 2 %.

3.4.4.15 Aesculin Agar Medium. (Adapted from Cowan, 1974).

SENA Medium	100 ml
Aesculin (BDH)	0.5 g

The medium was sterilized and 1 ml 5 % (w/v) sterile ferric ammonium citrate (Ajax) was added.

3.4.4.16 Egg Albumen Agar Medium.

Sterile Molten SENA Medium	100 ml
Egg Albumen/MPBS, sterile, (1:1)	10 ml

3.4.4.17 Arbutin Agar Medium.

SENA Medium	100 ml
Arbutin (Sigma)	0.5 g

The medium was sterilized and 1 ml 5 % (w/v) sterile ferric ammonium citrate (Ajax) was added.

3.4.4.18 Arylsulphatase Agar Medium (Adapted from Whitehead, Morrison and Young, 1952).

Sterile Molten SENA	100 ml
FS Tripotassium phenolpthalein disulphate (0.01 M) (Sigma)	10 ml

3.4.4.19 Casein Agar Medium (Adapted from Cowan, 1974).

SENA Medium	100 ml
Casein (BDH)	1 g

pH was adjusted to 7.6 and the medium sterilized.

3.4.4.20 Chitin Agar Medium.

Sterile molten SENA Medium	100 ml
Hydrated Chitin Suspension	10 ml

Crab chitin flakes, 10 g, (Sigma) were placed in a ceramic mortar and

immersed in 100 ml ortho-phosphoric acid (Ajax) for hydration and held at 4°C. for 7d. The chitin was macerated to a smooth paste and suspended in 500 ml water. The suspension was transferred to a dialysis sac (**Union Carbide** AW 12 000 - 14 000) and dialyzed against tap water until the pH was neutral, and then overnight against distilled water. The suspension was diluted to 500 ml and sterilized. Ten ml suspension was combined aseptically with 100 ml sterile molten SENA.

3.4.4.21 Sodium-Enriched DNA Agar Medium, (SEDNA).

DNA Test Agar (Oxoid CM 321)	40 g
Sodium Chloride (Ajax)	15 g
Distilled Water	1 l

Oxoid DNA agar base was supplemented with salt and sterilized.

3.4.4.22 Gelatin Agar Medium.

SENA Medium	100 ml
Gelatin (Oxoid L 8)	1 g

pH was adjusted to 7.6 and the medium sterilized.

3.4.4.23 Egg Yolk Agar Medium (Adapted from Willis, 1960).

Sterile Molten SENA Medium	100 ml
Egg yolk in sterile MPBS (1:1)	10 ml

Mixed and dispensed aseptically.

3.4.4.24 Phosphatase Agar Medium. (Adapted from Smibert and Krieg, 1981).

Sterile Molten SENA Medium	100 ml
FS Sodium phenolpthalein diphosphate 1% (Sigma)	1 ml

3.4.4.25 Starch hydrolysis.

Soluble Starch, 10 ml 2 %, (Ajax) was sterilized and combined with 100 ml sterile molten SENA.

3.4.4.26 DL-3-Hydroxybutyrate Medium, (HBSENA).

SENA Medium		100 ml
Glucose	(Ajax)	0.5 g
Sucrose	(Ajax)	0.5 g
DL-3-Hydroxybutyrate (Sodium Salt)	(Sigma)	0.2 g

The pH was adjusted to 7.6 and the medium sterilized.

3.4.4.27 Sulphide Production (Adapted from Jegathesan and Paramasivam, 1976).

Sterile Molten SENA Medium	100 ml
FS Lead Acetate, 10 % (BDH)	1 ml
Sterile Sodium Thiosulphate, 2 % (BDH)	1 ml

The pH was adjusted to 7.6 and the medium sterilized.

3.4.4.28 Serum Agar Medium.

Sterile Molten SENA	100 ml
Horse Serum (CSL)	10 ml

3.4.4.29 Tyrosine Agar Medium (Adapted from Cowan, 1974).

SENA Medium	100 ml
Tyrosine (BDH)	1 g

The pH was adjusted to 7.6 and the medium sterilized.

3.4.4.30 Xanthine Hydrolysis (Adapted from Cowan, 1974).

SENA Medium	100 ml
Xanthine (Sigma)	1 g

The pH was adjusted to 7.6 and the medium sterilized.

3.4.4.31 Congo Red Medium.

Sterile Molten SENA Medium	100 ml
Congo Red 5 % FS (Sigma)	2 ml

3.4.4.32 Growth in 0-10 % Sodium Chloride.

SEN broth was dispensed in 100 ml aliquots sans sodium chloride and to each bottle 1 g agar and 0,0.5,1,3,6, or 10 g salt was added and the combined media were sterilized.

3.4.4.33 pH 10 Agar Medium.

(a) Sterile Molten Double Strength SENA	50 ml
(b) Valine Buffer.	50 ml
[0.2 M Valine (BDH) (23.5 g/l)	25 ml]
[0.4 M Sodium Hydroxide	24 ml]
[Distilled Water	1 ml]

The temperatures of solutions (a) and (b) were equilibrated in a waterbath at 60°C., combined and the pH of a sample checked. If necessary the pH was adjusted to 10 with N hydrochloric acid.

3.4.4.34 pH 4.5 Agar Medium.

(a) Sterile Molten Double Strength SENA Medium 50 ml

(b) Succinate Buffer (Gomori 1955). 50 ml

[0.2 M Succinic Acid (Sigma) (23.6 g/l) 25 ml]

[0.4 M Sodium Hydroxide 10 ml]

[Distilled Water 15 ml]

Solutions (a) and (b) were held in a waterbath at 60°C. and combined and the pH of a sample checked. If necessary the pH was adjusted to 4.5 with N hydrochloric acid or N sodium hydroxide.

3.4.4.35 Marine Phosphate Buffered Glucose Salts, (MPBGS), Medium.

Part (a)

Sodium Sulphate (Ajax)	4 g
di-Potassium Hydrogen Phosphate (Ajax)	15.94 g
Potassium diHydrogen Phosphate (Ajax)	1.16 g
Sodium Chloride (Ajax)	15 g
Distilled Water	750 ml

pH adjusted to 7.8

Part (b)

Magnesium Chloride, Hexahydrate (Ajax)	4 g
Magnesium Sulphate (Ajax)	0.1 g
d+Glucose (BDH)	2 g
Distilled Water	250 ml

Parts (a) and (b) were prepared separately, sterilized and aseptically combined.

3.4.4.36 Inhibitory Media.

Stock solutions of compounds at active strengths as specified in Table 3.1 were diluted into sterile molten SENA to achieve the listed working strengths. All compounds were prepared in sterile distilled water excepting

quinolines which were prepared in 95 % ethanol; and dichlorophene and deferrated EDDA (Rogers 1973) which were dissolved in 0.0005 N sodium hydroxide. Inhibitor stock solutions were presumed as sterile except fast yellow which was autoclaved and pyronin-Y which was filter sterilized. Solutions were stored at 4°C. and new antibiotic solutions prepared every three months.

Table 3.1: Active Strengths of Sterile Inhibitor Solutions and Respective Dilutions and Assumed (Working) Strengths in Agar Media.

Compound	Source	Active Strength	dilu -tion	Work Strength
Novobiocin sodium salt	(Upjohn)	10 mg/ml	0.001	10 µg/ml
Penicillin-G	(Sigma)	10 000 unit/ml	0.0015	15 unit/ml
Polymyxin B sulphate	(Sigma)	10 mg/ml	0.0005	5 µg/ml
Streptomycin sulphate	(Sigma)	10 mg/ml	0.001	10 µg/ml
Sulphamethoxazole	(Sigma)	5 mg/ml	0.001	5 µg/ml
Tetracycline HCl	(Sigma)	10 mg/ml	0.001	10 µg/ml
Trimethoprim	(Sigma)	10 mg/ml	0.001	10 µg/ml
2,4-diamino-6,7-diisopropyl-pteridine phosphate (0/129)	(BDH)	25 mg/ml	0.0004	10 µg/ml
(as 0/129 phosphate)			0.06	150 µg/ml
Basic Fuchsin	(BDH)	1 %	0.025	0.025 %
Brilliant Green	(BDH)	0.1 %	0.0125	0.0013 %
Dichlorophene	(BDH)	0.1 %	0.005	0.0005 %
EDDA	(ICN)	0.01 M	0.1	10 µM/ml
Fast Yellow	(Sigma)	0.1 %	0.01	0.01 %
Haloquinol	(Sigma)	0.1 %	0.005	0.0005 %
8-hydroxyquinoline	(Ajax)	0.1 %	0.005	0.0005 %
Methyl Violet	(Gurr)	0.1 %	0.02	0.005 %
Pyronin-Y	(Sigma)	0.2 %	0.01	0.002 %
Sodium Dodecylsulphate	(Ajax)	5.0 %	0.04	0.2 %
Thionine	(Gurr)	2.5 %	0.01	0.025 %
TTC	(BDH)	0.1 %	0.01	0.001 %

3.4.4.37 Carbohydrate Media.

Carbohydrates were prepared in aqueous solution, filter sterilized and stored at 4°C. Stock solutions, (Table 3.2), were added aseptically to a 1 % concentration in the sterile molten support medium used for O/F tests (3.4.4.12) supplemented to contain 1% agar (Oxoid L 11).

Table 3.2: Concentrations (%) of Aqueous Filter Sterilized Carbohydrates and Their Origins.

Carbohydrate	Stock solution (%)
D(+)Xylose (Sigma)	10
L(+)Arabinose (BDH)	10
Mannose (Sigma)	10
Sucrose (Ajax)	10
Maltose (Sigma)	10
D(+)Cellobiose (BDH)	10
D(-)Salicin (BDH)	5
Mannitol (BDH)	10
Sorbitol (BDH)	10
m-Inositol (BDH)	2.5
Dulcitol (BDH)	2.5

3.4.4.38 Carbon Assimilation Medium.

Carbon assimilation tests were performed on the medium of Lee *et al.*, (1981) prepared as below.

(a) Marine Salts Solution.

Ammonium Chloride (Ajax)	5 g
Ammonium Nitrate (Ajax)	1 g
Sodium Sulphate (Ajax)	2 g
di-Potassium Hydrogen Phosphate (Ajax)	3 g
Potassium di-Hydrogen Phosphate (Ajax)	1 g
Magnesium Sulphate Heptahydrate (Ajax)	0.1 g
Sodium Chloride (Ajax)	10 g
Distilled Water	250 ml

(b) Magnesium Agar Base.

Magnesium Chloride Hexahydrate (Ajax)	4 g
Agar (Oxoid L 28)	10 g
Distilled Water	750 ml

Table 3.3: Carbon Substrates, their Origins, Forms, and Final Stock Concentrations Equivalent, Usually to 10 % Pure Base.

Carbon Source	Origin	Form	10% equivalent.
Glycine	(Ajax)	Crystalline	10 %
l-α-Alanine	(Sigma)	Crystalline	10 %
d-α-Alanine	(Sigma)	Crystalline	10 %
dl-β-Alanine	(Sigma)	Crystalline	10 %
Serine	(Sigma)	Crystalline	10 %
l-Leucine	(Sigma)	Crystalline	10 %
l-Valine	(BDH)	Crystalline	10 %
l+Glutamic acid	(Sigma)	Crystalline	10 %
l-Lysine	(Sigma)	Hydrochloride	12 %
l-Arginine	(Sigma)	Hydrochloride	12 %
l-Ornithine	(Sigma)	Hydrochloride	13 %
l-Citrulline	(Sigma)	Crystalline	10 %
γ-Amino-butyric acid	(Sigma)	Crystalline	10 %
δ-Amino-valerate	(Sigma)	Hydrochloride	13 %
l-Norvaline	(Sigma)	Crystalline	10 %
l-Proline	(Sigma)	Crystalline	10 %
Putrescine	(Sigma)	Hydrochloride	18 %
Sarcosine	(Sigma)	Crystalline	10 %
l-Glutamine	(Sigma)	Crystalline	10 %
d+Glucosamine	(Sigma)	Hydrochloride	12 %
n-Acetylglucosamine	(Sigma)	Crystalline	10 %
d-Ribose	(Sigma)	Crystalline	10 %
Xylose	(Sigma)	Crystalline	10 %
l-Arabinose	(Sigma)	Crystalline	10 %
l-Rhamnose	(Sigma)	Crystalline	10 %

Table 3.3; (Cont.): Carbon Substrates, Origins, Forms, and Final Stock Concentrations Equivalent, Usually to 10 % Pure Base.

Carbon Source	Origin	Form	10% equivalent.
d-Glucose	(Ajax)	Crystalline	10 %
d-Mannose	(Sigma)	Crystalline	10 %
d-Galactose	(Sigma)	Crystalline	10 %
Sucrose	(Ajax)	Crystalline	10 %
d+Trehalose	(Sigma)	Crystalline	10 %
Maltose	(Sigma)	Crystalline	10 %
Cellobiose	(Sigma)	Crystalline	10 %
Lactose	(BDH)	Crystalline	10 %
Raffinose	(BDH)	Crystalline	10 %
Galactarate	(Unknown)	Crystalline	10 %
d-Galacturonic acid	(Sigma)	Monohydrate	11 %
Gluconate	(BDH)	Sodium salt	11 %
d-Glucuronic acid	(Sigma)	Crystalline	10 %
Salicin	(Sigma)	Crystalline	5 % = 5%
Starch	(Ajax)	Polymer	10 %
Dextrin	(BDH)	Polymer	2.5 % = 2.5 %
Polygalacturonic acid	(Sigma)	Sodium salt	10 %
Inulin	(BDH)	Polymer	10 %
Pullulan	(Sigma)	Polymer	10 %
Formate	(BDH)	Sodium salt	15 %
Acetate	(May and Baker)	Sodium salt	14 %
Propionate	(BDH)	Sodium salt	13 %
n-Butyrate	(Fluka)	Free acid	10 %
Hexanoate	(Sigma)	Free acid	10 %
Heptanoate	(Sigma)	Free acid	10 %
Caprylate	(Sigma)	Free acid	10 %
Nonanionate	(Sigma)	Free acid	10 %
Caprate	(Sigma)	Sodium salt	11 %
Malonate	(BDH)	Sodium salt	12 %

Table 3.3; (Cont.): Carbon Substrates, Origins, Forms, and Final Stock Concentrations Equivalent, Usually to 10 % Pure Base.

Carbon Source	Origin	Form	10% equivalent.
Succinate	(BDH)	Sodium salt	14 %
Maleate	(BDH)	Sodium salt	14 %
Adipic acid	(Sigma)	Crystalline	10 %
Malate	(BDH)	Sodium salt	14 %
Tartarate	(BDH)	Sodium salt	13 %
DL-3-Hydroxybutyrate	(Sigma)	Sodium salt	12 %
6-Hydroxycaproate	(Unknown)	Crystalline	10 %
Lactate 70 %	(BDH)	Sodium salt	18 %
dl-Glyceric acid	(Sigma)	Hemicalcium salt	12 %
Poly-β-Hydroxybutyrate	(Sigma)	Polymer	10 %
Citrate	(Ajax)	Sodium salt	14 %
α-Ketoglutarate	(Sigma)	Acid	10 %
Pyruvate	(BDH)	Sodium salt	13 %
Erythritol	(Sigma)	Crystalline	10 %
Dulcitol	(BDH)	Crystalline	2.5 % = 2.5 %
Mannitol	(BDH)	Crystalline	10 %
Sorbitol	(BDH)	Crystalline	10 %
Inositol	(Sigma)	Crystalline	10 %
Ethanol	(Ajax)	95% aqueous	10 %
Benzoate	(BDH)	Sodium salt	12 %
p-Hydroxybenzoate	(Sigma)	Sodium salt	11 %
Phenyl-acetic acid	(Sigma)	Crystalline	10 %

Parts (a) and (b) were sterilized separately; and Part (b) held at 60°C. All carbon sources, (Table 3.3) except starch and poly-β-hydroxybutyrate which were sterilized at 121°C. 15 min, were prepared as filter-sterilized stock solutions, (insoluble organic acids were neutralized with sodium hydroxide before filtration), and stored at 4°C. Stock solutions were added separately to (a) and mixed with (b) to produce a final 0.1% concentration of carbon source.

3.5 Study Area.

The principal study area (Figure 3.2) centered at 24° 35'S. and 151° 55'E and extended approximately in a 30 km radius. The area consists primarily of deep marine sediments. In some places these have been subject to intrusions by volcanic activity with the consequent occurrence of granites and metamorphic rocks (Ellis and Whitaker, 1976). All these rocks contain considerable salts, principally as sodium, and magnesium, and bicarbonate ions which are leached into soils, underlying aquifers and the Baffle Creek and Kolan River systems. (S.Macnish Pers.Comm.). Annual rainfall in the region (Queensland Department Primary Industry) is about 1 100 mm.

In the coastal sedimentary basin *(ca* 2 km) there are some sugarcane farms, macadamia plantations and market gardens. Inland to the sources of the watercourses high soil salinities preclude such intensive cropping and the catchments are used instead for cattle grazing and to grow natural or pine forests. Examination of a 1985 infra-red Landsat image indicated substantial areas (*ca* 30 %) of brown-red colouration, indicating bare rock or sparsely vegetated ground. The region also supports a small number of hobby farms (S.Macnish Pers.Comm.) which are assumed to impose only a minimal pollution load.

Environmental sampling was primarily from Baffle Creek at Winfield [Site 1.], (Plate 3.1, top) a small holiday resort at the mouth of Baffle Creek; and at Wartburg [Site 2.], (Plate 3.1, bottom), a brackish-water site *ca* 10 km upstream. Plankton was collected from brackish-water in Oyster Creek [Site 3.] and Site [1.]. A series of samples were taken from fish caught from the Baffle Creek system at the locations as numbered. An additional single fish specimen was obtained from the Kolan River.

The principal study area has relatively insignificant exposure to man-made stresses such as from fertilizers, pesticides and sewage. Additional, intrinsic stresses such as resulting from increased suspended solids in run-off into sparsely vegetated catchments would also be less here than into

neighbouring (cultivated) river catchments.

The secondary (marine) site investigated was Moreton Bay. This is more heavily polluted than the Baffle Creek system but RSD incidence is quite low.

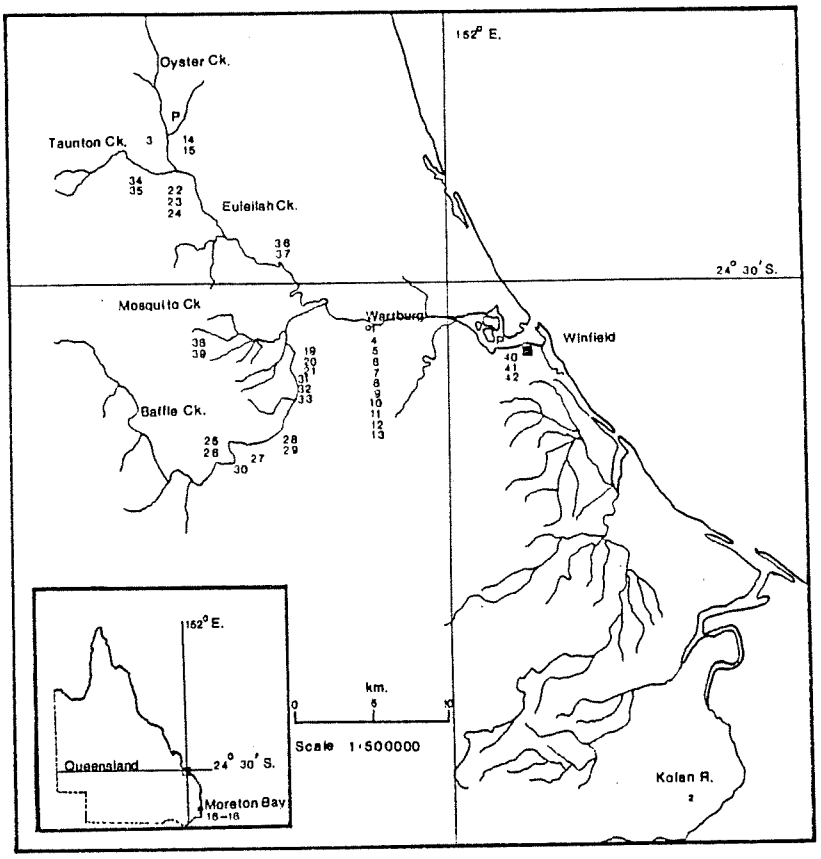
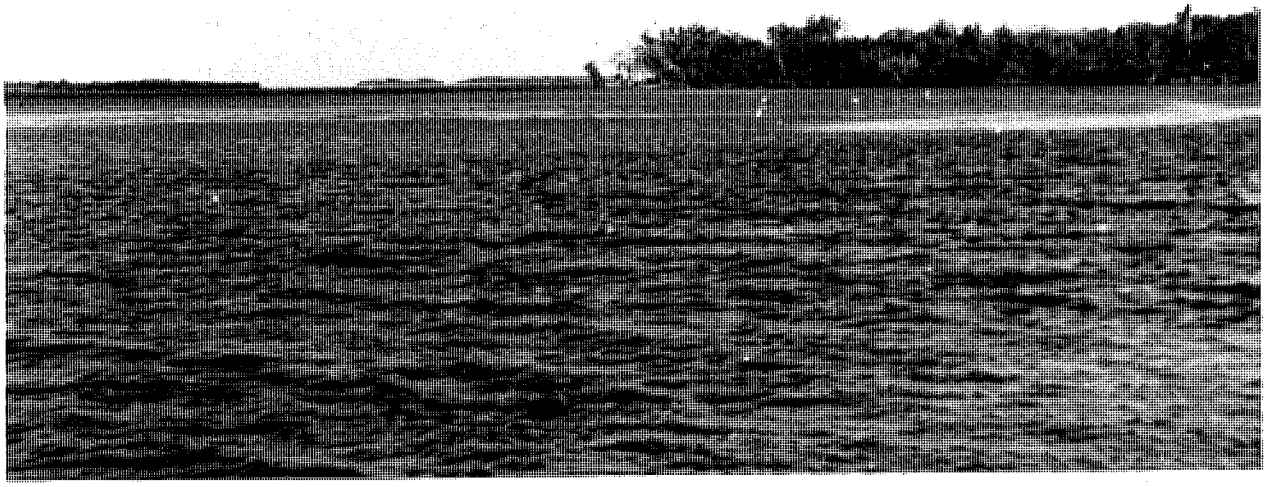


Figure 3.2: **Map of the Study Area.**

Plankton collection sites are indicated by a **"P"**; and **Fish** specimens by numbers **1-42.**

Plate 3.1: *Top,* Site [1.], Winfield, an Estuarine Habitat; *Bottom,* Site [2.], Baffle Ck., at Wartburg, a Brackish-Water Habitat.



3.6 Environmental Sampling.

3.6.1 Sample Collection.

Collection sites are shown on Figure 2.1. Environmental material was collected only once. Samples from fish and their epifauna were taken over a period of about fourteen months.

3.6.2 Abiota.

3.6.2.1 Water.

Portable salinity **(T.P.S, 2-03-17)** and pH **(Jones Scientific Instruments)** meters were used to measure temperature, salinity and pH, on site at Winfield [Site 1.], Wartburg [Site 2.] and Oyster Ck [Site 3.]. All water samples were taken at a depth of one meter using a ZoBell (1941) type depth sampler. Samples for dissolved oxygen were collected without agitation in 250 ml ground glass-stoppered bottles. Dissolved oxygen was fixed immediately by adding 2 ml of each of the more dense alkali-iodide azide, and manganous sulphate reagents (3.4.4.01) and the bottles mixed by inverting 30 times. Samples of water for bacteriological examination were collected from Sites [1.] and [2.] aseptically in 2.5 l sterile bottles.

3.6.2.2 Sediments.

Inverted gamma-sterilized polythene bags were used to take and store *ca* 100 g of the oxidized layer from the top 1cm of the inter-tidal sedimentary profile from Sites [1.] and [2.].

3.6.3 Biota.

3.6.3.1 Benthos.

Aquatic plants (algae), detritivores (polychaete, crabs and prawn) and filter feeders (bivalve molluscs) were taken from Sites [1.] and [2.], and placed in separate labeled sterile polythene bags or enriched directly (3.7.2.4.1).

3.6.3.2. Plankton.

Plankton including plants, hervivores and detritivores, (diatoms and copepods) was gathered at Sites [1.] and [3.] in a 200 mesh (*ca* 0.01 mm grid) plankton-net trawled for 500 meters at *ca* 5 knots and decanted to sterile 750 ml jars.

3.6.3.3 Fish and Epifauna.

Infected and control fish representing carnivorous *[P.sheridani* & *N.come]* and herbivorous *[M.cephalus]* species were numbered and identified, and placed whole into sterile polythene bags or the skin swabbed directly. Swabs were stored on Amies transport medium, **(Medical Wire and Equipment)**, (Appendix 2). Parasitic epifauna taken from fish were transferred directly to enrichment media, or placed in a vial of transport medium. Most swabs from fish, or vials with epifauna (see below) were collected by a field agent, (C. Kirchner), who specified origins, prevailing weather conditions, estimated salinity by taste, identified affected fish species, described location and development stage of lesion(s), made qualitative assessment of "disease" incidence, and noted if external parasites were present, according to the schedule shown in Plate 3.2.

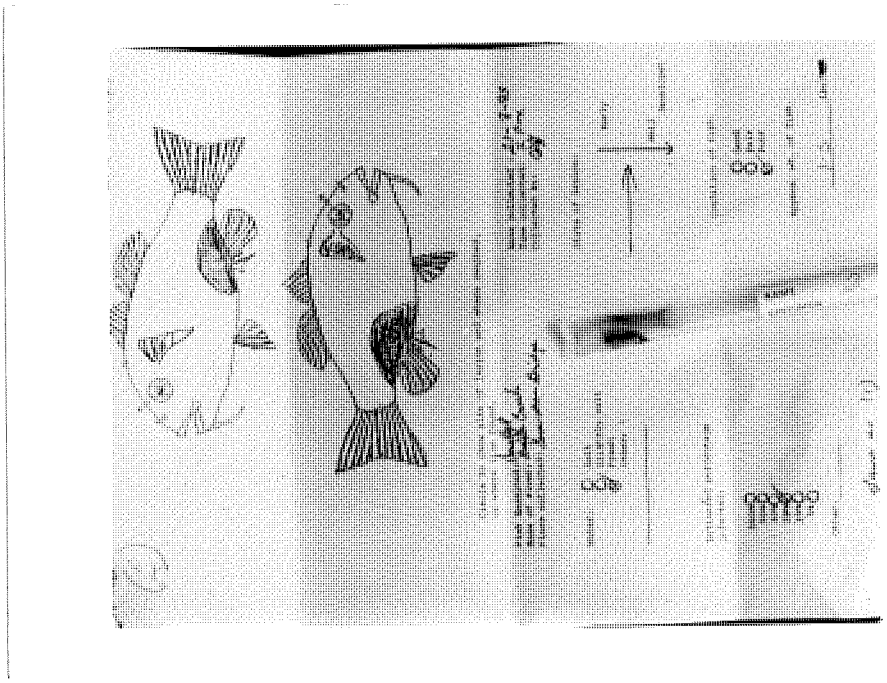


Plate 3.2: Transport Medium Vial with Field Pathology Report.

Specimen descriptions accompanying fish isopod epifauna detailed the same environmental observations, and noted if fish hosts were RS affected or unaffected, and usually if the isopods were feeding, or engorged.

Details of fish, their pathology, and of loci for bacteriological sampling and prevailing environmental conditions are shown in Table 3.6.

3.6.4 Sample Storage.

Environmental material collected by the author was stored at 4°C. and processed at the laboratory within 40h. Specimens of unidentified biota were collected by C.Kirchner were dispatched by normal post in transport medium usually within 8h, but always within 24h, received within 48h of collection and processed immediately upon receipt, (Appendix 2.).

3.6.5 Determination of Dissolved Oxygen, (Adapted from APHA, 1975).

The water sample with fixed dissolved oxygen was acidified with 2 ml concentrated sulphuric acid, and 200.0 ml of solution was taken and stained with *ca* 1 ml starch indicator, and titrated against freshly prepared 0.0250 N sodium thiosulphate (3.4.4.01) until clear. The volume (ml) of titrant consumed was equivalent to the original concentration (mg/l) of dissolved oxygen.

3.7 Bacterial Isolation and Presumptive Identification of *Vibrionaceae*.

3.7.1 Experimental Design.

Because media for isolation of enteropathogenic *Vibrionaceae* have only limited reported value for recovery of the known fish pathogen *V.anguillarum* (2.10.2), and because there is little or no information regarding recoveries of other unidentified or potential environmental fish pathogens, a variety of different isolation protocols were used, initially for comparison of selectivity against non-*Vibrionaceae,* and then for examination of comparative species distributions amongst the classified samples of isolates.

Four direct isolation media were used. The selective criteria relative to "non-specific" nutrient agar for the first two of these, TCBS (3.4.2.2), and Simidu (3.4.2.1), were described in (2.10.2). Later isolation media were based on SENA (with 2 % salt), and SENA also with variable concentrations of the iron-chelator (and potential virulence-selective agent) EDDA (3.4.2.3) incubated aerobically and anaerobically.

Two enrichment broths were used in conjunction with all of the above media;

BTB-Teepol (3.4.2.5) selective by the presence of salt, sucrose and a surfactant, and ATW (3.4.2.4) selective only by alkaline and saline conditions.

All bacterial cultures were obtained by the general protocol shown in Figure 3.3. Strain origins, isolation protocols as set out below and results of primary characterization as defined below are set out in Table 3.7.1-2. Parallel isolations were performed initially onto Simidu (Plate 3.3) and TCBS and later also onto SENA, and SENA with EDDA. Unless otherwise specified incubations on aerobic and anaerobic, media were for 48 h.
Pure cultures of resultant provincial isolates were assayed for their postures toward twelve morphological and biochemical characters, Gram reaction, fermentation, oxidase reaction, motility, polar flagellation, cell shape, gas from glucose, weak or strong catalase reaction, agar hydrolysis, luminescence and presence of poly-β-hydroxybutyrate granules. Attribute profiles from these provincial isolates were encrypted according to the octal system shown in Table 3.7.1 to produce the first four digits of a code. A final binary digit indicated for the purposes of this study whether a strain was accepted as a presumptive member of the *Vibrionaceae.* These results were tabulated together with isolation protocols in Table 3.7.2.

Provincial isolates intrinsically all grew in the presence of 2 % salt and were further classified as presumptive *Vibrionaceae,* according to flexible criteria. All strains which conformed to the general pattern for the family i.e. Gram-negative rods which are fermentative and have a positive oxidase reaction were included, but additional latitude was permitted to include profiles such as would be generated by atypical species e.g. from oxidase-negative species such as *V.metschnikovii* and *V.gazogenes* which can also produce gas from glucose, and the similarly aerogenic but oxidase positive species *A.hydrophila* and the non-motile species *A.punctata.* Peritrichous flagellation was considered as a negative indicator only when accompanied by gas production or by the absence of polar flagella and oxidase activity.

Presumptively identified provincial isolates recovered from each of the selective media were compared by the Chi-square test (χ^2) to determine which protocol most successfully isolated presumptive *Vibrionaceae.* Later diagnosis (3.8) of different protocols was made according to the distributions of species obtained.

To assay selective inhibition and non-specific recovery of *Vibrionaceae* by Simidu and TCBS media these were compared with SENA for their ability to support growth of 86 *Vibrionaceae* type and reference cultures.

3.7.2 Bacterial Isolation Methods.

3.7.2.1 Waters.

3.7.2.1.1 Portions of water (100 ml) were filtered through sterile 0.2 µm pore size 47 mm membrane filters *(Millipore)* and the membranes placed in ATW (3.4.2.4) and BTB-Teepol broths and incubated 24 h and growth was taken and swabbed to half plates of Simidu and TCBS isolation media, streaked and incubated 48 h.

3.7.2.1.2 A 0.1 ml sample was swabbed to half plates of TCBS and Simidu media and streaked with an inoculation loop.

3.7.2.2 Sediments.

3.7.2.2.1 Two gram of sediment were placed in 20 ml of ATW and BTB-teepol enrichment broth and incubated 24 h and a loopful of growth was streaked onto TCBS and Simidu agar media.

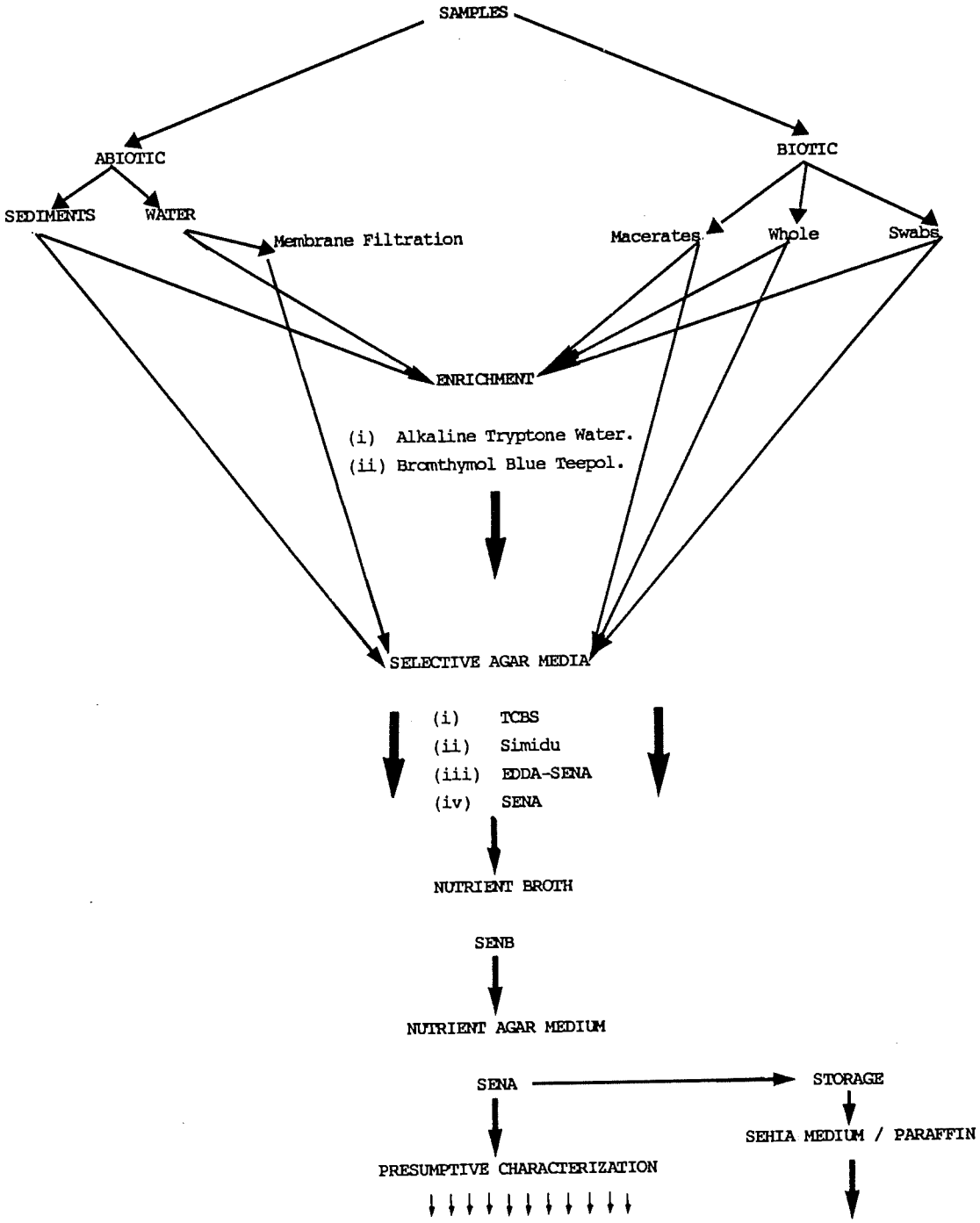
3.7.2.2.2 Swabs from the sediment were streaked to half plates of Simidu and TCBS selective agar media, and incubated 48 h.

3.7.2.3 Plankton.

Copepods and diatoms predominated in plankton from Sites [1.] and [3].

3.7.2.3.1 Raw Plankton from Sites [1.] and [3.] was filtered onto a number 4 filter **(Whatmans)** and *ca* 0.5 g incubated in each of ATW and BTB-teepol enrichment media for 24 h when a loopful of growth was streaked to Simidu and TCBS media.

Figure 3.3.1: **Isolation and Enrichment Protocol.**



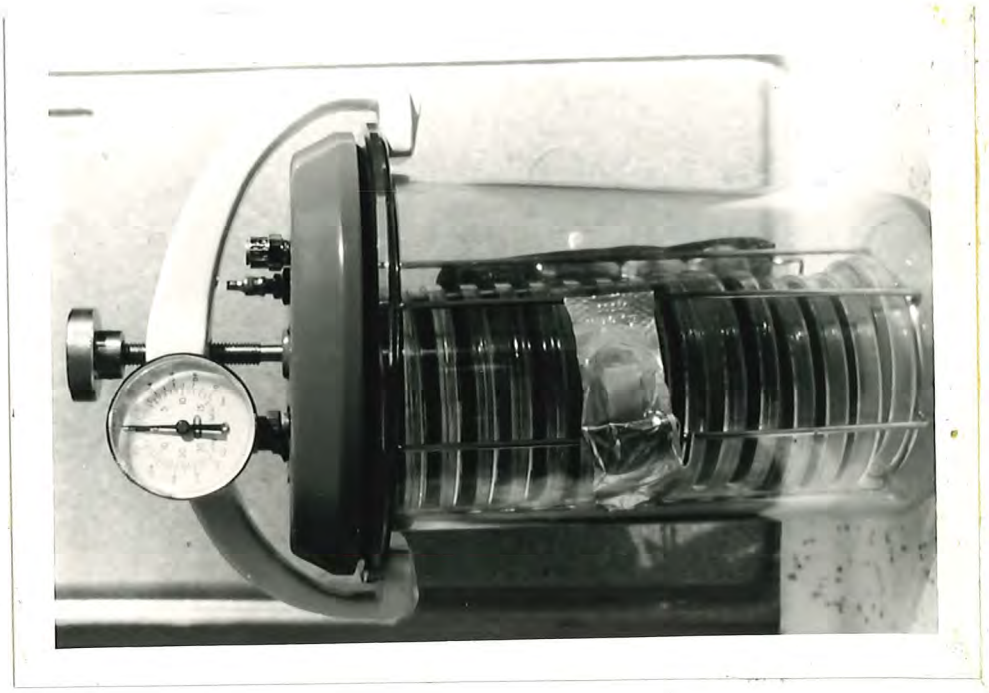


Plate 3.3: Anaerobic Chamber with Plates of Simidu Medium.

3.7.2.3.2 Filtered Plankton from 3.7.2.3.1 was swabbed onto half-plates of TCBS and Simidu media and these inoculum smears were streaked.

3.7.2.3.3 Copepods.

Copepods were identified by J.Greenwood. Site [1.] copepods were principally from *Order Calanoidea* and comprised the species *Acrocalanus gibber, Acrocalanus gracilis, Paracalanus parvus, Pseudodiaptomus mertoni, Bestiola similis;* and a Cyclopoid species *Oithona* sp. Only two species of copepods, both calanoid were isolated from Site [3.] these were identified as *Gladioferens pectinatus* and a taxonomically distinct species from *Family Centropagidae.*

The raw plankton sample from Site [1.] was placed into a beaker in the path of a narrow-beamed bench-lamp. Copepods attracted to this light were drawn off with a pasteur pipette. Twenty of these were placed into separate centrifuge tubes with 10 ml marine phosphate buffered saline (MPBS-2.4.3.1) and washed by centrifugation for 10 min at 800 g. The copepods were drawn into fresh pasteur pipettes and separately placed 5 per plate on two plates of TCBS and Simidu agar media.

3.7.2.4 Benthos.

3.7.2.4.1 Algae.

The alga *Gracilaria verrucosa*, (identified by A.B.Cribb), from Site [2.] was placed into ATW immediately upon sampling and its flora subcultured after 24 h by streaking to TCBS and Simidu media.

3.7.2.4.2 Polychaete.

An unidentified nereid polychaete, from Site [2.] was triturated directly in a sterile mortar containing *ca* 5 g of sterile quartz sand. The macerate was suspended in an equal volume of MPBS and swabbed to half plates of TCBS and Simidu media, streaked and incubated 48 h. The macerate suspension was then diluted in an equal volume of ATW and BTB-teepol double strength enrichment media with incubation periods and subculturings onto selective media as in (3.7.2.1.2).

3.7.2.4.3 Lamellibranch Molluscs.

Lamellibranch molluscs (bivalves) were taken from Site [1.], *[Crassostrea commercialis*, (Sydney rock oyster)], and Site [2.], *[Modiolus* sp. (mussels, identified by J.Stanisic)]. Shells were opened and discarded and the soft tissues from individual bivalves treated as in (3.7.2.4.2).

3.7.2.4.4 Decapod Crustaceans.

Decapod crustaceans were obtained from Sites [1.] and [2.]. At Site [1.] two soldier crabs, *Mictyris longicarpus,* were captured from a mudbeach. At Site [2.] a swimming crab, *Varruna littorata,* (Identified by P.Davy), and a banana prawn, *Penaeus merguiensis,* were taken.

Carapaces were removed and digestive glands aseptically punctured, swabbed and half plates of TCBS and Simidu agar media inoculated and streaked. Digestive glands swabs were then inoculated to enrichment media and sub-cultured as in (3.7.2.1.2).

3.7.2.5 Fish and Epifauna.

3.7.2.5.1 Whole Fish.

Diseased and control fish were humanely sacrificed, weighed, measured and their overall condition was described. Also noted, if present were lesion location(s) and development stage(s), production of excess mucus, erosion of fins, scale erection or scale loss, swelling or reddening of vent, opacening of eyes and oedema. Swabs were taken from surface lesions of affected fish or from the unaffected skin of control fish. Gills were examined for paleness (anaemia), physical condition, and the presence of large isopod parasites.

The body cavity was aseptically opened and the condition of organs and presence or absence of epi-visceral parasites and fat deposits was noted.

The gall bladder was examined for colour and turbidity, and where atypical (turbid, or not green), it was punctured and swabbed. Usually the posterior kidney was aseptically punctured and a swabbed. Swabs for bacterial inocula were also taken from the liver, spleen, and with moribund inappetant fish, also from the posterior gut.

The fish's tail was swabbed with 70 % alcohol and severed with a sterile scalpel and the stub blotted to deposit blood and interstitial fluid on TCBS agar.

Direct microscopic examination for bacteria, and of blood cell age, were undertaken respectively from posterior kidney, and spleen smears from moribund fish.

3.7.2.5.2 Fish Swabs.

Swabs inoculated from Fish (1-13), (Table 3.6) were incubated in enrichment media 24 h and a loopful of culture was transferred to plates of Simidu and TCBS media and streaked. Paired enrichment was by interchanging the swab and vial with components from a fresh vial of transport medium, and decanting *ca* 5 ml sterile ATW and BTB-teepol enrichment media separately to each vial and incubating. Sub-cultures to selective agar media were as in (3.7.2.1.2).

For Fish (14-42), swabs were smeared directly onto selective media and incubated after streaking as follows: Simidu for 48 or 72 h, TCBS 48 h, SENA 48 h, and EDDA (3.4.2.3) in the presence and absence of oxygen 48 and 72 h respectively. Enrichments from these swabs were for 24h in ATW, but sub-cultures were made to all selective media with incubation periods as for primary inoculations.

3.7.2.5.3 Epifauna.

Two isopod species of fish epifauna were sampled, these were a cymothoid species, *Cymothoa indica* (Identified by C.Jones), taken from whiting, *Sillago ciliata,* and a cyralanid species, *Pseudolana dactylosa,* (Plate 3.4), (Identified by A.J.Bruce) from mullet, *Mugil cephalus;* boney bream, *Nemataloşa come;* king salmon, *Polydactylus sheridani,* and from a brackish-water sand bank.

Internal bacterial flora from the female cymothoid Isopod (ex Fish 16) was obtained by aspirating fluid from its gut with a 27 gauge sterile needle, into a 1 ml syringe (Terumo) and spotting this inoculum onto plates of TCBS and Simidu media and streaked.

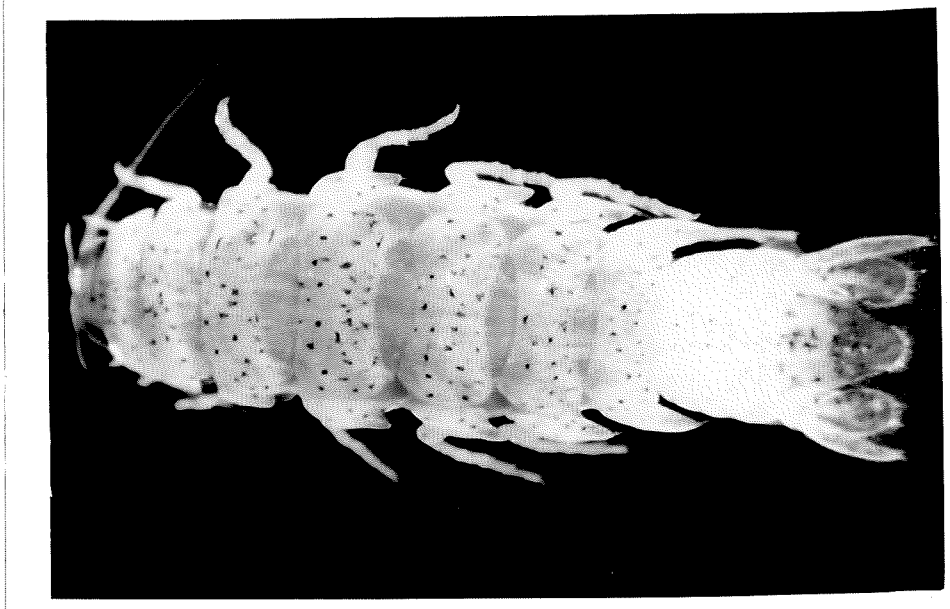


Plate 3.4: Engorged Cyralanid Isopod, *Pseudolana dactylosa.*

Cyralanid Isopod (1) was captured from a brackish-water sand bank and transferred directly to ATW. Isopod (1a) was taken from a mullet (Fish 12) and submitted for identification. Cyralanid Isopods (2) and (3) were taken from an unaffected king salmon, *P.sheridani,* at the same time as the RS affected Fish (22-24), no mention was made by C.Kirchner as to whether these isopods were feeding. Isopod (2) was macerated as in (3.7.2.4.2) and the Isopod (3) transferred whole to separate enrichment in ATW for 24 h and a loopful of growth was sub-cultured to TCBS agar.

Isopod (4) was taken from the RS affected mullet (Fish 31) the gut contents were aseptically aspirated as above, swabbed to half plates of TCBS, SENA, and EDDA media; streaked, and incubated 48 h.

Four engorged Isopods (4a-8) were selected from 30 recovered from two unaffected fish, (boney bream, and mullet), taken at the same time as when swabs from RS affected mullet Fish (34) and (35) and their gut contents aspirated as above. Aspirate from Isopod (4a) was inoculated to both SENA and TCBS media. Other aspirates were inoculated only to SENA.

Isopods (9), [engorged] and (10), [unfed] were obtained from an unaffected boney bream. The gut of the engorged isopod was aspirated as above while the unfed isopod was first infused with MPBS before drawing off an inoculum. Parallel inoculations of 0.1ml aspirate were made to ATW and BTB-teepol enrichment broths and these were incubated 18 h before dilution streaking growth to SENA and incubating a further 24 h. Direct inoculations were also made to half plates of 0.5, 0.75 and 1.0 μM EDDA (3.4.2.3.). These were streaked and incubated 48 h.

3.7.2.6 Presumptive Screening.

Representative colony types from selective agar media either obtained by direct or secondary isolation after enrichment, were picked by loop to SENB and incubated 24 h. These were streaked to plates of SENA confirm purity and restreaked where necessary. Growth from these pure plates was used to ascertain strain posture according to the following presumptive screening criteria, and to inoculate sodium-enriched heart infusion agar, SEHIA, storage slopes.

3.7.2.6.01 Gram Reaction (Gregersen, 1978).

A nichrome loopful of growth was emulsified in 0.05 ml 3 % potassium hydroxide on a microscope slide and slowly withdrawn. The presence of a DNA string was recorded as an indication of Gram-negative bacteria. No string was recorded as a Gram-positive bacterium.

3.7.2.6.02 Glucose Fermentation.

Paired 12 X 75 mm tubes containing 3 ml marine oxidation fermentation (3.4.4.12) were held at 60°C. for 2 h to expel dissolved oxygen, cooled for 20 min and stab inoculated with growth. One tube from each pair was overlaid with a sterile molten agar plug (3.4.4.10 (c)). Fermentative strains produced an acid (yellow) reaction in both sealed and open tubes in up to 7 d. Oxidation of glucose was indicated by yellow colour at the air interface of open tubes only. Strains which produced blue colouration were scored as oxidative. Repeat determinations with a heavier inoculum were performed for cultures which produced intermediate yellow green colouration or no colour change.

3.7.2.6.03 Oxidase Reaction.

Growth was spread onto oxidase test strips (3.4.4.04) with sterile tooth-picks, a purple colouration within 10 s was scored as oxidase-positive.

3.7.2.6.04 **Motility.**

Growth was used to inoculate SEHIA slopes in bijoux and these were incubated *ca* 3 weeks. A drop of SENB was run over the growth and incubated 2 h. From this drop a loopful of growth was taken and placed on a microscope cover glass, inverted and the hanging drop viewed by oil immersion phase contrast microscopy for motility at X 1 000. When motility could not be readily determined, growth was taken from SEHIA slopes and inoculated to 2 ml SENB and incubated and examined as above. Results were recorded according to the presence (positive) or absence (negative) of motility.

3.7.2.6.05 **Swarming Growth.**

Cultures on SENA were examined for occurrence of swarming growth.

3.7.2.6.06 **Polar Flagellation.**

Cells from 3.7.2.6.04 were further observed to determine if motility derived from the action of peritrichal- or polar- distributed flagella . Peritrichous flagellation was distinguished by producing smooth and uniform cellular movements compared to polar flagellation which caused random, individually jerky and collectively seething (elixate) movements of cells.

3.7.2.6.07 **Cell Shape.**

Cells from 3.7.2.6.04 were classified according to whether they were rod shaped or coccoid.

3.7.2.6.08 **Gas from Glucose.**

The sealed tubes from above were examined for agar splitting. Strains which caused splitting in up to 7 d were scored as gas producers.

3.7.2.6.09 **Strong Catalase Reaction.**

Growth was flooded with 3 % hydrogen peroxide (Ajax) and the presence of immediate strong effervescence from colonies was scored as positive. No effervescence or only a weak reaction was coded as negative.

Table 3.4: Reference and Type Cultures.

Species	UQM Accession	Other Codes	Source	Sender	Ref. No.	Batch No.
**Aeromonas hydrophila*	UQM 2768	ATCC 7966 NCTC 8049	Canned milk.	M. O'Brien	[1]	2
A.hydrophila anaerogenes	UQM 2769	ATCC 15467	Oil Emulsions.	M. O'Brien	[1]	1,6
A.punctata	UQM 156		CBVNCC			ND
Escherichia coli	UQM 1803	ATCC 11775 B		NCTC		ND
**Photobacterium angustum*	UQM 2893	ACMM 57 ATCC 25915	Seawater.	ACMM	[1]	4
**P.fischeri*	UQM 2889	ACMM 123 ATCC 7744	Seawater.	ACMM	[1]	4,7
**P.leiognathi*	UQM 2892	ACMM 59 ATCC 25521	Leiognathid Light organ.	ACMM	[1]	4
P.phosphoreum	UQM 0140		Spoiled fish.	Unknown	[1]	4
**P.phosphoreum*	UQM 2482	ATCC 11040 NCMB 1282	Seawater.	Unknown		
**Plesiomonas shigelloides*	UQM 1134	NCIB 1282 ATCC 14029		NCTC	[1]	5
P.shigelloides	UQM 1617		Creek.	G.H.G. Davis		7
Streptococcus agalactiae	UQM 1737			NIH		ND
**Vibrio aestuarianus*	UQM 2920	ATCC 35048	Oyster, Oregon.	ATCC	[2]	8
V.alginolyticus	UQM 2675	ACMM 87 CDC B4185	Blister Infection, Florida.	ACMM	[1]	4
**V.alginolyticus*	UQM 2770	ATCC 17749	Spoiled Fish.	M. O'Brien		1,6,6a,
**V.anguillarum*	UQM 2628	ACMM 114	*Gadus morhua,* Nares	C. Garland	[1]	6,6a
**V.anguillarum*	UQM 2771	ATCC 19264	*Gadus morhua,* Nares	M. O'Brien		All
**V.campbellii*	UQM 2543	ATCC 25920	Seawater,	ATCC	[1]	4
**V.campbellii*	UQM 2543	ATCC 25920	800m Hawai.	M. O'Brien	[1]	1,6
**V.carchariae*	UQM 2919	ATCC 35084	*Charcarhinus plumbeus.*	ATCC	[3]	6
V.cholerae	UQM 2441		Logan River, Q.	QHD	[1]	2
V.cholerae	UQM 2442		Blood Culture, Q.	QHD		2
V.cholerae	UQM 2772	P688 (West)	Sediment.	M. O'Brien		4
**V.cholerae*	UQM 2773	ATCC 14035	Human faeces.	M. O'Brien		6,6a
**V.costicola*	UQM 2888	NCMB 701	Bacon curing brine.	ACMM	[4]	4,7
V.damsela	UQM 2853	ATCC 33537 CDC 2588	Human puncture wound.	PMD	[5]	2,7
**V.diazotrophicus*	UQM 2780	ATCC 33466	Sea Urchin.	M. O'Brien	[6]	2,7
**V.fluvialis*	UQM 2774	NCTC 11327 ATCC 33809	Human faeces.	M. O'Brien	[1]	1,6
**V.furnissii*	UQM 2775	NCTC 11328	River water, England.	M. O'Brien	[1]	1,6
**V.harveyi*	UQM 2766	ACMM 172 ATCC 14126	Dead amphipod.	J.L.Reichelt	[1]	4
**V.harveyi*	UQM 2781	ATCC 14126	Dead amphipod.	M. O'Brien		1,6
V.hollisae	UQM 2852	ATCC 33565	Human faeces.	PMD	[7]	7
**V.mediterranei*	UQM 3076	CECT 621	Seawater, Spain.	E. Garay	[8]	8
**V.metschnikovii*	UQM 211	NCTC 8443	Fowl.	QIMR	[1]	1,6
**V.mimicus*	UQM 2954	ATCC 33653	Human ear.	PMD	[9]	7,8
**V.natriegens*	UQM 879	ATCC 14048	Salt-marsh mud.	ATCC	[1]	4,7
**V.natriegens*	UQM 2782	ATCC 14048	Salt-marsh mud.	M. O'Brien	[1]	1,6,6a
**V.nereis*	UQM 2783	ATCC 25917	Seawater, Hawaii.	M. O'Brien	[1]	1,6
**V.nigripulchritudo*	UQM 2784	ATCC 27043	Seawater, Hawaii.	M. O'Brien	[1]	1,6,7,8

Table 3.4; (Cont.): Reference and Type Cultures.

Species	UQM Accession	Other Codes	Source	Sender	Ref. No.	Batch No.
**V.ordalii*	UQM 2890	ACMM 121 ATCC 33509	*Oncorhynchus kisutch*, kidney.	ACMM	[1]	7,8
V.ordalii	UQM 2906	ACMM 117	*O.kisutch.*	ACMM		5,7
**V.orientalis*	UQM 2921	ATCC 33934	Water, Yellow Sea.	ATCC	[10]	6,7
V.parahaemolyticus	UQM 1399	RCBB DUHIG	Human?	J. O'Brien	[1]	6
V.parahaemolyticus	UQM 2125	QACL J113	Seawater.	QACL		4,6
V.parahaemolyticus	UQM 2126	QACL 3385	Borewater.	QACL		4,6
V.parahaemolyticus	UQM 2200	AGAL 145 NCTC 10884	Seafood.	AGAL		4,6
V.parahaemolyticus	UQM 2201	AGAL 136 NCTC 10885	Oysters.	AGAL		4,6
V.parahaemolyticus	UQM 2776	ATCC 17802	Boiled Sardines.	M. O'Brien	[1]	1,6
**V.pelagius*	UQM 2785	ATCC 25916	Seawater, Hawaii.	M. O'Brien	[1]	1,6,7,8
**V.proteolyticus*	UQM 2472	ATCC 15338	*Limnoria punctata* gut.	ATCC	[1]	7
**V.splendidus*	UQM 2786	ATCC 25914	Marine fish.	M. O'Brien	[1]	1
**V.tubiashii*	UQM 2923	ATCC 1909	Juvenile clams.	ATCC	[11]	6,7
**V.vulnificus*	UQM 2778	ATCC 27562	Human blood.	M. O'Brien	[1]	1,6a
V.vulnificus Biogroup II	UQM 2922	ATCC 33148	*Anguilla japonica.*	ATCC	[12]	6

* Denotes type culture.

Abbreviations for Originating and Concurrent Culture Collections.

ACMM Australian Collection of Marine Microorganisms.
AGAL Australian Government Analytical Laboratories.
ATCC American Type Culture Collection, USA.
CDC Center for Disease Control, USA.
CECT Collecion Espanola de Cultivos Tipo, Spain).
CBVNCC C.B.Van Niels Culture Collection, Ca. USA.
NCIB National Collection of Industrial Bacteria, UK.
NCMB National Collection of Marine Bacteria, UK.
NCTC National Collection of Type Cultures, UK.
NIH National Institute of Health, Wellington, NZ.
M. O'Brien M.O'Brien for R.R.Colwell, Department of Microbiology, University of Maryland, USA.
PMD P.M.Desmarchellier (Personal Collection).
QACL Queensland Agricultural College, Lawes.
QHD Queensland Health Department.
QIMR Queensland Institute of Medical Research.
RCBB Red Cross Blood Bank (Brisbane).
UQM University of Queensland Microbiology Department.

Published Descriptions [n] for Type and Reference Cultures.

[1] Baumann and Schubert (1984).
[2] Tison and Seidler (1983).
[3] Grimes, Stemmler, Hada, May, Maneval, Hetrick, Jones, Stoskopf and Colwell (1984).
[4] Garcia, Ventosa, Ruiz-Berraquero and Kocur (1987).
[5] Love, Teebkin-Fisher, Hose, Farmer, Hickman and Fanning (1981).
[6] Guerinot, West, Lee, and Colwell (1982).
[7] Hickman, Farmer, Hollis, Fanning, Steigerwalt, Weaver, and Brenner (1982).
[8] Pujalte and Garay (1986).
[9] Davis, Fanning, Madden, Steigerwalt, Bradford, Smith, and Brenner (1981).
[10] Yang, Yeh, Cao, Baumann, Baumann, Tang (1983).
[11] Hada, West, Lee, Stemmler and Colwell (1984).
[12] Tison, Nishibuchi, Greenwood and Seidler (1982).

Table 3.5: Tentatively Identified Bacteria Additionally Investigated.

Putative Identity	Source*	Sender Ref.	Strain Origin.	Access Code	Batch No.
Vibrio sp.	AFHRL	FD 57	Water, Singapore.	Be 04	5
Vibrio sp.	.	FD 151	Goldfish, Singapore.	Be 06	5
Vibrio sp.	.	FD 337	"Ramc" and "Barbs" Singapore.	Be 07	5
Vibrio sp.	.	FD 349	Lemon Tetras, Singapore.	Be 08	5
Vibrio sp.	.	FD 350	.	Be 09	5
Vibrio sp.	.	FD 351	Angelfish, Singapore.	Be 10	5
Vibrio sp.	.	FD 352	Guppies, Singapore.	Be 11	5
Vibrio sp.	.	FD 353	Guppies, Singapore.	Be 11	5
Vibrio sp.	.	FD 359	Angel fish, Singapore.	Be 13	5
Vibrio sp.	.	FD 360	.	Be 14	5
Vibrio sp.	.	FD 362	.	Be 16	5
Vibrio sp.	.	FD 363	.	Be 17	5
Vibrio sp.	.	FD 364	.	Be 18	5
Vibrio sp.	.	FD 365	.	Be 19	5
Vibrio sp.	.	FD 367	Oranders, Singapore.	Be 20	5
V.anguillarum	DAMP	85.3475.1	Rainbow Trout, Tas.	DA 1	7
V.anguillarum	.	85.3954.1	Rainbow Trout, Tas.	DA 2	7
V.anguillarum	.	85.4067	Rainbow Trout, Tas.	DA 3	7
V.splendidus I	.	85.3928.2	Atlantic Salmon, Tas.	DA 4	7
V.splendidus I	.	86.0062	Atlantic Salmon, Tas.	DA 5	7
V.splendidus I	.	86.420.12	Rainbow Trout, Tas.	DA 6	7
V.harveyi	.	85.3961.6	Rainbow Trout, Tas.	DA 7	7
V.diazotrophicus	.	85.3961.2	Rainbow Trout, Tas.	DA 9	7
V.pelagius I	.	86.186.7	Atlantic Salmon, Tas.	DA 11	7,8
V.damsela	.	86.665.H	Leather Back Turtle, Tas.	DA 12	7
V.pelagius	.	85.368/1	Unspecified, Tas.	DA 13	7
Vibrio sp.	DASUT	B1	Active 9d oyster larva, Bicheno, Tas.	UQM 3012	4,7
Vibrio sp.	.	B2	Inactive 9d oyster larva, Bicheno, T.	B2	4,7
Vibrio sp.	.	B51	Hatchery Seawater, Bicheno, Tas.	B51	4,7
Vibrio sp.	.	B55	Oyster Spat, Bicheno, Tas.	B55	4,7
V.anguillarum	.	HP3 S7	Marine sediment, S.E. Tas.	Gar 10	8
V.anguillarum	.	Bi3 S16	Marine sediment, S.E. Tas.	Gar 20	8
V.anguillarum	.	Bi3 W16	Seawater, S.E. Tas.	Gar 30	8
V.anguillarum	.	Nu1 S19	Marine sediment, S.E. Tas.	Gar 40	8
V.anguillarum	.	N1 F1	*Red-Spot*, Rainbow trout, S.E. Tas.	Gar 60	8
V.anguillarum	.	N1 F4	.	Gar 70	8
V.anguillarum	.	N1 F6	.	Gar 80	8
V.damsela	.	Sy3 S4	Marine sediment, S.E. Tas.	Gar 90	8
V.ordalii	.	H1 S11	Marine sediment, S.E. Tas.	Gar 110	8
V.ordalii	.	Sh3 W6	Seawater, S.E. Tas.	Gar 120	8
V.tubiashii	.	HP3 S5	Marine sediment, S.E. Tas.	Gar 140	8
V.tubiashii	.	Sy3 S6	Marine sediment, S.E. Tas.	Gar 160	8
V.tubiashii	.	Sy3 S10	Marine sediment, S.E. Tas.	Gar 170	8
V.tubiashii	.	Bi2 S14	Marine sediment, S.E. Tas.	Gar 180	8
V.vulnificus	DAW	11	*Red-Spot* affected fish; ,DC 11	UQM 2743	1,6
V.anguillarum	.	14	Clarence river NSW. ,DC 14	UQM 2744	1,6
V.vulnificus	.	18	. ,DC 18	UQM 2740	1,6
Vibrio sp.	.	21	. ,DC 21	UQM 2839	1,6
V.vulnificus	.	29	. ,DC 29	UQM 2745	1,6
V.vulnificus	.	32	. ,DC 32	UQM 2741	1,6
V.cholerae Non 01	.	35	. ,DC 35	UQM 2742	1,6
V.cholerae .	.	276	Advanced ulcer *M.cephalus* ,DC 276	UQM 3061	8
V.cholerae .	.	FD 160	Gut of ulcerated *M.cephalus*	FD 160	8
V.cholerae .	.	FD 161	Gut of control *M.cephalus*	FD 161	8
V.cholerae .	.	FD 162	Gut of ulcerated *M.cephalus*	FD 162	8
V.anguillarum	DMUQ	DMUQ 9131	Mud, Oxley Ck., Sherwood, Q.	DMUQ 9131	5
V.anguillarum	.	DMUQ 9221	Plant, Brisbane R. Chelmer, Q.	DMUQ 9221	5

Table 3.5: Tentatively Identified Bacteria Additionally Investigated. (Cont.)

Putative Identity	Source*	Sender Ref.	Strain Origin.	Access Code	Batch No.
V.anguillarum	.	DMUQ 9233	Mud, Oxley Ck., Sherwood, Q..	DMUQ 9233	5
V.anguillarum	.	DMUQ 9446	Mud, Brisbane R. Mt. Crosby, Q.	DMUQ 9446	5
V.anguillarum	.	DMUQ 9558	Mud, Oxley Ck., Sherwood, Q.	DMUQ 9558	5
Vibrio cholerae	FM	FM 05205	Shell Oysters, Woolowere Bay,NSW.	UQM 2729	2
V.cholerae	.	FM 05206	Marine Sediment, Woolowere Bay, NSW.	UQM 2730	1,6
V.cholerae	.	FM 05207	Shell Oyster, Murphy's Bay, NSW.	UQM 2731	4,7
V.cholerae	.	FM 05210	Seawater, Neverfail Bay, NSW.	UQM 2732	4,7
V.cholerae	.	FM 05213	Marine Sediment, Woolowere Bay, NSW.	UQM 2733	4
V.cholerae	.	FM 05214	Seawater, Woolowere Bay, NSW	UQM 2734	4
V.cholerae	.	FM 05228	Seawater, Murphy's Bay, NSW.	UQM 2735	4
Alteromonas sp.	MICRO 1	JC 1	Mercury enriched Seawater, Q.	JC 1	7
Alteromonas sp.	.	JC 2	Mercury enriched Seawater, Q.	JC 2	7
Alteromonas sp.	.	JC 3	Mercury enriched Seawater, Q.	JC 3	7
A.hydrophila	MICRO 2	α-72	*Red-Spot, M.cephalus,* Bundaberg, Q.	UQM 2838	1,2
V.anguillarum	.	Σ 1017	*M.cephalus,* Liver, Bundaberg, Q.	UQM 2843	1,2,6,8a
V.alginolyticus	.	Σ 47	Seawater,MoretonBay,Q.	47	4
.	.	Σ 182	Terebellidpolychaete,MoretonBay,Q.	182	4
.	.	Σ 200	Sediment,MoretonBay, Q.	200	4
.	.	Σ 273	Sediment,MoretonBay, Q.	273	4,7
.	.	Σ 391	*Phyllodoce* sp., Moreton Bay, Q.	391	4
.	.	Σ 392	.		ND
.	.	Σ 459	Sediment,MoretonBay, Q.	459	4
V.gazogenes	.	Stn 180	Estuarine sediment, Winfield,Q.	UQM 2840	1,1
V.gazogenes	.	Stn 170	Estuarine sediment, Winfield,Q.	UQM 2842	2,2
V.harveyi	.	ϕ 1	Plankton, Moreton Bay, Q.	ϕ 1	ND
V.harveyi	.	ϕ 29	Plankton, Moreton Bay, Q.	ϕ 29	4
V.harveyi	.	Σ 36	*Crassostrea commercialis,*Moreton Bay.	UQM 2849	2,2
V.tubiashii	.	Σ 126	*Nereis diversicolor,* Moreton Bay,Q.	UQM 3281	4
Pseudomonas sp.	.	Stn 8190a	*Crassius auratus,*kidney,Brisbane.	8190a	7a
Micrococcus sp.	.	Stn 8660	.	8660	5
Shewanella sp.	.	Stn 8640	.	UQM 8640	5,6
Vibrio harveyi	MICRO 3	UQM 2855	*Acanthopagurus australis,*kidney,Q.	UQM 2855	2
Vibrio sp.	MICRO 4	Stn 9099	*Penaeus esculentus* uropod lesion, Q.	UQM 3249	8
Vibrio sp.	MICRO 5	Stn 6490	Seawater, prawn pond, Yamba,NSW.	6490	6
Vibrio sp.	.	Stn 6500	Seawater, prawn pond, Yamba,NSW.	6500	5,5
Vibrio sp.	.	Stn 6501	Seawater, prawn pond, Yamba,NSW.	6501	5
Vibrio sp.	.	Stn 6510	Seawater, prawn pond, Yamba,NSW.	6510	5,8
Vibrio sp.	.	Stn 6520	Seawater, prawn pond, Yamba,NSW.	6520	5,6
V.alginolyticus	WAMRL	MdB81	Pearl Oysters, Broome.	UQM 2722	ND
V.alginolyticus	.	MdA81	Pearl Oysters, Broome.	UQM 2723	4
V.harveyi	.	Kb981	Pearl Oysters, Broome.	UQM 2724	4

Key to Strain Origins.

* AFHRL J.Humphreys;Australian Fish Health Reference Laboratory,Benalla,Vic.
DAMP J.Carson; Department of Agriculture, Mt. Pleasant, Launceston, Tasmania.
DASUT C.Garland; Department of Agricultural Science, University of Tasmania, Hobart, Tas.
DAW D.Callinan; Department of Agriculture, Wollongbar, NSW.
DMUQ D.Myatt; Department of Microbiology, University of Queensland, St. Lucia, Q.
FM C.Blimka; CSIRO Division of Food Research, Sydney, NSW.
MICRO[*n*] Isolations at UQM from material provided by persons numbered:
[1]J.Camilleri,[2]S.Nearhos;[3]R.Lester;[4]M.O'Donahue;[5]J.Paynter.
WAMRL M.Mannion; Western Australian Marine Research Laboratory, North Beach, WA.

3.7.2.6.10 **Agar Hydrolysis.**

White colonies on SENA which sank into the agar within 24 h, and which made the surrounding medium fragile rather than elastic, and which clarified agar at the margins of growth were recorded as agar hydrolytic.

3.7.2.6.11 **Luminescence.**

Cultures which glowed in the dark after allowing up to five minutes for eye acclimatization, were recorded as luminescent.

3.7.2.6.12 **Presence of Poly-β-Hydroxybutyrate (PHB).**

Cells from 3.7.2.6.04 which contained refractive inclusions were recorded as producing PHB granules.

3.7.2.7 **Comparative Vibrionaceae Recovery on TCBS and Simidu Media.**

The type and reference cultures used had origins listed in Table 3.4 and the following UQM accession codes 211, 879, 1617, 2125-6, 2200-1, 2472, 2543, 2628, 2768-86, 2852-3, 2888-90, 2892-3, 2906, 2919-23. Tentatively identified environmental and fish isolated strains had origins as shown in Table 3.5 and were coded as follows UQM 2722, 2729-35, 2740-44, 2839-40, 2843, 2849, 2855, 3061, DA 1-3, 5-7, 9, 11-13, Be 4, 6-9, 10-14, and Be 16-20 was examined.

A loopful of growth from the eighty-six above listed cultures from SENA were separately streaked onto half-plates of Simidu, and TCBS agar media and the plates were incubated 48 h. Results were recorded according to whether or not these media supported growth.

3.7.3 **Results.**

3.7.3.1 **Bacterial Recovery from Inoculated Media.**

Inoculations were undertaken from 79 fish preparations including 50, (14 internal and 36 external), from RS affected or previously affected fish and 17, (13 internal and 4 external), from asymptomatic (control) fish. Autopsied control and diseased fish (Table 3.6) both carried encysted larvae of trypanorrynch cestodes on the outer surface of internal organs. Twelve

additional, (7 internal and 5 external), diseased fish inocula were obtained indirectly from Moreton Bay. Some of these in direct microscopy of internal tissues indicated bacteraemia (Table 3.6). Twelve inocula were obtained from Isopods, (3) external and (9) from gut aspirates. Additionally inocula were taken from ten calanoid copepods and 16 further preparations, 4 (abiotic and 12 biotic) of environmental material.

Bacterial presence in samples, as indicated by growth on selective agar media, was shown for all fish and environmental samples, inoculated under all protocols except the following; Fish 1, *M.cephalus* skin (BTB-teepol/TCBS), liver (ATW/TCBS & Simidu), kidney (ATW & BTB-teepol/TCBS & Simidu); Fish 2, *M.cephalus* skin (BTB-teepol/TCBS & Simidu); Fish 3, *N.come* skin, liver, kidney and gall (BTB-teepol/TCBS & Simidu), gall (ATW & BTB-teepol/TCBS & Simidu); Fish 4, *M.cephalus* kidney (BTB-teepol/TCBS & Simidu); Fish 5, *M.cephalus* skin (ATW/TCBS & Simidu) & (BTB-teepol/TCBS); Fish 6, *M.cephalus* liver (ATW/TCBS) & (BTB-teepol/TCBS & Simidu); Fish 7, *M.cephalus* (ATW/TCBS); Fish 8, *M.cephalus* skin (ATW/Simidu) & (BTB-teepol/TCBS & Simidu), kidney (ATW/TCBS) & (BTB-teepol/TCBS & Simidu); Fish 10, *M.cephalus* liver (BTB-teepol/TCBS), kidney (BTB-teepol/Simidu & TCBS); Fish 11, *M.cephalus* kidney (BTB-teepol/Simidu & TCBS); Fish 12 *M.cephalus* skin, liver & kidney, (BTB-teepol/Simidu & TCBS); Fish 13 *M.cephalus* skin (BTB-teepol/Simidu), scale pocket & liver (BTB-teepol/TCBS & Simidu) kidney (ATW/TCBS) & (BTB-teepol/TCBS & Simidu), from Isopod (4a) (TCBS & SENA), fro any separate preparations of copepods on TCBS and from copepods 1,9 and 10 on Simidu, or from sediment directly inoculated to Simidu medium.

A total of 996 bacterial isolates were cultured. Of these 97 were from abiotic samples, 588 from fish, and the residue from various biota. From the Baffle creek and Kolan river systems 136 isolates were cultured from asymptomatic (control) fish and 353 from diseased fish. The remaining 99 fish isolates were cultured from diseased Moreton Bay fish.

Table 3.6: Diseased and Unaffected Fish Assayed for Presumptive *Vibrios*.

Fish, Species. [Sample]	Origin. [Agent]. [Date].	Prevailing Conditions.	Lesion.	Gross Appearance and Pathology.
1. *Mugil cephalus* [Scalepocket] [Liver] [Kidney] [Spleen]	Baffle Ck. [25.02.85]	T 26°C. pH 7.7 Salinity- 22 p.p.t. D.O. 7.6mg/l	Early dermal ulcer.	590 g and 360 mm long. Kidney liver and spleen were not anaemic, but the fish appeared lean, and its condition poor.
2. *Mugil cephalus* [Skin lesion] [Scale pocket] [Scale pocket] [Liver] [Kidney]	Kolan R. [C.Kirchner] [25.02.85]	ND.	Medially developed dermal ulcer.	590 g and 360 mm long. The ulcer was 60 x 25 mm. The Liver appeared mottled and oedematious all other organs appeared normal except that there were Trypanorrynch cysts their surfaces.
3. *Nematalosa come* [Skin] [Liver] [Kidney] [Gall Bladder]	Oyster Ck. [C.Kirchner] [25.02.85]	T 22°C. pH 5.6 Salinity- 6 p.p.t. D.O. 3.8mg/l	Asymptomatic Control. (For observation of normal fish flora).	120 g and 210 mm long. Fins, gills, skin, liver, kidney, gall bladder and spleen appeared normal. No pathologic symptoms were evident.
4. *Mugil cephalus* [Liver] [Kidney]	Baffle Ck. [25.02.85]	T 26°C. pH 7.6 Salinity- 22 p.p.t. D.O. 7.6mg/l	Asymptomatic.	490 g and 360 mm long. Fins, gills, skin, kidney, and spleen appeared normal. The liver appeared mottled. No trypanorrynch were observed. The fish seemed in good condition.
5. *Mugil cephalus* [Scalepocket] [Kidney]	Baffle Ck. [25.02.85]	T 26°C. pH 7.6 Salinity- 22 p.p.t. D.O. 7.6mg/l	Early lesion with blood in scale pockets.	440 g and 340 mm long. Gills, skin, liver, kidney and spleen appeared normal. Trypanorrhynch cysts as in fish (2) were present. Some bleeding was present under scales and the adipose fins were damaged, but this may have been a netting injury.

Table 3.6; (Cont.): Diseased and Unaffected Fish Assayed for Presumptive *Vibrios*.

Fish, Species. [Sample]	Origin. [Agent]. [Date].	Prevailing Conditions.	Lesion.	Gross Appearance and Pathology.
6. *Mugil cephalus* [Vent lesion] [Peri-cephalic Scale pocket] [Liver] [Spleen]	Baffle Ck. [25.02.85]	T 26°C. pH 7.6 Salinity- 22 p.p.t. D.O. 7.6mg/l	Healing of head and vent lesions.	472 g and 350 mm long. Gills, skin, liver, kidney liver and spleen appeared normal. Trypanorrhynchs were not observed. Lesions were encroached by greater vascularization and scale regrowth. The vent had been repositioned in healing.
7. *Mugil cephalus* [Liver] [Kidney]	Baffle Ck. [25.02.85]	T 26°C. pH 7.6 Salinity- 22 p.p.t. D.O. 7.6mg/l	Asymptomatic Control.	467 g and 345 mm long. Gills, skin, liver, kidney liver and spleen appeared normal. Trypanorrhynch cysts were present but immature. The fish appeared in good condition.
8. *Mugil cephalus* [Ecchymosis] [Liver] [Kidney]	Baffle Ck. [25.02.85]	T 26°C. pH 7.6 Salinity- 22 p.p.t. D.O. 7.6mg/l	3-4 ecchymoses were present.	450 g and 350 mm long. Gills, skin, liver, kidney liver and spleen appeared normal. Trypanorrhynch cysts were present on viscera.
9. *Mugil cephalus* [Liver] [Kidney]	Baffle Ck. [25.02.85]	T 26°C. pH 7.6 Salinity- 22 p.p.t. D.O. 7.6mg/l	Asymptomatic Control.	495 g and 350 mm long. Gills, skin, liver, kidney liver and spleen appeared normal. Trypanorrhynch cysts were present on heart. Lesions were healing, and condition was good.
10. *Mugil cephalus* [Liver] [Kidney]	Baffle Ck. [25.02.85]	T 26°C. pH 7.6 Salinity- 22 p.p.t. D.O. 7.6mg/l	Asymptomatic Control.	535 g and 335 mm long. Gills, skin, liver, kidney liver and spleen appeared normal. Condition was good.
11. *Mugil cephalus* [Liver] [Kidney]	Baffle Ck. [25.02.85]	T 26°C. pH 7.6 Salinity- 22 p.p.t. D.O. 7.6mg/l	Asymptomatic. Control	498 g and 335 mm long. Gills, skin, liver, kidney liver and spleen appeared normal. Cysts were present on viscera, but condition was good.

Table 3.6; (Cont.): Diseased and Unaffected Fish Assayed for Presumptive *Vibrios*.

Fish, Species. [Sample]	Origin. [Agent]. [Date].	Prevailing Conditions.	Lesion.	Gross Appearance and Pathology.
12.*Mugil cephalus* [Scalepocket] [Liver] [Kidney]	Baffle Ck. [25.02.85]	T 26°C. pH 7.6 Salinity- 22 p.p.t. D.O. 7.6mg/l	Small dermal lesions.	507 g and 360 mm long. Gills showed scarring. The kidney, liver and spleen appeared normal. Healed lesions were present. An isopod, *Pseudolana dactylosa*, not initially observed was attached externally.
13.*Mugil cephalus* [Skin] (On site) [Scalepocket] [Liver] [Kidney]	Baffle Ck. [25.02.85]	T 26°C. pH 7.6 Salinity- 22 p.p.t. D.O. 7.6mg/l	Small dermal lesions. Healing.	602 g and 365 mm long. Gills showed scarring. Skin lesions were almost healed. All organs seemed normal. Cysts were present on viscera.
14.*Mugil cephalus* [scale pocket] (On site)	Oyster Ck. [C.Kirchner] [13.03.85]	Slightly salty water (by taste)	Early dermal lesions. (3)	ND.
15.*Mugil cephalus* [scale pocket] (On site)	Oyster Ck. [C.Kirchner] [13.03.85]	Slightly salty water (by taste)	Early dermal lesions. (1)	ND.
16.*Sillago ciliata* [Blood] [Liver] [Kidney]	Moreton Bay [D. Fenwick] [18.03.85]	Acid pH ? in aquarium.	Extensive Dermal Lesions. Fish moribund.	72 g and 220 mm long. Fish had exopthalma, fin erosion and loss of mucous. Skin lesions were not deep. A pair of isopods, *Cymothoa indica* were present in the buccal cavity. One attached to the tongue the other to an eroded gill arch. Food was not present in gut. The gills and liver were pale and anaemic. Gall bladder was clear rather than green. By microscopic examination no bacteria were observed in blood from the liver.

Table 3.6; (Cont.): Diseased and Unaffected Fish Assayed for Presumptive *Vibrios*.

Fish, Species [Sample]	Origin [Agent] [Date]	Prevailing Conditions	Lesion	Gross Appearance and Pathology.
17.*Sillago ciliata* [Fluid from Vent] [Ecchymosis] [Liver] [Kidney] [Spleen]	Moreton Bay [D.Fenwick] [18.03.85]	Acid pH ? in aquarium.	Extensive Dermal Lesions. Fish dead for 1 h.	80 g and 210 mm long. Fish had exopthalma, fin erosion and loss of mucus. Skin lesions were not deep. Vent red and ecchymoses near tail. Gut with only a pale yellow viscous fluid. This by microscopy revealed single vibriod cells and non-motile chains up to 25 µm long. Like bacteriawere seen in a kidneysmear.Some blood cells had irregular margins.The liver was pale oedematiousand fragile.
18.*Sillago ciliata* [Mucus] [Gut 7 cm from Vent] [Gut 1 cm from Vent] [Kidney]	Moreton Bay [D.Fenwick] [18.03.85]	Acid pH ? in aquarium.	Extensive Dermal Lesions. Fish moribund.	80 g and 210 mm long. Gross symptoms as for fish (16) and (17). Extensive non-necrotic lesion around the vent.Liver was pale and anaemic, by microscopy malformed blood cells were seen. The spleen was normal but no mature red blood cells were seen. Mucus showed almost pure suspension of polar rods. More bacteria were present in gut near the vent than 7 cm from it.
19.*Mugil cephalus* [Skin lesion] (On site)	Baffle Ck. [C.Kirchner] [19.03.85]	ND.	ND.	ND.
20.*Mugil cephalus* [Skin lesion] (On site)	Baffle Ck. [C.Kirchner] [19.03.85]	ND.	ND.	ND.
21.*Mugil cephalus* [Skin lesion] (On site)	Baffle Ck. [C.Kirchner] [19.03.85]	ND.	ND.	ND.
22.*Polydactylus sheridani* [Skin lesion] (On site)	Euleilah Ck. [C.Kirchner] [29.05.85]	ND.	ND.	ND.

Table 3.6; (Cont.): Diseased and Unaffected Fish Assayed for Presumptive *Vibrios*.

Fish, Species. [Sample]	Origin. [Agent]. [Date].	Prevailing Conditions.	Lesion.	Gross Appearance and Pathology.
23.*Nematalosa come* [Skin lesion] (On site)	Euleilah Ck. [C.Kirchner] [29.05.85] [Date]	ND.	ND.	ND.
24.*Mugil cephalus* [Skin lesion] (On site)	Euleilah Ck. [C.Kirchner] [29.05.85]	ND.	ND.	ND.
25.*Mugil cephalus* [Scale pocket] [Skin lesion]	Baffle Ck. [C.Kirchner] [02.07.85]	Fresh (by taste) Recent winter chill	Atypical early Dermal lesions (2)	*Ca* 450 g fish in good condition. Scales were elevated and a gelatinous secretion? was also present.
26.*Mugil cephalus* [Dermal lesion] (On site)	Baffle Ck. [C.Kirchner] [02.07.85]	Fresh (by taste) Recent winter chill	Extensive dermal ulcer (1).	*Ca* 450 g fish in good condition. Less than 5 % of fish were affected.
27.*Mugil cephalus* [Dermal lesion] (On site)	Baffle Ck. [C.Kirchner] [02.07.85]	Fresh (by taste) Recent winter chill	Extensive dermal ulcer (1).	*Ca* 450 g fish in good condition. Less than 5 % of fish were affected.
28.*Mugil cephalus* [Dermal lesion] (On site)	Baffle Ck. [C.Kirchner] [19.08.85]	Slightly salt (by taste)	Extensive dermal ulcer (1).	*Ca* 750 g fish in good condition. Less than 5 % of fish were affected.
29.*Mugil cephalus* [Dermal lesion] (On site)	Baffle Ck. [C.Kirchner] [20.08.85]	Slightly salt (by taste)	Extensive dermal lesion (1).	*Ca* 450 g fish in good condition. Less than 5 % of fish were affected.
30.*Mugil cephalus* [Dermal lesion] (On site)	Baffle Ck. [C.Kirchner] [20.08.85]	Slightly salt (by taste)	Extensive dermal lesion (1).	*Ca* 750 g fish in good condition. Less than 5 % of fish were affected.
31.*Mugil cephalus* [Dermal lesion] (On site)	Baffle Ck. [C.Kirchner] [21.08.85]	Fresh (by taste) winter chill	Extensive dermal lesion (1).	*Ca* 750 g fish in poor condition. Up to 20 % of fish were affected. Lesion near vent. This fish carried a pseudolanid isopod.

Table 3.6; (Cont.): Diseased and Unaffected Fish Assayed for Presumptive *Vibrios*.

Fish, Species. [Sample]	Origin. [Agent]. [Date].	Prevailing Conditions.	Lesion.	Gross Appearance and Pathology.
32.*Mugil cephalus* [Dermal lesion] (On site)	Baffle Ck. [C.Kirchner] [21.08.85]	Fresh (by taste)	Extensive dermal lesion (1).	*Ca* 1 kg fish in good condition. *Ca* 20 % of fish were affected.
33.*Mugil cephalus* [Dermal lesion] (On site)	Baffle Ck. [C.Kirchner] [21.08.85]	Fresh (by taste)	Extensive dermal lesion (1).	*Ca* 900 g fish in good condition. *Ca* 5 % of fish were affected.
34.*Mugil cephalus* [Scale pocket] [Dermal lesion] (On site)	Taunton Ck. [C.Kirchner] [28.08.85]	Fresh (by taste)	Early dermal tail lesion. Advanced cephalic lesion.	*Ca* 750 g fish in good condition. Less than 5 % of fish affected.
35.*Mugil cephalus* [Dermal lesion] (On site)	Taunton Ck. [C.Kirchner] [28.08.85]	Fresh (by taste) winter chill	Developed cephalic dermal lesion.	*Ca* 750 g fish in good condition. Less than 5 % of fish affected.
36.*Mugil cephalus* [Scale pocket] [Unaffected skin]	Baffle Ck. [C.Kirchner] [19.02.86]	Fresh (by taste)	Skin lesion	*Ca* 750 g fish in good condition. less than 5 % of fish affected.
37.*Mugil cephalus* [Skin]	Baffle Ck. [C.Kirchner] [19.02.86]	Fresh (by taste)	Asymptomatic Control.	*Ca* 750 g fish in good condition. Less than 5 % of fish affected.
38.*Mugil cephalus* [Skin lesion] [Dermal lesion]	Mosquito Ck. [C.Kirchner] [04.03.86]	Fresh (by taste) Recent rain.	Early and advanced tail lesions	*Ca* 700 g fish in good condition. Less than 5 % of fish affected.
39.*Mugil cephalus* [Unaffected skin]	Mosquito Ck. [C.Kirchner] [04.03.86]	Fresh (by taste) Recent rain.	Asymptomatic Control.	*Ca* 1 kg fish in good condition. Less than 5 % of fish affected.
40.*Mugil cephalus* [Dermal lesion]	Baffle Ck.. Winfield [C.Kirchner] [13.05.86]	Salt (by taste)	Intermediate tail lesion.	*Ca* 1 kg fish in good condition. *Ca* 5 % of fish fish affected.

Table 3.6; (Cont.): Diseased and Unaffected Fish Assayed for Presumptive *Vibrios*.

Fish, Species. [Sample]	Origin. [Agent]. [Date].	Prevailing Conditions.	Lesion.	Gross Appearance and Pathology.
41.*Mugil cephalus* [Unaffected skin]	Baffle Ck. Winfield [C.Kirchner] [13.05.86]	Salt (by taste)	Asymptomatic Control.	*Ca* 500 g fish in good condition. *Ca* 5 % of fish fish affected.
42.*Mugil cephalus* [Skin lesion]	Baffle Ck. Winfield [C.Kirchner] 13.05.86	Salt (by taste)	Atypical early tail lesion.	*Ca* 750 g fish in good condition. *Ca* 5 % of fish fish affected.

From 996 cultures 728 or 73 % were classified as presumptive ***Vibrionaceae*** in accord with Table 3.7.1 and as depicted in Table 3.7.2. These data are summarized in Table 3.8. according to frequency and originating material. The incidence of cultures from selective media classified as presumptive ***Vibrionaceae*** ranged between *ca* 50 % for samples inoculated from engorged isopods and diseased and control fish, to 100 % for algae, copepods, Site [1.] crabs, isopod externae and Site [2.] water. Among fish isolates presumptively classified as ***Vibrionaceae,*** 69 came from control fish, 196 from river system isolates and 91 from Moreton Bay fish.

Statistical comparisons between these latter frequencies of presumptive ***Vibrionaceae,*** (Normal approximation to the Binomial distribution), indicated the only significant (very highly, $p < 0.001$) differences were between the control group and the diseased Moreton Bay fish group.

Such comparisons though may not be valid when it is considered that presumptive ***Vibrionaceae*** from river system fish were isolated from selective agar media only after enrichment, while those from diseased Moreton Bay fish were isolated directly.

Table 3.7.1: Key to Octal Encryption of Phenotypic Characters in Table 3.7.2.

1st digit	0 Gram +ve	0 Oxidative	0 Oxidase-negative
	1 Gram -ve	2 Fermentative	4 Oxidase-positive
2nd digit	0 Non-motile	0 Non-Swarming	0 Non-Polar flagellation.
	1 Motile	2 Swarming	4 Polar flagellation.
3rd digit	0 Coccoid	0 Anaerogenic	0 Catalase weak or negative.
	1 Rod shaped	2 Aerogenic	4 Catalase strong.
4th digit	0 No agar hydrolysis	0 Non-Luminescent	0 No PHB Granules
	1 Agar Hydrolysis	2 Luminescent	4 PHB Granules
5th digit	0 non-*Vibrionaceae*	1 *Vibrionaceae*	

Table 3.7.2: Strain Origins, Isolation Protocols, Octal Codes, and Batch-Key to Phenotypic Analyses.

Strain Number Stn	Site	Source	Type	Isolation Protocol	Octal Code	Batch No.
10	Site [1.]	Water	Swab	Simidu	70101	ND
20	.	.	.	.	75141	ND
30	.	.	.	.	45100	ND
40	.	.	.	.	47100	ND
41	.	.	.	.	47100	ND
4560	.	.	.	ATW24:Simidu	75101	ND
4561	.	.	.	.	25300	ND
4570	.	.	.	.	75101	7
4590	.	.	.	.	77101	ND
4610	.	.	.	BTB24:Simidu	75101	ND
4611	.	.	.	.	75101	7
50	.	.	.	TCBS	77121	ND
60	.	.	.	.	77121	ND
70	.	.	.	.	43100	ND
80	.	.	.	.	43100	ND
90	.	.	.	.	77101	ND
100	.	.	.	.	77101	ND
110	.	.	.	.	75101	ND
120	.	.	.	.	75101	ND
4640	.	.	.	ATW24:TCBS	75101	ND
4650	.	.	.	.	77101	7
4660	.	.	.	.	75101	7
4670	.	.	.	.	75101	ND
4620	.	.	.	BTB24:TCBS	75101	ND
4630	.	.	.	.	75101	7
4631	.	.	.	.	75101	8
910	Site [2.]	Water	.	Simidu	75101	ND
920	.	.	.	.	70101	ND
930	.	.	.	.	72101	3
5570	.	.	.	ATW24:Simidu	77101	8
5580	.	.	.	.	75101	8
5590	.	.	.	.	37101	8

3.7.2: Strain Origins, Isolation Protocols, Octal Codes, and Batch-Key to Phenotypic Analyses. (Cont.)

Strain Number Stn	Site	Source		Type	Isolation Protocol	Octal Code	Batch No.
5600	Site [2.]	Water		.	ATW24:Simidu	77101	8
5650	.	.		.	BTB24:Simidu	77101	8
5660	.	.		.	.	75101	ND
5670	.	.		.	.	75101	8
5680	.	.		.	.	75101	ND
940	.	.		.	TCBS	75101	ND
950	.	.		.	.	75101	ND
960	.	.		.	.	75101	6
970	.	.		.	.	75101	ND
5610	.	.		.	ATW24:TCBS	77101	ND
5620	.	.		.	.	77101	8
5630	.	.		.	.	77101	ND
5640	.	.		.	.	77101	ND
5690	.	.		.	BTB24:TCBS	75101	8
5700	.	.		.	.	75101	8
750	Site [1.]	Copepod	(2)	Whole	Simidu	75101	3
760	.	O.Calanoidea		.	.	75101	3,7
770	.	.	(3)	.	.	75101	3,7
780	.	.		.	.	75101	3
790	.	.	(5)	.	.	75101	ND
800	.	.		.	.	77101	ND
810	.	.	(6)	.	.	77301	ND
820	.	.		.	.	75101	3
830	.	.	(7)	.	.	75101	3
840	.	.		.	.	75101	3
850	.	.		.	.	75101	3
860	.	.		.	.	77101	3
870	.	.	(8)	.	.	77101	3
880	.	.		.	.	75101	ND
890	.	.	(8)	.	TCBS	77101	ND
670	Site [1.]	Plankton		Swab	Simidu	77101	3
671	.	.		.	.	77101	3
680	.	.		.	.	77101	3
690	.	.		.	.	77101	3
700	.	.		.	.	37101	3
4860	.	.		Paste	ATW24:Simidu	77101	ND
4870	.	.		.	.	77101	7
4880	.	.		.	.	77101	7
4890	.	.		.	.	77101	7
4950	.	.		.	BTB24:Simidu	77101	7
4960	.	.		.	.	75101	ND
710	.	.		Swab	TCBS	77101	3
720	.	.		.	.	77101	3
730	.	.		.	.	77101	ND
740	.	.		.	.	70101	ND
4920	.	.		Paste	ATW24:TCBS	77101	ND
4970	.	.		.	BTB24:TCBS	77301	7
4980	.	.		.	.	77301	7
4990	.	.		.	.	75101	7
5000	.	.		.	.	77101	8
1330	Site [3.]	Plankton		Swab	Simidu	75101	6
1340	.	.		.	.	75101	ND
1350	.	.		.	.	57100	ND
5830	.	.		Paste	ATW24:Simidu	75101	ND
5840	.	.		.	.	75101	8
5850	.	.		.	.	75301	ND
5900	.	.		.	BTB24:Simidu	75101	ND

3.7.2: Strain Origins, Isolation Protocols, Octal Codes, and Batch-Key to Phenotypic Analyses. (Cont.)

Strain Number Stn	Site	Source	Type	Isolation Protocol	Octal Code	Batch No.
5910	Site [3.]	Plankton	Paste	BTB24:Simidu	77101	ND
1370	.	.	Swab	TCBS	75101	ND
1380	.	.	.	.	75101	6
1390	.	.	.	.	75101	ND
1400	.	.	.	.	75101	ND
5860	.	.	Paste	ATW24:TCBS	75101	8
5870	.	.	.	.	77101	ND
5880	.	.	.	.	35101	ND
5890	.	.	.	.	75101	ND
5920	.	.	.	BTB24:TCBS	77101	8
5930	.	.	.	.	75101	ND
390	Site [1.]	Oyster (1)	.	Simidu	77101	3
400	.	*Crassostrea commercialis*	.	.	70101	ND
410	.	.	.	.	77101	3
420	.	.	.	.	77101	ND
421	.	.	.	.	?????	3
5310	.	.	.	ATW24:Simidu	77101	8
5320	.	.	.	.	77101	8
5330	.	.	.	.	77101	ND
5340	.	.	.	.	75101	ND
5390	.	.	.	BTB24:Simidu	77101	ND
5400	.	.	.	.	77101	ND
430	.	.	.	TCBS	77121	ND
440	.	.	.	.	75101	ND
450	.	.	.	.	77101	3
460	.	.	.	.	77101	ND
470	.	.	.	.	75101	ND
480	.	.	.	.	77101	3
5350	.	.	.	ATW24:TCBS	77101	ND
5360	.	.	.	.	77101	8
5370	.	.	.	.	75101	8
5371	.	.	.	.	77101	ND
5380	.	.	.	.	77121	ND
5410	.	.	.	BTB24:TCBS	73100	ND
5420	.	.	.	.	73100	ND
5430	.	.	.	.	77101	8
5440	.	.	.	.	77101	8
570	Site [1.]	*C.commercialis* (2)	.	Simidu	77101	3
580	.	.	.	.	77101	3
590	.	.	.	.	77101	ND
600	.	.	.	.	77101	ND
610	.	.	.	.	77101	ND
620	.	.	.	.	77101	ND
630	.	.	.	.	75101	ND
640	.	.	.	.	77101	3
650	.	.	.	.	77101	ND
5450	.	.	.	ATW24:Simidu	77101	8
5460	.	.	.	.	00000	ND
5510	.	.	.	BTB24:Simidu	77101	8
5520	.	.	.	.	75101	ND
5470	.	.	.	ATW24:TCBS	77121	8
5480	.	.	.	.	77101	8
5490	.	.	.	.	77101	8
5530	.	.	.	BTB24:TCBS	77101	8
5540	.	.	.	.	77001	8
5550	.	.	.	.	77101	8
5560	.	.	.	.	75101	8

3.7.2: Strain Origins, Isolation Protocols, Octal Codes, and Batch-Key to Phenotypic Analyses. (Cont.)

Strain Number Stn	Site	Source	Type	Isolation Protocol	Octal Code	Batch No.
1000	Site [2.]	Mussel (1)	Swab	Simidu	75101	ND
1010	.	*Modiolus sp.*	.	.	77101	6
1020	.	.	.	.	35101	6
6150	.	.	.	ATW24:Simidu	77301	ND
6160	.	.	.	.	77101	ND
6190	.	.	.	BTB24:Simidu	77101	ND
6200	.	.	.	.	77101	ND
1040	.	.	.	TCBS	55100	ND
1050	.	.	.	.	55100	ND
6170	.	.	.	ATW24:TCBS	77301	ND
6180	.	.	.	.	77101	ND
6210	.	.	.	BTB24:TCBS	77101	ND
6220	.	.	.	.	77101	8
1060	Site [2.]	Mussel (2)	.	Simidu	75101	6
1070	.	*Modiolus sp.*	.	.	75101	ND
1080	.	.	.	.	35501	6
1090	.	.	.	.	35501	6
6230	.	.	.	ATW24:Simidu	72101	ND
6240	.	.	.	.	77101	ND
6250	.	.	.	.	77101	ND
6260	.	.	.	.	77101	ND
6300	.	.	.	BTB24:Simidu	23300	ND
6310	.	.	.	.	23300	ND
6320	.	.	.	.	75101	ND
6330	.	.	.	.	77101	ND
1100	.	.	.	TCBS	70101	ND
1110	.	.	.	.	70101	ND
6270	.	.	.	ATW24:TCBS	75101	ND
6280	.	.	.	.	75101	ND
6290	.	.	.	.	77101	ND
6340	.	.	.	BTB24:TCBS	23300	ND
6350	.	.	.	.	31300	ND
6360	.	.	.	.	75101	ND
6370	.	.	.	.	35101	ND
1450	Site [2.]	Algae	.	ATW24:Simidu	75101	6
1460	.	*Gracilaria verrucosa*	.	.	75101	6
1470	.	.	.	.	77101	6,8a
1480	.	.	.	.	77101	6
1410	.	.	.	BTB24:Simidu	77101	6
1420	.	.	.	.	77101	6,8a
1430	.	.	.	BTB24:TCBS	77101	ND
1440	.	.	.	.	77101	ND
1490	.	.	.	ATW24:TCBS	77101	ND
1500	.	.	.	.	77101	6
130	Site [1.]	Sediment	.	Simidu	77101	3
140	.	.	.	.	70301	ND
141	.	.	.	.	75301	ND
150	.	.	.	.	77101	6
160	.	.	.	.	75301	ND
170	.	.	.	.	35301	3,3
171	.	.	.	.	77161	3
180	.	.	.	.	35101	1,1
181	.	.	.	.	77161	3
190	.	.	.	.	77141	3
200	.	.	.	.	77121	3
4680	.	.	.	ATW24:Simidu	77101	ND
4690	.	.	.	.	70101	ND

3.7.2: Strain Origins, Isolation Protocols, Octal Codes, and Batch-Key to Phenotypic Analyses. (Cont.)

Strain Number Stn	Site	Source	Type	Isolation Protocol	Octal Code	Batch No.
4700	Site [1.]	Sediment	Swab	ATW24:Simidu	75101	ND
4710	.	.	.	.	75101	7
4790	.	.	.	BTB24:Simidu	75101	ND
4800	.	.	.	.	75101	7
4801	.	.	.	.	70101	ND
4810	.	.	.	.	30100	ND
210	.	.	.	TCBS	32100	ND
220	.	.	.	.	77141	ND
230	.	.	.	.	77301	ND
240	.	.	.	.	77101	ND
250	.	.	.	.	77101	3
260	.	.	.	.	77101	3
270	.	.	.	.	77101	3
4720	.	.	.	ATW24:TCBS	75101	ND
4730	.	.	.	.	77101	7
4731	.	.	.	.	77101	7
4740	.	.	.	.	75101	7
4750	.	.	.	.	75101	ND
4760	.	.	.	.	70101	ND
4770	.	.	.	.	75101	ND
4820	.	.	.	BTB24:TCBS	75101	7
4830	.	.	.	.	75101	7,8
4840	.	.	.	.	75101	ND
4850	.	.	.	.	75101	ND
5710	Site [2.]	Sediment	.	ATW24:Simidu	75101	8
5720	.	.	.	.	75101	8
5730	.	.	.	.	77101	ND
5740	.	.	.	.	75101	8
5770	.	.	.	BTB24:Simidu	31300	ND
5780	.	.	.	.	31300	ND
5790	.	.	.	.	31100	ND
5800	.	.	.	.	75101	8
5801	.	.	.	.	35301	ND
5750	.	.	.	ATW24:TCBS	75101	8
5760	.	.	.	.	77101	ND
5810	.	.	.	BTB24:TCBS	71001	8
5820	.	.	.	.	71001	8
1510	Site [2.]	Nereid polychaete	.	ATW24:Simidu	75101	6
1520	.	.	.	.	75101	ND
1530	.	.	.	.	75101	ND
1540	.	.	.	.	75101	ND
1550	.	.	.	ATW24:TCBS	77101	ND
1551	.	.	.	.	77101	6
1560	.	.	.	.	75101	ND
1570	.	.	.	.	75101	ND
1580	.	.	.	.	77101	6
280	Site [1.]	Crab (1)	.	Simidu	77101	3
281	.	*Mictyrus longicarpus*	.	.	77101	3
290	.	.	.	.	77101	3
300	.	.	.	.	75101	3
5010	.	.	.	ATW24:Simidu	77101	8
5020	.	.	.	.	77101	ND
5070	.	.	.	BTB24:Simidu	77101	ND
5080	.	.	.	.	72101	ND
5090	.	.	.	.	77101	8
320	.	.	.	TCBS	75101	ND
321	.	.	.	.	77101	ND

3.7.2: Strain Origins, Isolation Protocols, Octal Codes, and Batch-Key to Phenotypic Analyses. (Cont.)

Strain Number Stn	Site	Source	Type	Isolation Protocol	Octal Code	Batch No.
330	Site [1.]	Crab (1)	Swab	TCBS	77101	ND
340	.	*M.longicarpus*	.	.	37101	ND
350	.	.	.	.	77101	3
360	.	.	.	.	77101	3
370	.	.	.	.	77101	3
380	.	.	.	.	77101	3
5030	.	.	.	ATW24:TCBS	77101	ND
5040	.	.	.	.	77101	ND
5050	.	.	.	.	77301	8
5060	.	.	.	.	77101	ND
5110	.	.	.	BTB24:TCBS	77101	ND
5120	.	.	.	.	77101	ND
5130	.	.	.	.	72101	ND
5140	.	.	.	.	77101	ND
490	Site [1.]	Crab (2)	.	Simidu	77101	3
500	.	*M.longicarpus*	.	.	77101	3
510	.	.	.	.	75101	ND
520	.	.	.	.	77121	3
5150	.	.	.	ATW24:Simidu	77101	ND
5160	.	.	.	.	77101	8
5170	.	.	.	.	77101	ND
5180	.	.	.	.	77101	ND
5190	.	.	.	ATW24:TCBS	77101	8
5200	.	.	.	.	77101	8
5201	.	.	.	.	77101	8
5210	.	.	.	.	77101	ND
5220	.	.	.	.	77101	8
530	.	.	.	TCBS	77101	ND
540	.	.	.	.	37101	ND
550	.	.	.	.	77101	ND
560	.	.	.	.	75101	ND
5230	.	.	.	BTB24:Simidu	77101	8
5240	.	.	.	.	77101	8
5250	.	.	.	.	77101	ND
5260	.	.	.	.	77101	8
5270	.	.	.	BTB24:TCBS	77101	8
5280	.	.	.	.	77101	8
5290	.	.	.	.	77101	8
5300	.	.	.	.	77101	8
1120	Site [2.]	Crab	Swab	Simidu	37101	ND
1140	.	*Varuna littorata*	.	.	17100	ND
1150	.	.	.	.	75101	6
5950	.	.	.	ATW24:Simidu	77101	8
5980	.	.	.	BTB24:Simidu	77301	ND
5990	.	.	.	.	75301	ND
1160	.	.	.	TCBS	75101	ND
1170	.	.	.	.	75101	6
1180	.	.	.	.	75101	ND
1190	.	.	.	.	75101	ND
5960	.	.	.	ATW24:TCBS	77101	ND
5970	.	.	.	.	77101	ND
6000	.	.	.	BTB24:TCBS	75301	ND
6010	.	.	.	.	75301	ND
6020	.	.	.	.	75301	6
6030	.	.	.	.	77101	ND
1210	Site [2.]	Banana Prawn	Swab	Simidu	55100	ND
1211	.	*Penaeus merguiensis*	.	.	75101	ND

3.7.2: Strain Origins, Isolation Protocols, Octal Codes, and Batch-Key to Phenotypic Analyses. (Cont.)

Strain Number Stn	Site	Source		Type	Isolation Protocol	Octal Code	Batch No.
1220	Site [2.]	Banana Prawn		Swab	Simidu	75101	ND
1230	.	*P.merguiensis*		.	.	55100	ND
1231	.	.		.	.	75101	ND
1240	.	.		.	.	75101	6
1250	.	.		.	.	31100	ND
1260	.	.		.	.	55100	ND
1261	.	.		.	.	31100	ND
6040	.	.		.	ATW24:Simidu	77101	ND
6050	.	.		.	.	77101	ND
6090	.	.		.	BTB24:Simidu	77301	ND
6100	.	.		.	.	77301	ND
6110	.	.		.	.	30100	ND
6120	.	.		.	.	77301	ND
1280	.	.		.	TCBS	75101	6
1281	.	.		.	.	75101	ND
1290	.	.		.	.	75101	ND
1291	.	.		.	.	55100	ND
1300	.	.		.	.	77101	ND
1310	.	.		.	.	75101	ND
1320	.	.		.	.	55100	ND
1321	.	.		.	.	57100	ND
6060	.	.		.	ATW24:TCBS	77301	ND
6070	.	.		.	.	77301	ND
6080	.	.		.	.	77301	ND
6130	.	.		.	BTB24:TCBS	77301	ND
6140	.	.		.	.	77321	ND
1690	Site [2.]	Fish (1)	[Scale Pocket]	Swab	ATW24:Simidu	37101	ND
1700	.	*Mugil cephalus*		.	.	77121	2
1710	.	.		.	BTB24:Simidu	77121	1
1720	.	.		.	.	77121	ND
1730	.	.		.	ATW24:TCBS	77101	ND
1740	.	.		.	.	77101	ND
1750	.	.		.	.	77121	1
1760	.	.		.	.	77301	1
1770	.	.		.	BTB24:TCBS	77101	ND
1790	.	.		.	.	77121	1
1800	.	.		.	.	77321	1
1810	.	.	[Liver]	Swab	ATW24:Simidu	75101	ND
1820	.	.		.	.	75101	6
1830	.	.		.	.	77301	ND
1840	.	.		.	.	35301	ND
1841	.	.		.	.	75101	ND
1850	.	.		.	ATW24:TCBS	75121	ND
1860	.	.		.	.	75121	ND
1870	.	.		.	.	75101	6
1871	.	.		.	.	75101	ND
1880	.	.		.	.	75321	ND
1890	.	.	[Spleen]	Swab	ATW24:Simidu	75101	6
1900	.	.		.	.	75101	ND
1910	.	.		.	.	75101	ND
1911	.	.		.	.	75101	ND
1920	.	.		.	.	21200	ND
1921	.	.		.	.	75101	ND
1930	.	.		.	BTB24:Simidu	31200	ND
1940	.	.		.	.	31200	ND
1950	.	.		.	ATW24:TCBS	75101	6
1960	.	.		.	.	75101	6

3.7.2: Strain Origins, Isolation Protocols, Octal Codes, and Batch-Key to Phenotypic Analyses. (Cont.)

Strain Number Stn	Site	Source	Type	Isolation Protocol	Octal Code	Batch No.
1970	Site [2.]	Fish (1) [Spleen]	Swab	ATW24:TCBS	75121	7
1980	.	*M.cephalus*	.	.	75121	ND
1990	.	.	.	.	75101	6
2000	.	.	.	.	31300	4
2001	.	.	.	.	31300	ND
2020	Kolan R.	Fish (2) [Skin Lesion]	Swab	ATW24:Simidu	75101	ND
2001	.	*M.cephalus*	.	.	31300	ND
2020	.	.	.	.	31300	ND
2030	.	.	.	.	75101	ND
2031	.	.	.	.	31300	ND
2040	.	.	.	.	31300	ND
2050	.	.	.	BTB24:Simidu	31300	ND
2051	.	.	.	.	31300	ND
2060	.	.	.	.	31300	ND
2061	.	.	.	.	31300	ND
2090	.	.	.	ATW24:TCBS	05100	ND
2100	.	.	.	.	75101	6
2101	.	.	.	.	31300	ND
2071	.	.	.	BTB24:TCBS	31300	ND
2080	.	.	.	.	31300	ND
2110	.	.	[Scale Pocket] Swab	ATW24:Simidu	31300	ND
2120	.	.	.	.	31300	ND
2130	.	.	.	BTB24:Simidu	31300	ND
2131	.	.	.	.	31300	ND
2140	.	.	.	.	31300	ND
2150	.	.	.	.	31300	ND
2151	.	.	.	.	31300	ND
2160	.	.	.	.	31300	ND
2170	.	.	.	.	75101	6
2180	.	.	.	.	31300	ND
2190	.	.	.	.	31300	ND
2200	.	.	.	.	31300	ND
2210	.	.	[Scale Pocket] Swab	ATW24:Simidu	31300	ND
2211	.	.	.	.	31300	ND
2220	.	.	.	.	31300	ND
2230	.	.	.	.	31300	ND
2240	.	.	.	.	31100	ND
2250	.	.	.	.	31100	ND
2260	.	.	.	.	70101	ND
2270	.	.	.	.	70101	ND
2280	.	.	.	.	75301	6
2290	.	.	[Liver] Swab	ATW24:Simidu	75101	7,8a
2300	.	.	.	.	75101	ND
2301	.	.	.	.	75301	ND
2310	.	.	.	.	75301	ND
2320	.	.	.	.	75301	ND
2330	.	.	.	BTB24:Simidu	31300	ND
2340	.	.	.	.	31300	ND
2350	.	.	.	.	31300	ND
2360	.	.	.	.	31300	ND
2361	.	.	.	.	75101	ND
2370	.	.	.	ATW24:TCBS	75301	6
2380	.	.	.	.	77101	ND
2390	.	.	.	BTB24:TCBS	75101	ND
2410	.	.	.	.	35301	ND
2411	.	.	.	.	75101	ND
2420	.	.	.	.		

3.7.2: Strain Origins, Isolation Protocols, Octal Codes, and Batch-Key to Phenotypic Analyses. (Cont.)

Strain Number Stn	Site	Source	Type	Isolation Protocol	Octal Code	Batch No.
2430	Kolan R.	Fish (2) [Kidney]	Swab	ATW24:Simidu	75101	ND
2440	.	*M.cephalus*	.	.	75101	6,6
2450	.	.	.	.	55100	ND
2460	.	.	.	.	77121	ND
2470	.	.	.	BTB24:Simidu	77101	6
2480	.	.	.	.	31300	ND
2490	.	.	.	.	75101	6
2500	.	.	.	.	31300	ND
2510	.	.	.	ATW24:TCBS	75101	6
2520	.	.	.	.	77101	ND
2530	.	.	.	.	75101	ND
2540	.	.	.	.	75101	ND
2550	.	.	.	BTB24:TCBS	75101	ND
2560	.	.	.	.	75101	6
2570	Site [3.]	Fish (3) [Skin]	Swab	ATW24:Simidu	31300	ND
2571	.	*Nematalosa come*	.	.	75101	ND
2572	.	.	.	.	55100	ND
2580	.	.	.	.	31300	ND
2590	.	.	.	ATW24:TCBS	31300	ND
2600	.	.	.	.	31300	ND
2610	.	. [Liver]	Swab	ATW24:Simidu	31300	ND
2620	.	.	.	.	31300	ND
2630	.	.	.	BTB24:TCBS	31300	ND
2631	.	.	.	.	55100	ND
2640	.	.	.	.	31300	ND
2641	.	.	.	.	31300	ND
2650	.	. [Kidney]	Swab	ATW24:Simidu	31300	ND
2651	.	.	.	.	51100	ND
2660	.	.	.	.	31300	ND
2670	.	.	.	ATW24:TCBS	31300	ND
2680	.	.	.	.	31300	ND
2690	.	. [Gall]	Swab	ATW24:Simidu	31300	ND
2691	.	.	.	.	31300	ND
2700	.	.	.	.	31300	ND
2710	.	.	.	ATW24:TCBS	31300	ND
2720	.	.	.	.	75301	ND
2730	.	.	.	.	75301	ND
2731	.	.	.	.	31300	ND
2740	.	.	.	.	35301	ND
2741	.	.	.	.	35301	ND
2750	Site [2.]	Fish (4) [Liver]	Swab	ATW24:Simidu	75101	ND
2760	.	*M.cephalus*	.	.	75101	ND
2770	.	.	.	BTB24:Simidu	75101	6
2780	.	.	.	.	35101	6
2790	.	.	.	ATW24:TCBS	75101	ND
2800	.	.	.	.	75101	6
2830	.	.	.	.	15100	ND
2840	.	.	.	.	31100	ND
2810	.	.	.	BTB24:TCBS	75101	6
2820	.	.	.	.	75101	6
2850	.	. [Kidney]	Swab	ATW24:Simidu	31100	ND
2860	.	.	.	.	31100	ND
2870	.	.	.	ATW24:TCBS	31100	ND
2871	.	.	.	.	51100	ND
2872	.	.	.	.	31300	ND
2880	.	.	.	ATW24:TCBS	31300	ND
2881	.	.	.	.	11100	ND

3.7.2: Strain Origins, Isolation Protocols, Octal Codes, and Batch-Key to Phenotypic Analyses. (Cont.)

Strain Number Stn	Site	Source		Type	Isolation Protocol	Octal Code	Batch No.
2890	Site [2.]	Fish (5)	[Scale Pocket]	Swab	BTB24:Simidu	31300	ND
2891	.	*M.cephalus*		.	.	31300	ND
2900	.	.		.	.	71300	ND
2910	.	.	[Kidney]	Swab	ATW24:Simidu	75101	ND
2920	.	.		.	.	75101	6
2930	.	.		.	.	71300	ND
2931	.	.		.	.	37101	ND
2940	.	.		.	.	35301	ND
2950	.	.		.	.	35301	ND
2951	.	.		.	.	75101	ND
2960	.	.		.	BTB24:Simidu	75101	ND
2970	.	.		.	.	31300	ND
2980	.	.		.	.	31300	ND
2990	.	.		.	.	31300	ND
3000	.	.		.	ATW24:TCBS	75101	6
3010	.	.		.	.	75101	6
3020	.	.		.	.	30100	ND
3030	.	.		.	.	30100	ND
3035	.	.		.	.	31300	ND
3040	.	.		.	BTB24:TCBS	31300	ND
3050	.	.		.	.	31300	ND
3060	Site [2.]	Fish (6)	[Vent Lesion]	Swab	ATW24:Simidu	31100	4
3070	.	*M.cephalus*		.	.	31100	ND
3080	.	.		.	ATW24:TCBS	51100	ND
3081	.	.		.	.	31300	ND
3090	.	.		.	.	51100	ND
3091	.	.		.	.	31300	ND
3100	.	.		.	BTB24:TCBS	55100	ND
3101	.	.		.	.	57100	ND
3110	.	.		.	.	55100	ND
3120	.	.	[Skin Lesion]	Swab	ATW24:Simidu	75101	ND
3130	.	.		.	.	37101	6
3140	.	.		.	BTB24:Simidu	37101	6
3150	.	.		.	.	37101	ND
3160	.	.		.	ATW24:TCBS	77101	7
3170	.	.		.	.	77101	ND
3180	.	.		.	BTB24:TCBS	77101	7
3190	.	.		.	.	77101	7
3200	.	.	[Liver]	Swab	ATW24:Simidu	30100	ND
3210	.	.		.	.	30100	ND
3220	.	.	[Spleen]	Swab	ATW24:Simidu	35301	7
3230	.	.		.	.	35301	1
3240	.	.		.	.	75101	ND
3250	.	.		.	.	75101	ND
3260	.	.		.	BTB24:Simidu	75301	7
3270	.	.		.	.	75301	7
3280	.	.		.	ATW24:TCBS	75101	ND
3290	.	.		.	.	75101	ND
3310	.	.		.	BTB24:TCBS	75301	7
3320	.	.		.	.	00000	ND
3321	.	.		.	.	75101	7
3330	.	.		.	.	75301	7,8a
3340	.	.		.	.	75301	ND
3350	.	.		.	.	75301	7
3360	Site [2.]	Fish (7)	[Liver]	Swab	ATW24:Simidu	55100	ND
3370	.	*M.cephalus*		.	.	55100	ND
3380	.	.		.	BTB24:Simidu	75101	7

3.7.2: Strain Origins, Isolation Protocols, Octal Codes, and Batch-Key to Phenotypic Analyses. (Cont.)

Strain Number Stn	Site	Source	Type	Isolation Protocol	Octal Code	Batch No.
3390	Site [2.]	Fish (7) [Liver]	Swab	BTB24:Simidu	77101	2
3400	.	*M.cephalus*	.	BTB24:TCBS	33100	ND
3410	.	.	.	.	37101	ND
3420	.	. [Kidney]	Swab	ATW24:Simidu	37101	ND
3430	.	.	.	.	77101	ND
3440	.	.	.	BTB24:Simidu	71101	ND
3450	.	.	.	.	37101	2
3460	.	.	.	.	37101	7
3470	.	.	.	.	77101	7
3480	.	.	.	ATW24:TCBS	37101	ND
3490	.	.	.	.	77101	ND
3500	Site [2.]	Fish (8) [Petechium]	Swab	ATW24:TCBS	?????	ND
3510	.	*M.cephalus*	.	.	?????	8
3520	.	. [Liver]	Swab	ATW24:Simidu	77101	ND
3521	.	.	.	.	77101	7
3610	.	.	.	.	31300	ND
3530	.	.	.	BTB24:Simidu	77101	ND
3540	.	.	.	.	31300	ND
3550	.	.	.	ATW24:TCBS	73300	ND
3560	.	.	.	.	75301	7
3570	.	.	.	.	77101	ND
3571	.	.	.	.	77101	7,8a
3580	.	.	.	.	75301	ND
3590	.	.	.	BTB24:TCBS	31000	ND
3600	.	.	.	.	31300	ND
3620	.	. [Kidney]	Swab	ATW24:Simidu	17100	ND
3630	.	.	.	.	17100	ND
3640	Site [2.]	Fish (9) [Liver]	Swab	ATW24:Simidu	70101	ND
3641	.	*M.cephalus*	.	.	75101	ND
3650	.	.	.	.	75101	ND
3651	.	.	.	.	70101	ND
3660	.	.	.	.	75101	ND
3670	.	.	.	.	75101	ND
3680	.	.	.	BTB24:Simidu	50100	ND
3681	.	.	.	.	75101	7,8
3690	.	.	.	.	70101	ND
3691	.	.	.	.	50100	ND
3692	.	.	.	.	50100	ND
3700	.	.	.	ATW24:TCBS	70101	2
3710	.	.	.	.	70101	ND
3720	.	.	.	BTB24:TCBS	70101	ND
3730	.	.	.	.	35101	ND
3740	.	. [Kidney]	Swab	ATW24:Simidu	31300	ND
3750	.	.	.	.	33300	ND
3761	.	.	.	BTB24:Simidu	55100	ND
3770	.	.	.	.	75101	ND
3880	.	.	.	ATW24:TCBS	35100	ND
3890	.	.	.	.	35100	ND
3900	.	.	.	.	35100	ND
3910	.	.	.	.	70100	ND
3920	.	.	.	BTB24:TCBS	75101	7
3930	.	.	.	.	75101	ND
3780	Site [2.]	Fish (10) [Liver]	Swab	ATW24:Simidu	75101	7
3781	.	*M.cephalus*	.	.	75101	7
3790	.	.	.	.	75101	ND
3800	.	.	.	.	75101	8
3801	.	.	.	.	30300	7

3.7.2: Strain Origins, Isolation Protocols, Octal Codes, and Batch-Key to Phenotypic Analyses. (Cont.)

Strain Number Stn	Site	Source	Type	Isolation Protocol	Octal Code	Batch No.
3810	Site [2.]	Fish (10) [Liver]	Swab	ATW24:Simidu	75101	ND
3811	.	*M.cephalus*	.	.	31300	ND
3820	.	.	.	BTB24:Simidu	75101	7
3830	.	.	.	.	75101	ND
3840	.	.	.	ATW24:TCBS	75101	7
3841	.	.	.	.	55100	ND
3850	.	.	.	.	35100	ND
3870	.	.	.	.	35100	ND
3940	.	. [Kidney]	Swab	ATW24:Simidu	70101	ND
3950	.	.	.	.	75101	7
3960	.	.	.	.	75101	8
3970	.	.	.	.	75101	8
3980	.	.	.	ATW24:TCBS	75101	ND
3990	.	.	.	.	70101	ND
4000	.	.	.	.	70101	ND
4010	.	.	.	.	75101	ND
4020	Site [2.]	Fish (11) [Liver]	Swab	ATW24:Simidu	33300	ND
4021	.	*M.cephalus*	.	.	55100	ND
4030	.	.	.	.	31300	ND
4040	.	.	.	.	31300	ND
4050	.	.	.	.	31300	ND
4060	.	.	.	BTB24:Simidu	31300	ND
4070	.	.	.	.	31300	ND
4071	.	.	.	.	75301	2
4080	.	.	.	ATW24:TCBS	35301	ND
4100	.	.	.	.	75301	ND
4101	.	.	.	.	71301	ND
4110	.	.	.	.	75301	2
4111	.	.	.	.	75301	ND
4120	.	.	.	BTB24:TCBS	33300	ND
4130	.	.	.	.	33300	ND
4140	.	. [Kidney]	Swab	ATW24:Simidu	33300	ND
4150	.	.	.	.	33300	ND
4160	.	.	.	.	33300	ND
4170	.	.	.	.	31300	ND
4180	.	.	.	ATW24:TCBS	31300	ND
4190	.	.	.	.	31300	ND
4200	.	.	.	.	11100	ND
4201	.	.	.	.	31300	ND
4210	.	.	.	.	31300	ND
4220	Site [2.]	Fish (12) [Scale Pocket]	Swab	ATW24:Simidu	00000	ND
4221	.	*M.cephalus*	.	.	00000	ND
4230	.	.	.	.	00000	ND
4240	.	.	.	ATW24:TCBS	00000	ND
4250	.	.	.	.	00000	ND
4260	.	. [Liver]	Swab	ATW24:Simidu	31300	ND
4270	.	.	.	.	30300	ND
4290	.	.	.	ATW24:TCBS	00000	ND
4300	.	. [Kidney]	Swab	ATW24:Simidu	10100	ND
4301	.	.	.	.	10100	ND
4310	.	.	.	.	10100	ND
4320	.	.	.	ATW24:TCBS	00000	ND
4330	.	.	.	.	00000	ND
4340	.	.	.	.	31300	ND
4350	.	.	.	.	31300	ND
4360	.	.	.	.	75101	ND
4370	.	.	.	.	75101	ND

3.7.2: Strain Origins, Isolation Protocols, Octal Codes, and Batch-Key to Phenotypic Analyses. (Cont.)

Strain Number	Site	Source	Type	Isolation Protocol	Octal Code	Batch No.
Stn						
4380	Site [2.]	Fish (12) [Kidney]	Swab	BTB24:TCBS	31100	ND
4390	.	*M.cephalus*	.	.	71300	ND
4400	Site [2.]	Fish (13) [Skin]	Swab	ATW24:Simidu	75301	7
4401	.	*M.cephalus*	.	.	55100	ND
4410	.	.	.	.	55100	ND
4411	.	.	.	.	31300	ND
4420	.	. [Scale Pocket]	Swab	ATW24:Simidu	31300	ND
4430	.	.	.	.	31300	ND
4440	.	.	.	.	00000	ND
4450	.	.	.	.	00000	ND
4460	.	.	.	ATW24:TCBS	00000	ND
4470	.	.	.	.	00000	ND
4480	.	. [Liver]	Swab	ATW24:Simidu	75101	ND
4490	.	.	.	.	75121	ND
4500	.	.	.	.	75121	ND
4501	.	.	.	.	35321	7
4510	.	.	.	.	75301	7
4520	.	.	.	ATW24:TCBS	75101	ND
4530	.	.	.	.	75101	ND
4540	.	. [Kidney]	Swab	ATW24:Simidu	00000	ND
4550	.	.	.	.	00000	ND
6450	Baffle	Fish (14) [Skin Lesion]	Swab	Simidu	75101	6
6460	Ck.	*M.cephalus*	.	.	75101	6
6390	.	.	.	TCBS	75101	1
6400	.	.	.	.	75101	2
6470	Baffle	Fish (15) [Skin Lesion]	Swab	Simidu	77101	6
6480	Ck.	*M.cephalus*	.	.	77101	6
6410	.	.	.	TCBS	75101	6
6411	.	.	.	.	?????	4
6420	.	.	.	.	75101	ND
6421	.	.	.	.	77101	6,6
6430	.	.	.	.	77101	6,6
6440	.	.	.	.	77101	6
6650	Moreton	Fish (16) [Blood]	Tail	TCBS	75101	6
6660	Bay	*Sillago ciliata*	. blot	.	75101	6
6670	.	.	.	.	75101	6,6
6680	.	.	.	.	75101	5
6690	.	. [Liver]	.	TCBS	75101	1
6700	.	.	.	.	75101	5
6710	.	. [Kidney]	.	SENA	75101	5,8
6720	.	.	.	.	75101	5,6,7
6730	.	.	.	.	75101	5,8
6740	.	.	.	.	00000	ND
6750	.	.	.	TCBS	00000	ND
6760	.	.	.	.	75101	5
6770	.	.	.	.	75101	5
6780	.	.	.	.	75101	5,7
6790	Moreton	Fish (17) [Vent]	Swab	SENA	75101	ND
6791	Bay	*S.ciliata*	.	.	55100	ND
6800	.	.	.	.	75101	ND
6801	.	.	.	.	75101	ND
6810	.	.	.	.	75101	4
6820	.	.	.	.	75101	5
6830	.	.	.	.	75101	5,6
6840	.	.	.	.	75101	5
6850	.	.	.	Simidu	75101	5
6860	.	.	.	.	75101	5
6870	.	.	.	.	75101	5

3.7.2: Strain Origins, Isolation Protocols, Octal Codes, and Batch-Key to Phenotypic Analyses. (Cont.)

Strain Number Stn	Site	Source	Type	Isolation Protocol	Octal Code	Batch No.
6880	Moreton	Fish (17) [Vent]	Swab	Simidu	75101	5
6890	Bay	*S.ciliata*	.	TCBS	75101	5
6900	.	.	.	.	75101	5,5
6910	.	.	[Sub-dermal	Swab	SENA	75101	5
6920	.	.	Lesion]	.	.	75101	5
6930	.	.		.	.	75101	5
6940	.	.		.	.	75101	5
6941	.	.		.	.	75101	5
6950	.	.		.	.	75101	5
6980	.	.		.	Simidu	75101	ND
6990	.	.		.	.	75101	5
7000	.	.		.	TCBS	75101	1
7010	.	.		.	.	75101	5
7020	.	.		.	.	75101	ND
7030	.	.		.	.	75101	5
7040	.	.	[Liver]	Swab	SENA	55100	ND
7050	.	.		.	.	55100	ND
7060	.	.		.	.	75101	5
7070	.	.		.	.	75101	5
7080	.	.		.	.	75101	ND
7090	.	.		.	Simidu	75101	5
7100	.	.		.	.	75101	5,6
7110	.	.		.	TCBS	75101	5
7120	.	.		.	.	75301	5,6
7130	.	.	[Kidney]	Swab	SENA	55100	ND
7140	.	.		.	.	55100	ND
7150	.	.		.	.	75101	ND
7160	.	.		.	.	75101	5
7170	.	.		.	.	75101	5,6
7180	.	.		.	Simidu	75101	5
7190	.	.		.	.	75101	5
7210	.	.		.	TCBS	75101	5
7220	.	.		.	.	75101	ND
7230	.	.	[Spleen]	Swab	SENA	75101	ND
7240	.	.		.	.	75101	4
7241	.	.		.	.	75101	4
7250	.	.		.	.	75101	4
7260	.	.		.	.	75101	4,7a
7270	.	.		.	.	75101	ND
7280	.	.		.	Simidu	75101	4,7
7281	.	.		.	.	75101	4
7290	.	.		.	.	75101	2
7291	.	.		.	.	55100	ND
7300	.	.		.	.	75101	4,7a
7310	.	.		.	.	75101	4,7a
7320	.	.		.	TCBS	75101	4,4,5
7330	.	.		.	.	75101	4
7340	.	.		.	.	75101	4
7350	.	.		.	.	75101	4
7360	Moreton	Fish (18) [Mucus]		Swab	SENA	75101	2,2
7370	Bay	*S.ciliata*		.	.	77101	4
7380	.	.		.	Simidu	75101	2
7400	.	.		.	.	75101	4
7420	.	.		.	.	75141	ND
7430	.	.		.	.	75101	4
7440	.	.		.	.	75101	ND
7450	.	.		.	.	75101	5,6,7

3.7.2: Strain Origins, Isolation Protocols, Octal Codes, and Batch-Key to Phenotypic Analyses. (Cont.)

Strain Number Stn	Site	Source	Type	Isolation Protocol	Octal Code	Batch No.
7460	Moreton	Fish (18) [Gut (7 cm to	Swab	SENA	75141	1
7470	Bay	*S.ciliata* vent)]	.	.	75101	4
7480	.	.	.	Simidu	75141	2
7490	.	.	.	.	70141	ND
7500	.	.	.	.	70141	ND
7510	.	.	.	TCBS	75141	1
7520	.	.	.	.	75101	2
7530	.	.	.	.	75101	4
7540	.	.	.	.	75101	ND
7550	.	. [Gut (1 cm to	Swab	.	?????	ND
7560	.	. vent)]	.	.	?????	ND
7570	.	. [Kidney]	Swab	SENA	70141	4
7580	.	.	.	.	75101	ND
7600	.	.	.	Simidu	75101	4,5,5a,7,8a
7610	.	.	.	TCBS	75101	4
7620	.	.	.	.	70101	ND
7630	.	.	.	.	75101	4,7
7640	.	.	.	.	75101	4
7641	.	.	.	.	33301	ND
7700	Baffle	Fish (19) [Skin Lesion]	Swab	Simidu	75101	5,7
7710	Ck.	*M.cephalus*	.	.	75301	2,5
7711	.	.	.	.	70301	2
7720	.	.	.	.	70301	ND
7730	.	.	.	.	77101	4
7740	.	.	.	.	75101	4
7650	.	.	.	TCBS	77111	1
7660	.	.	.	.	70151	ND
7661	.	.	.	.	77101	2
7680	.	.	.	.	75101	4
7681	.	.	.	.	75101	5
7800	Baffle	Fish (20) [Skin Lesion]	Swab	Simidu	77101	1
7810	Ck.	*M.cephalus*	.	.	75301	ND
7820	.	.	.	.	75101	1
7830	.	.	.	.	75101	ND
7750	.	.	.	TCBS	75101	4
7760	.	.	.	.	75301	4,7a
7770	.	.	.	.	75301	1
7780	.	.	.	.	50100	ND
7790	.	.	.	.	77301	7
7791	.	.	.	.	77101	1
7880	Baffle	Fish (21) [Skin Lesion]	Swab	Simidu	55100	ND
7900	Ck.	*M.cephalus*	.	.	55100	ND
7910	.	.	.	.	55100	ND
7840	.	.	.	TCBS	77101	2
7841	.	.	.	.	70301	ND
7850	.	.	.	.	71101	2
7860	.	.	.	.	77301	ND
7870	.	.	.	.	55100	6
7871	.	.	.	.	30300	ND
7920	Euleilah	Fish (22) [Skin Lesion]	Swab	ATW24:TCBS	77101	2,2
7930	Ck.	*Polydactylus sheridani*	.	.	77101	2
7940	.	.	.	.	70101	5,8
7941	.	.	Swab	ATW24:TCBS	75101	2
7950	.	.	.	.	75101	5
7960	Euleilah	Fish (23) [Skin Lesion]	Swab	ATW24:TCBS	77101	2
7970	Ck.	*N.come*	.	.	77101	ND

3.7.2: Strain Origins, Isolation Protocols, Octal Codes, and Batch-Key to Phenotypic Analyses. (Cont.)

Strain Number Stn	Site	Source	Type	Isolation Protocol	Octal Code	Batch No.
7980	Euleilah	Fish (24) [Skin Lesion]	Swab	ATW24:TCBS	55000	ND
7990	Ck.	*M.cephalus*	.	.	75101	5
8000	.	.	.	.	75101	ND
8010	.	.	.	.	75101	5,5
8060	Baffle	Fish (25) [Scale Pocket]	Swab	ATW24:EDDA	75301	ND
8061	Ck.	*M.cephalus*	.	.	75101	2
8070	.	.	.	.	23300	ND
8240	.	.	.	ATW24:Simidu72	25300	ND
8250	.	.	.	.	21300	ND
8080	.	. [Skin Lesion]	Swab	ATW24:EDDA	20100	ND
8090	.	.	.	.	75301	ND
8100	.	.	.	.	20100	ND
8110	.	.	.	.	50000	ND
8260	.	.	.	ATW24:Simidu72	75341	2,2
8270	.	.	.	.	75341	2,2
8271	.	.	.	.	75341	2
8120	Baffle	Fish (26) [Dermal Lesion]	Swab	ATW24:EDDA	75501	4
8130	Ck.	*M.cephalus*	.	.	75501	4
8180	.	.	.	ATW24:Anoxic EDDA	75101	ND
8181	.	.	.	.	75101	2
8190	.	.	.	.	75101	4
8280	.	.	.	ATW24:Simidu72	75301	ND
8290	.	.	.	.	75101	5
8300	.	.	.	.	21300	ND
8310	.	.	.	.	21300	ND
8140	Baffle	Fish (27) [Dermal Lesion]	Swab	ATW24:EDDA	75501	4
8150	Ck.	*M.cephalus*	.	.	55100	ND
8160	.	.	.	.	75301	ND
8170	.	.	.	.	20300	ND
8200	.	.	.	ATW24:Anoxic EDDA	70301	5,5
8210	.	.	.	.	70301	5
8220	.	.	.	.	75301	5
8230	.	.	.	.	75141	5
8320	.	.	.	ATW24:Simidu72	71301	4
8321	.	.	.	.	21300	ND
8330	.	.	.	.	21300	ND
8340	.	.	.	.	31100	ND
8350	.	.	.	.	21300	ND
8290a	Baffle	Fish (28) [Dermal Lesion]	Swab	Simidu	75101	ND
8210a	Ck.	*M.cephalus*	.	TCBS	35101	ND
8220a	.	.	.	.	75401	ND
8310a	Baffle	Fish (29) [Dermal Lesion]	Swab	Simidu	31300	ND
8320a	Ck.	*M.cephalus*	.	.	31300	ND
8330a	.	.	.	.	31300	ND
8230a	.	.	.	TCBS	30700	ND
8240a	.	.	.	.	00700	ND
8340a	Baffle	Fish (30) [Dermal Lesion]	Swab	Simidu	75501	4
8350a	Ck.	*M.cephalus*	.	.	30300	ND
8360	.	.	.	.	31300	ND
8250a	.	.	.	TCBS	10700	ND
8260a	.	.	.	.	10700	ND
8270a	.	.	.	.	55700	ND
8280a	.	.	.	.	75101	ND
8450	Baffle	Fish (31) [Dermal Lesion]	Swab	EDDA	51500	ND
8460	Ck.	*M.cephalus*	.	.	55500	ND
8430	.	.	.	TCBS	55500	ND
8440	.	.	.	.	55500	ND

3.7.2: Strain Origins, Isolation Protocols, Octal Codes, and Batch-Key to Phenotypic Analyses. (Cont.)

Strain Number	Site	Source	Type	Isolation Protocol	Octal Code	Batch No.
Stn						
8510	Baffle	Fish (32) [Dermal Lesion]	Swab	EDDA	75101	5
8520	Ck.	*M.cephalus*	.	.	75101	4
8470	.	.	.	TCBS	75101	4
8480	.	.	.	.	75101	4
8490	.	.	.	.	75101	4,7
8500	.	.	.	.	75101	4
8590	Baffle	Fish (33) [Dermal Lesion]	Swab	EDDA	55100	ND
8600	Ck.	*M.cephalus*	.	.	75101	7
8591	.	.	.	.	50100	ND
8550	.	.	.	TCBS	75101	4,7,8a
8551	.	.	.	.	55100	ND
8560	.	.	.	.	75501	4
8570	.	.	.	.	75101	4,7a
8580	.	.	.	.	70101	ND
8700	Tawnton	Fish (34) [Scale Pocket]	Swab	EDDA	55100	ND
8680	Ck.	*M.cephalus*	.	TCBS	55100	4
8690	.	.	.	.	50100	ND
8720	.	. [Dermal Lesion]	.	TCBS	55540	ND
8730	.	.	.	.	55100	ND
8740	.	.	.	EDDA	15100	ND
8750	.	.	.	.	15100	ND
8790	Tawnton	Fish (35) [Dermal Lesion]	.	EDDA	51100	ND
8800	Ck.	*M.cephalus*	.	.	35401	4
8760	.	.	.	TCBS	75101	ND
8770	.	.	.	.	75141	ND
8780	.	.	.	.	75101	ND
9110	Baffle	Fish (36) [Scale Pocket]	Swab	SENA	03000	ND
9120	Ck.	*M.cephalus*	.	.	15000	ND
9130	.	. [Unaffected skin]	.	SENA	15000	ND
9140	.	.	.	.	05000	ND
9150	.	.	.	.	31100	ND
9160	Baffle	Fish (37) [Unaffected	Swab	SENA	75301	ND
9170	Ck.	*M.cephalus* skin]	.	.	25300	ND
9180	.	.	.	.	75101	ND
9190	Mosquito	Fish (38) [Skin Lesion]	Swab	SENA	75301	ND
9200	Ck.	*M.cephalus* [Dermal Lesion]	.	.	75301	7,8
9210	.	.	.	.	75301	7
9220	Mosquito	Fish (39) [Unaffected	Swab	SENA	77101	7
9230	Ck.	. skin]	.	.	77101	ND
9250	Winfield	Fish (40) [Dermal Lesion]	Swab	SENA	77101	ND
9260	.	*M.cephalus*	.	.	77101	ND
9270	.	.	.	.	77101	ND
9280	.	.	.	.	77101	ND
9290	Winfield	Fish (41) [Unaffected	Swab	SENA	77101	ND
9300	.	M. cephalus. skin]	.	.	77101	ND
9310	.	.	.	.	77101	ND
9320	.	.	.	.	77101	ND
9330	Winfield	Fish (42) [Skin Lesion]	Swab	SENA	77101	ND
9340	.	*M.cephalus*	.	.	77101	ND
9350	.	.	.	.	77101	ND
9360	.	.	.	.	77301	ND
6550	Moreton	Cymothoid Isopod	Streak	SENA	75101	1,4,5,5a
6551	Bay	*Cymothoa indica*	.	.	75101	5
6560	.	[Gut aspirate]	.	.	75101	5,8
6561	.	.	.	.	75101	4,8
6570	.	.	.	.	75101	5
6580	.	.	.	.	75101	5,8
6590	.	.	.	TCBS	75101	5,8

3.7.2: Strain Origins, Isolation Protocols, Octal Codes, and Batch-Key to Phenotypic Analyses. (Cont.)

Strain Number Stn	Site	Source	Type	Isolation Protocol	Octal Code	Batch No.
6600	.	Cymothoid Isopod	.	TCBS	75101	8,8
6610	.	*Cymothoa indica*	.	.	75101	2,8
6611	.	.	.	.	75101	5,8
6620	.	.	.	.	75101	8
6630	.	.	.	.	75101	8
6640	.	.	.	.	75101	ND
1650	Baffle	Cyralanid Isopod	(1) Whole	ATW24:Simidu	75101	6
1660	Ck.	*Pseudolana dactylosa*	.	.	75101	ND
1670	.	.	.	.	75101	1
1680	.	.	.	.	75101	ND
1590	.	.	.	ATW24:TCBS	75101	ND
1600	.	.	.	.	75101	ND
1610	.	.	.	.	75101	6
1620	.	.	.	.	75121	1,8
1630	.	.	.	.	75101	6
1640	.	.	.	.	75101	1
8020	.	*Pseudolana dactylosa*	(2) .	ATW24:TCBS	75101	ND
8030	.	[Mascerate]	.	.	75101	2,2
8040	.	*P.dactylosa*	(3) .	ATW24:TCBS	75101	2
8050	.	[Whole]	.	.	75101	2
8390	Baffle	*P.dactylosa*	(4) Swab	SENA	35101	4,7a
8400	Ck.	[Gut aspirate]	.	.	35101	ND
8420	.	.	.	EDDA	55100	ND
8370	.	.	.	TCBS	75101	4,7a
8380	.	.	.	.	75101	4,7a,8a
8810	Baffle	*P.dactylosa*	(5) .	SENA	35101	4,7a
8820	Ck.	[Gut aspirate]	.	.	75101	4
8830	.	*P.dactylosa*	(6) .	.	75101	4,7a
8840	.	[Gut aspirate]	.	.	00000	ND
8850	.	*P.dactylosa*	(7) .	.	35100	ND
8860	.	[Gut aspirate]	.	.	35100	ND
8870	.	*P.dactylosa*	(8) .	.	35100	ND
8880	.	[Gut aspirate]	.	.	75101	4
8900	Euleilah	*P.dactylosa* (fed)	(9) Streak	SENA	55100	ND
8910	Ck.	[Gut aspirate]	.	.	75101	ND
8920	.	.	.	ATW24:SENA	70100	ND
8921	.	.	.	.	00000	ND
8930	.	.	.	.	75101	ND
8940	.	.	.	BTB24:SENA	75101	ND
8950	.	.	.	.	75101	8,8
8960	.	.	.	0.5 µM EDDA	75101	8
8970	.	.	.	.	00400	ND
8980	.	.	.	0.75 µM EDDA	55100	ND
8981	.	.	.	.	00400	ND
8990	.	.	.	1.0 µM EDDA	55100	8
9000	.	*P.dactylosa* (unfed)	(10) Streak	SENA	75101	ND
9010	.	[Gut rinse]	.	.	77301	8
9020	.	.	.	ATW24:SENA	75101	ND
9030	.	.	.	.	11100	ND
9040	.	.	.	.	75101	ND
9050	.	.	.	BTB24:SENA	75101	ND
9060	.	.	.	.	75501	8
9061	.	.	.	.	31100	ND
9070	.	.	.	0.5 µM EDDA	75101	ND
9080	.	.	.	.	75301	ND
9090	.	.	.	0.75 µM EDDA	75301	ND
9100	.	.	.	1.0 µM EDDA	75301	ND

Table 3.8: Frequency of Presumptive *Vibrionaceae* Cultures Originating from Different Biotic and Abiotic Sources.

		Total	Number Presumptive *Vibrios*	Number further Tested	Number Internal	Number External
Algae	Site [2.]	10	10	7	-	-
Plankton	Site [1.]	20	20	15	-	-
Plankton	Site [3.]	18	17	5	-	-
Copepod	Site [1.]	15	15	10	-	-
Oyster	Site [1.]	45	42	22	-	-
Mussel	Site [2.]	34	28	6	-	-
Prawn	Site [2.]	28	19	2	-	-
Crab	Site [1.]	50	50	26	-	-
Crab	Site [2.]	16	15	4	-	-
Polychaete	Site [2.]	11	11	3	-	-
River Fish	Control	136	69	26	24	2
River Fish	Diseased	353	196	114	31	83
Moreton Bay	*[S.ciliata]*	99	91	73	50	23
Isopods	*[C.indica]*	13	13	12	12	-
	[P.dactylosa]	14	14	9	-	9
Internal	fed	25	13	10	10	-
Internal	unfed	12	10	3	3	-
Water	Site [1.]	26	20	6	-	-
Water	Site [2.]	21	21	11	-	-
Sediment	Site [1.]	37	35	18	-	-
Sediment	Site [2.]	13	10	7	-	-

Table 3.9: Comparative Recoveries of Presumptive *Vibrionaceae* from Different Isolation Protocols.

	Total Isolates.	% Presumptive *Vibrio*
Simidu	132	86
ATW24:Simidu	204	62
BTB24:Simidu	102	60
Total Simidu	**438**	**68**
TCBS	153	85
ATW24:TCBS	178	76
BTB24:TCBS	89	79
Total TCBS	**421**	**79**
SENA	85	80
ATW24:SENA	6	50
BTB24:SENA	5	80
Total SENA	**96**	**78**
EDDA	22	40
ATW24:EDDA	13	54
ATW24:Anoxic EDDA	7	100
Total EDDA	**42**	**55**

Table 3.10: Results of χ^2 Comparisons from 2 X 2 Contingency Tables.

(i)	Simidu	ATW24 Simidu	HIB24 Simidu
Simidu	x		
ATW24 Simidu	22.2**	x	
HIB24 Simidu	20.1**	0.11	x

(ii)	TCBS	ATW24 TCBS	HIB24 TCBS
TCBS	x		
ATW24 TCBS	4.3*	x	
HIB24 TCBS	1.6	0.26	x

(iii)	SENA	ATW24 SENA	HIB24 SENA
SENA	x		
ATW24 SENA	2.94	x	
HIB24 SENA	0.00	0.35	x

(iv)	EDDA	ATW24 EDDA	ANO2 EDDA
EDDA	x		
ATW24 EDDA	0.00	x	
ATW24 AnO2 EDDA	0.55	0.21	x

(v)	Simidu	TCBS	SENA	EDDA
Simidu	x			
TCBS	0.02	x		
SENA	1.17	0.96	x	
EDDA	22.9**	19.7**	13.2**	x

* $\chi^2_{0.05} > 3.84$ Result is "significant."

** $\chi^2_{0.01} > 6.63$ Result is "highly significant."

Of the 996 environmental and fish isolates, 438 were taken from Simidu medium, 420 from TCBS medium, 96 from SENA medium, and 42 from media containing EDDA. Results for comparative percentages of presumptive *Vibrionaceae* recovered by different isolation protocols are shown in Table 3.9. Tables 3.10 (i-v) summarize results from a series of statistical comparisons, (χ^2 from 2 X 2 contingency tables), between proportions of cultures designated as presumptive *Vibrionaceae* but from different isolation protocols. No significant difference was found between percentage recoveries of presumptive *Vibrionaceae* from SENA, Simidu or TCBS primary isolation media however each of these media, when compared with the EDDA medium, had "very highly significantly" greater proportions of isolates classified as presumptive *Vibrionaceae.*

Presumptive *Vibrionaceae* obtained from the same isolation media but first enriched in ATW or BTB-teepol were not significantly different, but both broths in combination with TCBS and particularly Simidu media had significantly lesser recoveries than these from primary isolation media. This effect was less clear in the smaller samples for secondary isolation from broths onto SENA and EDDA media.

A comparison between treatments of the small numbers of presumptively designated *Vibrionaceae* from EDDA media, indicated no significant differences.

3.7.3.2 Comparative Recoveries of Stored Cultures from Selective Media.

Both Simidu and TCBS media failed to support growth by the Tasmanian *Vibrio* sp. DA 7 and *Plesiomonas shigelloides* UQM 1617, but all remaining strains grew on Simidu medium. *V.metschnikovii* UQM 211, *V.gazogenes* UQM 2840, *V.costicola* UQM 2888, *V.anguillarum* DA 1-3, *V.pelagius* DA 09, and the *Vibrio* sp. DA 11, did not grow on TCBS medium.

Crossed-comparisons of recoveries of these 85 strains of *Vibrionaceae* from 2 X 2 contingency tables indicated no significant difference between SENA and Simidu media, but the χ^2 for Simidu and TCBS medium was calculated

as 5.2, indicating a "significantly" (i.e. χ^2 tab.$^{6.63}_{0.01}$ > χ^2 calc. > χ^2 tab.$^{3.84}_{0.05}$) greater inhibition of different *Vibrionaceae* by TCBS than by Simidu agar media (and SENA medium).

3.7.4 Discussion.

SENA medium, containing as selective criteria only 2 % salt and a nutrient base, was found to be less inhibitory but at least as selective as TCBS agar medium and comparably inhibitory and as selective as Simidu agar medium. Findings for SENA medium were however based upon smaller and less diverse samplings than the other two isolation media and might not be extensible to more diverse environmental isolations of bacteria such as from organically polluted water. Simidu medium which differs from other media principally by using facultative anaerobicity as a selective criterion was also less inhibitory than TCBS medium and was shown in parallel isolations from ecologically diverse sources, to be as selective for presumptive *Vibrionaceae.* However this medium appeared less selective than TCBS when used in concert with enrichment broths. Media which contained EDDA were generally not as selective for presumptive *Vibrios* as SENA, Simidu, and TCBS media however the small sample of cultures isolated from EDDA medium and anaerobically incubated might with more diverse evaluation indicate an appropriate means to simultaneously select for iron chelating (potentially virulent) *Vibrionaceae* (See 3.9).

While comparative percentage recoveries of presumptive *Vibrionaceae* from both isolation and enrichment media provide a general indication of the facility to support different species and selectivity for a group, without examination of the resultant cultures as measured by species distribution and relative abundance on different media (3.9) no further evaluation of media suitability can be made.

The experimental data from Appendix 2. for culture survival on Swabs in Amies transport medium seems to indicate that culture attrition between sampling and receival times uniformly affects different species of

Vibrionaceae, and so on the basis of that work it seems that unavoidable delays between sampling and inoculation of isolation or enrichment media may not have seriously affected the composition and relative mix of different species as they were *in situ.*

The presence of bacteria including presumptive *Vibrionaceae* in the internal organs of control river system fish might have reflected the onset of putrefaction in these fish before autopsy, or that these species comprise part of the flora of previously diseased fish, but which are held in stasis by the now recovered fish. The concurrent presence of trypanorrynch cysts on internal organs of RS affected and asymptomatic fish and the diversity of different bacterial groups present on these samples suggests the most likely explanation was that these larvae, during migration through the gut wall and encystation on internal organs, acted as mechanical vectors for the release of normal fish gut flora into the mesenteric cavity such as was earlier reported for nematode larvae by Shiply (1909) (2.5.1). Independent of these possibilities is the observation that the *Vibrionaceae* was present as a substantial component in the normal bacterial flora of diseased and control river system fish and as a dominant component of the flora of diseased Moreton Bay fish. The composition of the comparative bacterial flora from diseased and control fish might then provide an indication as to whether there are distinct *Vibrionaceae* floral communities on diseased and control fish or whether the same species are present from both fish groups. Likewise the composition of the demonstrated substantial *Vibrionaceae* flora from abiotic and biotic sources across different trophic levels may show distinct or common floral communities.

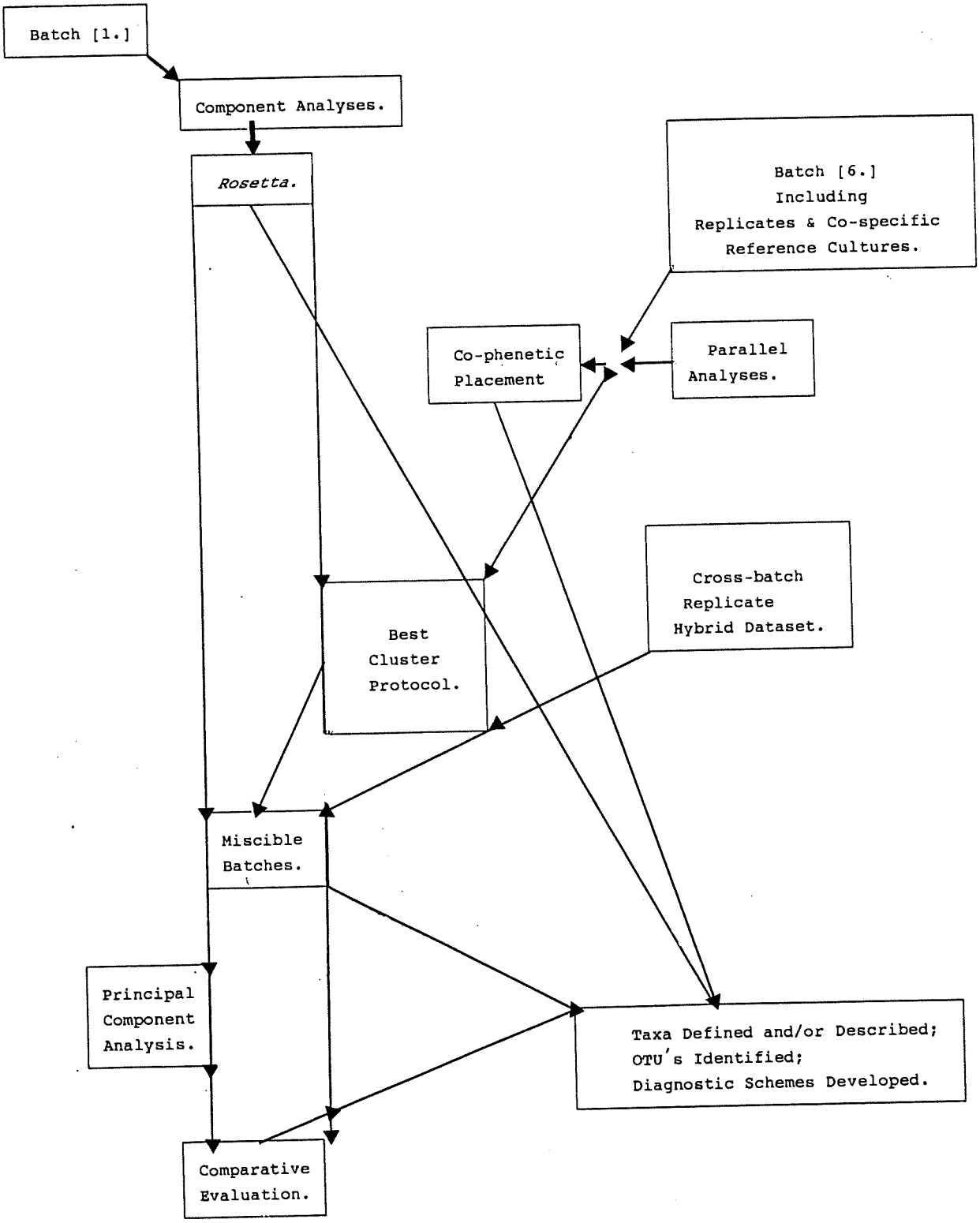
3.7.5 Conclusion.

It appears that different not normally invasive bacteria might be carried from the guts of RS prone fish into the mesentery through the actions of internal mechanical vectors i.e. migrating parasite larvae.

Different selective media were shown to yield similar proportions of presumptive *Vibrionaceae* from fish and the environment, but no determination

could be made as to which medium or protocol was the most suitable without first knowing the relative species distributions as produced by different media and protocols and the relative contributory significance of these species to RSD.

Figure 3.3.2: Schematic Overview of Numerical Analyses.



3.8.0 Numerical Analysis.

3.8.1 Experimental Design.

Presumptively identified *Vibrionaceae* were subjected to a series of detailed phenotypic and genotypic taxonomic analyses in order to further define their environmental distributions and appropriate isolation procedures. For logistical reasons it is often impossible to comply with the ideal situation for numerical analysis where all tests are conducted simultaneously. Two solutions are available; the first and most often used, and that recommended by O'Brien and Colwell (1987), relies upon having all strains available at experimental outset and progressively building a library of test results by fragmented simultaneous testing. This approach makes no concession to requirements for characterization of new strains, and logistical problems associated with the prolonged maintenance of many i.e. > 100 test cultures make this approach unfeasible for large analyses.

The second approach requires that analyses are conducted in a series of discrete batches each with manageable numbers of OTU's, which are later pooled and concurrently examined. This procedure suffers principally from artifactual noise i.e. experimental error from preparation and test procedures (2.9.3.6.1). Baumann, Furniss and Lee (1984) recommended against batch-wise diagnosis of *Vibrionaceae* i.e. by isolated comparison of test results with published attributes of reference cultures. However this batch approach may be validly implemented, if analyses are properly planned i.e. with datum-cultures included within- and across- batches to permit cross-correlation of test results. Attributes from these replicated datum-cultures, not corresponding between batches can then be eliminated from analyses and results standardized to the extent that crossed-comparisons become valid (See 3.8.5).

Numerical analyses were based around the premise that a *Rosetta* of well characterized OTU's could be used repeatedly as a diagnostic master-key in concurrent analyses with data for different OTU's gathered from successive batches. Apart from initially phenetically defining the *Rosetta,* (3.8.3.0)

analyses were conducted in a stepped-series, (Figure 3.3.2) which successively investigated procedures for assimilation of missing values and clustering (3.8.4.0); and then tested the extensibility of chosen best methods by concurrent cluster analysis of partial and complete replicates (3.8.5.0). Final analyses (3.8.6.1-3) were with pooled datasets formulated accordingly, and also partially tested by multivariate procedure (3.8.6.4).

3.8.2 Repetitive Methods.

Other problems of large-scale bacterial phenetic assay, from cross-feeding (2.9.3.6.1) and swarming *Vibrionaceae,* (Appendix 4.) were addressed by physically isolating OTU's in multiwell trays. Linbro$^{r.}$ **(Flo)** trays were chosen because of design suitability, (see Plate 3.7). In these, 24 separate wells were contained in a rectangular array. The lids possessed rims which when closed, overlapped each well to minimize gaseous diffusion and prevent contaminating overgrowth between wells via condensates.

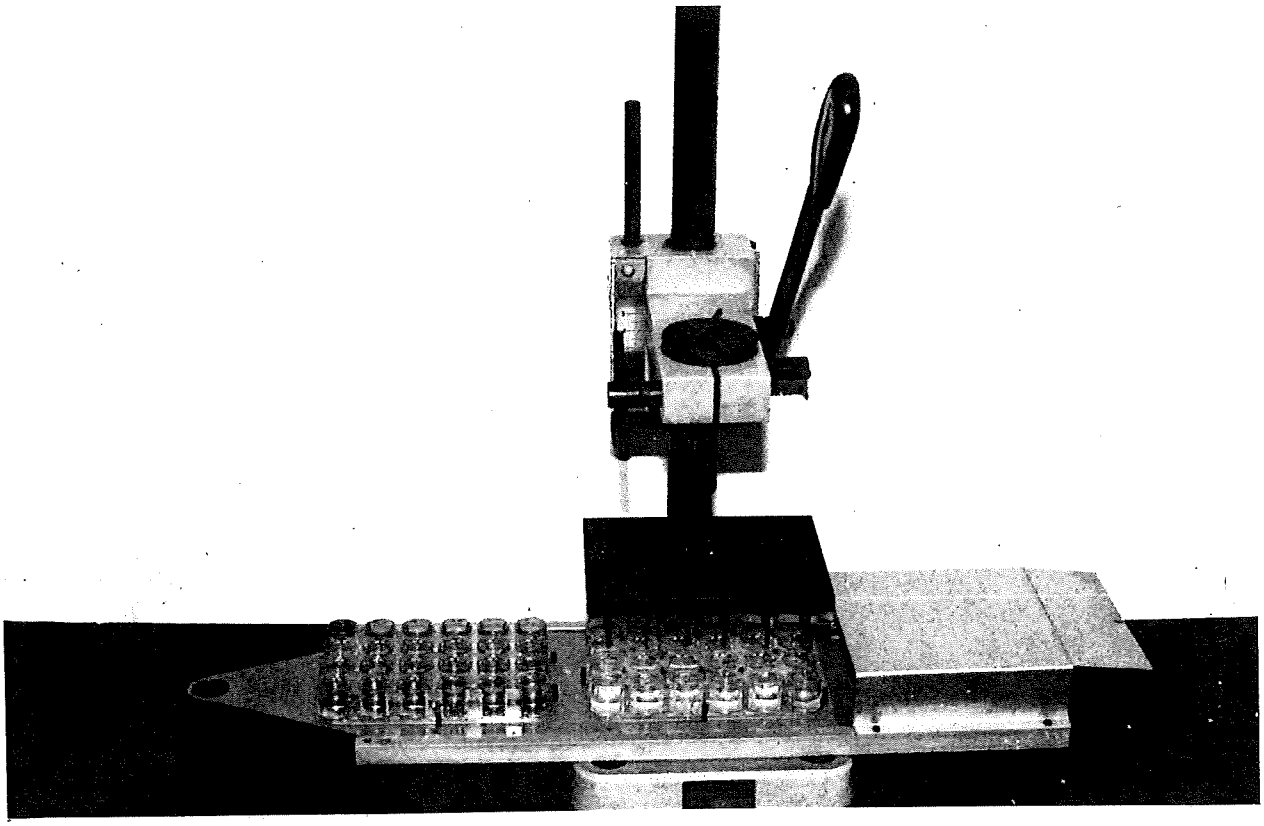
3.8.2.1 Preparation and Inoculation of Cultures.

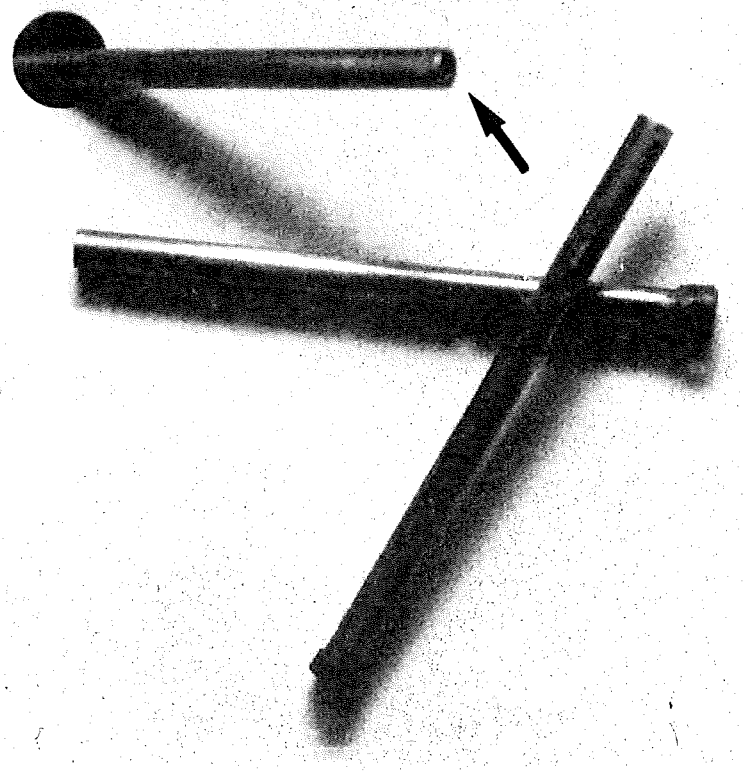
Strains were resuscitated from storage (3.3.4) in batches of 55 or 110. The type strain for *V.anguillarum* (UQM 2771) was included in all batches. Within each batch at least two strains were assigned as blind duplicates under different OTU numbers. Growth from 24 h plates of SENA was suspended in 5 ml sterile marine phosphate buffered saline to a turbidity equivalent of 3 on the MacFarland scale. Tubed media were inoculated by pasteur pipette and a further *ca* 1 ml of each culture suspension transferred to cupules in a sterile multi-well tray for simultaneous multiple inoculation.

The inoculator used was a modified drill press (Plate 3.5, *Top)* carrying a brass template with twenty-four sterile, free sliding, hemispherically excavated, stainless steel, 2mm diameter inoculation pins, calculated to deposit *ca* 1µl inoculum per well (Plate 3.5, *Bottom).*

Inoculation procedure using this device was as for tests of swarming inhibitors (Appendix 4.). Control trays of carbon assimilation (3.4.4.38) medium were inoculated first, followed by trays of this medium with single

Plate 3.5: *Top,* 24 Locus Inoculator with Trough and Target Trays on Carriage; *Bottom,* Stainless Steel Inoculation Pin with Excavation Indicated by Arrow.





carbon sources, in any order except that volatile organic acids were inoculated last. Of the remainder, multiwell trays lacking inhibitory supplements were inoculated before more stringent media.
Inoculated trays were placed in plastic bags, sealed and incubated. A loopful of each of the inocula, from initial culture suspension and from the multiwell inoculation trough were streaked to half plates of SENA to check purity after 24 and 48 h incubation. OTU's which appeared impure in either streak-pattern were replicated in subsequent batches but included in computer analyses as a consideration to the possibility of colony pleomorphism.

The data-sets, and number of OTU's phenetically characterized were: Batches [1.], [2.], & [3.]; 48, Batches [4.], [5.], [6.] & [7.]; 96, and Batch [8.], 100. This gives a total of 628 OTU's so tested. The different batch sizes here reflect the numbers of OTU's simultaneously examined. The number of partially replicated OTU's in supplementary tests conducted in parallel with these batches was 92 (See 3.8.5). Separately these comprised the following numbers of OTU's Batches [5a.], [6a.], [7a.], 24; and Batch [8a], 20 OTU's. OTU's for *V.anguillarum* UQM 2771 were included in all batches.

3.8.2.2.0 Numerical Analysis Test Procedures.

Phenetic test characters (190), were based upon morphological, physiological, biochemical, and restricted nutritional facets. For third party verification strains which produced positive and negative reactions within the context of these protocols and which may be useful as positive- and negative-controls in future studies are listed:

3.8.2.2.1 Morphological Observations.

Streak plates of SENA were incubated 24h and the following morphological observations were made.

3.8.2.2.1.1 Colony Diameter more than 3mm.

Positive: *Vibrio natriegens* UQM 0879. **Negative:** *Vibrio anguillarum* UQM 2771.

3.8.2.2.1.2 Colony Mucoid or stringy to loop contact.

Positive: *Vibrio natriegens* UQM 3077. **Negative:** *Vibrio anguillarum* UQM 2771.

3.8.2.2.1.3 Colony Surface Matt to incident light.

Positive: *Vibrio cholerae* UQM 2773. **Negative:** *Vibrio anguillarum.* UQM 2771.

3.8.2.2.1.4 Colony Opaque to transmitted light, measured by legibility of printed text through growth on SENA, under standard lighting conditions. Opaque colonies were recorded as positive.

Positive: *Vibrio natriegens* UQM 879. **Negative:** *Vibrio anguillarum* UQM 2771.

3.8.2.2.1.5 Colony Margin Entire.

Positive: *Vibrio natriegens* UQM 2782. **Negative:** *Vibrio alginolyticus* UQM 2770.

3.8.2.2.1.6 Colony Convex.

Positive: *Vibrio anguillarum* UQM 2771. **Negative:** *Vibrio alginolyticus* UQM 2770.

3.8.2.2.1.7 Swarming Growth.

See 3.7.2.6.05

Positive: *Vibrio alginolyticus* UQM 2770. **Negative:** *Vibrio anguillarum* UQM 2771.

3.8.2.2.1.8 Luminous Growth.

See 3.7.2.6.11.

Positive: *Vibrio harveyi* UQM 2781. **Negative:** *Vibrio anguillarum* UQM 2771.

3.8.2.2.2 Delayed Colony Morphology Features.

Growth from these plates were used to score tests 3.8.2.2.2.1-4 and the plates were reincubated for a further 6d and the following binary attributes were coded.

3.8.2.2.2.1 Presence of Red Pigment.

Positive: *Vibrio gazogenes* UQM 2840. **Negative:** *Vibrio anguillarum* UQM 2771.

3.8.2.2.2.2 Presence of Brown Pigment.

Positive: *Aeromonas hydrophila* UQM 2769. **Negative:** *Vibrio anguillarum* UQM 2771.

3.8.2.2.2.3 Presence of Black Pigment.

Positive: *Vibrio nigripulchritudo* ATCC 27043. **Negative:** *Vibrio anguillarum* UQM 2771.

3.8.2.2.2.4 Presence of Yellow-Orange Pigment.

Positive: *Vibrio cholerae* UQM 2742. **Negative:** *Vibrio anguillarum* UQM 2771.

3.8.2.2.3 Cell Morphology.

3.8.2.2.3.1 Gram State.

Clean microscope slides were divided into 8 sectors with a diamond pencil. A loopful of growth from 24h SENA was deposited on each sector, emulsified in a drop of MPBS and allowed to air dry. The preparations were heat fixed and stained according to Burke's modification (Doetsch 1981) of the Gram stain procedure i.e. slides were flooded with aq. crystal violet (3.4.4.02(a)) 30 s, rinsed with tap-water, flooded with Lugol's iodine (3.4.4.02.(b)) and mordanted 60 s. Preparations were drained and 95% ethanol was added dropwise and until no more colour eluted, flooded with 2% aq. safranin-O 10 s., rinsed with water, blotted dry and examined under oil at X 1000.

Positive: *Streptococcus agalactiae* UQM 1737. **Negative:** *Vibrio anguillarum* UQM 2771.

3.8.2.2.3.2 Straight Rods, as observed in Gram-stained preparations.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.metschnikovii* UQM 211.

3.8.2.2.3.3 Variable Length Rods, as observed in Gram-stained preparations.

Positive: *V.metschnikovii* UQM 211. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.3.4 Cell Length Greater than Two Times Width, as observed in Gram-stained preparations.

Positive: *V.metschnikovii* UQM 211. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.4 Metabolite Production.

3.8.2.2.4.1 Catalase.

The butt of a sterile swab stick was placed onto the surface of a 24 h colony and the stick transferred to 1 ml catalase test reagent (3.4.4.03) in 12 x 75 mm tubes. Results were scored as positive if foam was generated within 20 s.

Positive: *V.anguillarum* UQM 2771. **Negative:** *S.agalactiae* UQM 1737.

3.8.2.2.4.2 Strong Catalase.

Tubes from above were examined after 5 min, results where more than 0.5 cm foam was produced were scored as positive. Less than 0.5 cm foam was scored as negative.

Positive: *V.ordalii* UQM 2890. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.4.3 Oxidase.

As in 3.7.2.6.03 except that a platinum loop was used to spread growth onto test strips.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.metschnikovii* UQM 211.

3.8.2.2.4.4 Oxidase after Toluene Extraction.

Growth from OTU's which were oxidase-negative or produced delayed reactions in (3.8.2.2.4.3) was emulsified on a microscope slide in a drop of toluene (Baumann, Baumann, Mandel and Allen, 1972) and spread to oxidase strips and scored as above.

Positive: *V.damsela* UQM 2853. **Negative:** *V.metschnikovii* UQM 211.

3.8.2.2.4.5 Cholera-Red Reaction.

Multi-well trays of SENB were inoculated and incubated 24 h. To each well 0.5 ml 1 N hydrochloric acid was added. Strains which produced a diffuse red colouration were scored as positive. No colouration was scored as negative.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.natriegens* UQM 2782.

3.8.2.2.5 Broth Based Tests.

SENB (3 ml), were inoculated and incubated for periods as below and the following characters were recorded.

3.8.2.2.5.01 Motility.

SENB (3 h), culture was examined as in 3.7.2.6.04.

Positive: *V.anguillarum* UQM 2771. **Negative:** *A.punctata* UQM 156.

3.8.2.2.5.02 Polar Flagellation.

As above also checking for the characteristic darting, and collectively elixate motility (3.7.2.6.06) of polar flagellate bacteria. Non-motile and atypical OTU's were examined using the flagella staining procedure of Rhodes (1958) as follows: A sample (40 µl) of the inoculum suspension prepared in MPBS was placed on a microscope slide. The slide was tilted to produce a cell density gradient as the inoculum drop flowed down the slide, and air dried. The slide was immersed in mordant solution for *ca* 6 min and then into hot, *ca* 90°C., ammoniacal silver nitrate in a petri dish on a hot plate for 5 min. The slide was removed, the under surface wiped and the

preparation examined for the type of flagellation using oil immersion microscopy at 1 000 X, (See Plate 3.6).

Positive: *V.anguillarum* UQM 2771; *A.punctata* UQM 156. **Negative:** *E.coli* UQM 1803.

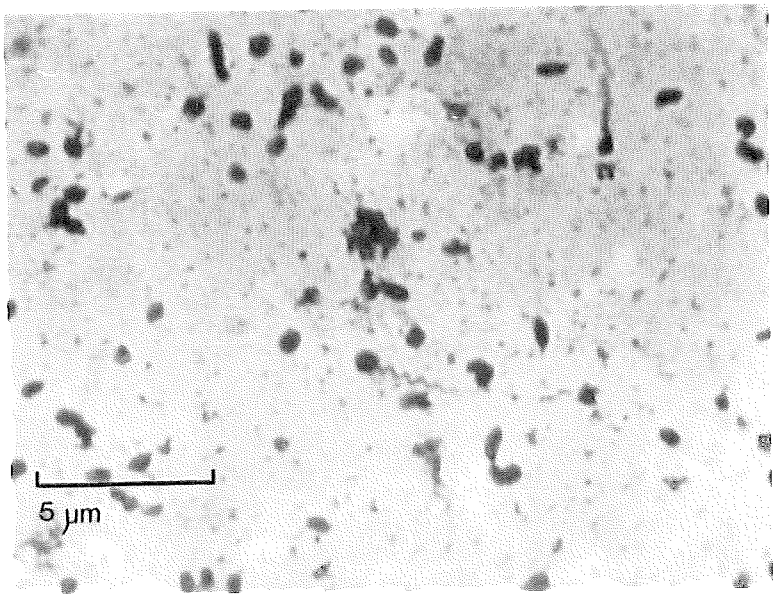


Plate 3.6: Polar and Fascicles of Lateral Flagella of a Swarming *Vibrio* sp.

3.8.2.2.5.03 24 h SENB Uniform Turbidity.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.zobellii* UQM 3231.

3.8.2.2.5.04 SENB (24 h) with Sediment.

Sediment formed at bottom of tube.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.metschnikovii* UQM 211.

3.8.2.2.5.05 SENB (24 h) Sediment Mucoid.

Tubes of SENB were subjected to a short twisting motion. Mucoid OTU's presented a rising filamentous plume.

Positive: *V.orientalis* UQM 2921. **Negative:** *V. ordalii* UQM 2890.

3.8.2.2.5.06 SENB (24 h) Growth Moderate to Heavy.

Where text was not legible through growth, under standard illumination was scored positive for moderate to heavy growth.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.metschnikovii* UQM 211.

3.8.2.2.5.07 SENB (24 h) Growth with Pellicle.

Positive: *V.anguillarum* UQM 2771 **Negative:** *V.metschnikovii* UQM 211.

3.8.2.2.5.08 Indole.

Kovacs' (1928) reagent, (0.5 ml), (3.4.4.05.1) was added to tubes of SENB (24 h). Positive cultures showed a 2mm deep magenta layer at the surface. Negative OTU's produced only a faint pink or yellow layer.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.ordalii* UQM 2890.

3.8.2.2.5.09 Indole Weak.

The medium (3.4.4.05.2) with additional tryptophan was tested as above after 24 h.

Positive: *V.nereis* UQM 2783. **Negative:** *V.damsela* UQM 2853.

3.8.2.2.5.10 pH greater than 7.05 (5 d).

A 40 µl sample from MRVP (3.4.4.06.2) culture suspension was withdrawn after 5 d incubation and placed on a pH 6.05 - 7.1 range indicator paper (Merck). The pH was recorded and scored as positive if above 7.05. (Adapted from Davis and Park, 1962).

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.damsela* UQM 2853.

3.8.2.2.5.11 pH greater than 5.15 (5 d).

When the pH from MRVP medium incubated above was less than 6.05 a further 40 µl culture of suspension was put to indicator paper pH 3.5 - 5.4 (Merck). The pH was recorded and scored as positive if above 7.05. (Adapted from Davis and Park, 1962).

Positive: *V.damsela* UQM 2853. **Negative:** *V.hollisae* UQM 2852.

3.8.2.2.5.12 Acetoin Production (Barritt, 1936).

MRVP medium from above was further tested for acetoin production by vigorous addition of 1 ml 5 % alcoholic α-napthol and 1 ml 20 % aq. potassium hydroxide (3.4.4.06.1) and allowed to stand 15 min. at 37°C. Positive tests produced a red layer at the air liquid interface.

Positive: *V.anguillarum* UQM 2771. **Negative:** *(V.tubiashii)* UQM 2923.

3.8.2.2.5.13 Nitrate Reduction.

Nitrate reduction medium (3 ml) (3.4.4.07.2) was inoculated and incubated for 5 d. One ml solution (a) and 1 ml of solution (b) (3.4.4.07.1) were added. If no red colour developed *ca* 0.05 g zinc powder was added. Tubes colouring red initially were scored as positive as were those which showed

no colouration upon addition of zinc. Colouration which occurred only after the addition of zinc was scored as negative.

Positive: *V.natriegens* UQM 2782; *V.nereis* UQM 2783. **Negative:** *V.metschnikovii* UQM 211.

3.8.2.2.5.14 Nitrite Reduction.

Nitrite reduction medium (3ml) (3.4.4.07.3) were inoculated and incubated 5d. Solutions (a) and (b) were added as above. Tubes showing red colouration were scored as negative and uncoloured tubes were scored positive.

Positive: *V.natriegens* UQM 2783. **Negative:** *V.nereis* UQM 2783.

3.8.2.2.5.15 Gluconate Oxidation.

Gluconate medium (3ml) (3.4.4.09.2) was inoculated and incubated 5 d. Benedict's reagent (1ml) (3.4.4.09.1) was added and the medium heated to 80°C. for 30 min. OTU's which produced a thick flocculant precipitate were scored as positive, those for which no or light precipitation was observed were scored as negative.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.gazogenes* UQM 2840.

3.8.2.2.5.16 Arginine dihydrolase Thornley (1960).

After stab inoculation of test and control tubes of arginine dihydrolase medium (3.4.4.10) tubes were sealed with 2 cm sterile agar plugs (3.4.4.10 (c)) and incubated 7 d. Positive tests showed a greater alkaline reaction (red) compared with the control.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.parahaemolyticus* UQM 2776.

3.8.2.2.5.17 Lysine Decarboxylase, (LDC).

The method of Moller (1955) was adopted with the prior addition of mineral oil (1ml) (Cowan, 1974). A heavy inoculum, (0.15) ml was added atop the paraffin to test and control tubes of decarboxylase media (3ml) (3.4.4.11) and these incubated 7 d. Control tubes without lysine were checked for an acid (yellow) reaction; if this was not evident the corresponding lysine test tube was scored as ND. Strains producing an alkaline reaction (blue) in the tube with lysine were scored as positive.

Positive: *V.parahaemolyticus* UQM 2776. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.5.18 Ornithine Decarboxylase, (ODC).

As above except ornithine was used in place of lysine.

Positive: *V.parahaemolyticus* UQM 2776. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.5.19 Glucose Fermentation.

Tubes containing sodium-enriched oxidation fermentation medium, (3ml) (3.4.4.12) (Hayward 1964) were stab inoculated in duplicate and one tube was overlaid with a 2 cm sterile agar plug. These were incubated 7 d and scored positive if both tubes were entirely yellow and negative if only the aerobic tube was yellow.

Positive: *V.anguillarum* UQM 2771. **Negative:** *(Beneckea hyperoptica)* UQM 469.

3.8.2.2.5.20 Gas from Glucose.

Fermentation tests which produced sufficient gas to displace the agar plug were scored as positive.

Positive: *Aeromonas hydrophila* UQM 2838. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.6 Hydrolytic Enzyme Production.

3.8.2.2.6.01 ONPG Hydrolysis (o-Nitrophenyl-β-D-galactopyranosidase Production).

ONPG/Tryptone water (1 ml) (3.4.4.13) was inoculated and incubated 24 h. Positive tests showed bright or moderate yellow colouration. Negative tests showed little or no yellow colouration.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.hollisae* UQM 2852.

3.8.2.2.6.02 Urea Hydrolysis.

Slopes of modified Christensen's urea medium (3 ml) (3.4.4.14) were inoculated and incubated 24h. Positive OTU's produced a red colouration to the butt and slope.

Positive: *V.parahaemolyticus* UQM 2776. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.6.03 Aesculin Hydrolysis.

Multiwell trays of SENA were overlaid with 0.5 ml aesculin agar (3.4.4.15), inoculated and incubated 24 h. Strains were scored as positive if a dark brown halo was produced around colonies (See Plate 3.7, top).

Positive: *V.natriegens* UQM 2782. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.6.04 Aesculin Hydrolysis Weak.

As above with less intense colouration were scored positive.

Positive: *V.pelagius* UQM 2785. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.6.05 Agar Hydrolysis.

Strains were inoculated to multiwell trays of SENA and incubated 24 h. Strains which subsided into the medium and which clarified agar surrounding colonies were scored as positive.

Positive: *V.pelagius* UQM 3245. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.6.06 Egg Albumen Hydrolysis.

Multiwell trays of SENA were overlaid with egg albumen agar (3.4.4.16) were inoculated, incubated 48 h and flooded with 1 N hydrochloric acid to precipitate protein. Obstructive growth (especially from swarming OTU's) was removed with a cotton wool swab and the trays washed in tap water. Positive OTU's produced a cleared halo around original growth or its locus.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.vulnificus* UQM 2740.

3.8.2.2.6.07 Arbutin Hydrolysis.

Multiwell trays of SENA were overlaid with arbutin agar (3.4.4.17) were inoculated and incubated 24 h. Positive OTU's produced a brown diffusible pigment.

Positive: *V.gazogenes* UQM 2742. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.6.08 Aryl-sulphatase.

Multiwell trays of SENA were overlaid with agar containing tri-potassium phenolpthalein disulphate (3.4.4.18) inoculated and incubated 14 d. Wells in which OTU's produced aryl-sulphatase were coloured bright pink.

Positive: *V.pelagius* UQM 2785. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.6.09 Casein Hydrolysis.

Multiwell trays of SENA were overlaid with casein medium (3.4.4.19), inoculated and incubated 24 h. Trays were flooded with N hydrochloric acid and where necessary, after precipitation, wells were swabbed and washed as for albumen hydrolysis.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.ordalii* UQM 2906.

3.8.2.2.6.10 Chitin Hydrolysis.

Multiwell trays of SENA were overlaid with chitin agar (3.4.4.20), inoculated and incubated 14 d. Trays were inverted and examined under transmitted illumination for hydrolysis and clearing of the chitin suspension.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.gazogenes* UQM 2840.

3.8.2.2.6.11 Collagen Hydrolysis.

Multiwell trays of SENA were overlaid with collagen agar (100 ml sterile molten SENA with 10 ml of a 10 % aqueous gamma sterilized suspension of collagen (Sigma). Trays were inoculated and incubated 7 d. Positive OTU's were scored for their clarification of collagen suspension.

Positive: *V.anguillarum* UQM 3250. **Negative:** *Photobacterium* sp. UQM 3269.

3.8.2.2.6.12 DNA Hydrolysis.

Multiwell trays of SEDNA (3.4.4.21) were inoculated and incubated 24 h. Trays were flooded with N hydrochloric acid to precipitate DNA. Where necessary wells were swabbed and washed as for albumen hydrolysis. Positive OTU's were scored for their clarification of DNA in wells.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.ordalii* UQM 2906.

3.8.2.2.6.13 Gelatin Hydrolysis.

Multiwell trays of SENA were overlaid with gelatin agar (3.4.4.22) inoculated and incubated 24 h. To each well was added 0.5 ml N hydrochloric acid and *ca* 0.5 g ammonium sulphate crystals. Where necessary, after gelatin precipitation, wells were swabbed and washed as for albumen hydrolysis. Positive OTU's were scored for their clarification of gelatin.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.diazotrophicus* UQM 2780.

3.8.2.2.6.14 Lecithinase.

Multiwell trays of SENA were overlaid with egg-yolk agar (3.4.4.23) inoculated and incubated for 7d. After 24 and 48 h the colony margins were examined for a nacreous appearance.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.gazogenes* UQM 2840.

3.8.2.2.6.15 Lipase.

The trays from 3.8.2.2.6.1.14 were examined for the presence of a precipitant halo after incubation for up to 4d.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.ordalii* UQM 2890.

3.8.2.2.6.16 Esterase (SDS), 0.2%.

Wells from in trays from 3.8.2.2.9.18 which contained growth were also scored according to whether or not a precipitant halo encircled growth. Results were coded as ND where no growth was observed.

Positive: *V.natriegens* UQM 2782. **Negative:** *V.anguillarum* UQM 2843.

3.8.2.2.6.17 Egg Proteolysis.

Trays from 3.8.2.2.6.14-15 were further examined after 7 d for clearing of the egg layer.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.ordalii* UQM 2906.

3.8.2.2.6.18 Phosphatase.

Multiwell trays of SENA were overlaid with phenolpthalein diphosphate agar (3.4.4.24), inoculated and incubated for up to 7 d. Wells in which OTU's produced phosphatase were coloured bright pink.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.gazogenes* UQM 2842.

3.8.2.2.6.19 Starch Hydrolysis.

Multiwell trays of SENA overlaid with starch agar (3.4.4.25) were inoculated and incubated 48 h. To each well 0.1 ml Lugol's iodine (3.4.4.02) was added and OTU's which produced clear zones were scored as positive. Growth was swabbed from tests with swarming or mucoid OTU's.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.nereis* UQM 2783.

3.8.2.2.6.20 Sudanophilic Inclusions.

Growth (48 h) from trays of HBSENA (3.4.4.26) were emulsified and stained according to Burdon (1946).

Positive: *V.orientalis* UQM 3196. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.6.21 Many Sudanophilic Inclusions.

Positive: *V.nereis* UQM 2783. **Negative:** *V.orientalis* UQM 3196.

3.8.2.2.6.22 Sulphide Production.

Multiwell trays containing SENA supplemented with lead acetate and sodium thiosulphate (3.4.4.27) were inoculated and incubated 24 h. OTU's which produced a black discolouration and a metallic sheen were scored as positive.

Positive: *S.putrefaciens* UQM 3373. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.6.23 Serum Hydrolysis.

Multiwell trays of SENA were overlaid with horse serum agar (3.4.4.28) and incubated 48 h. Serum protein was precipitated by adding 0.5 ml N hydrochloric acid and *ca* 0.5 g ammonium sulphate crystals to each well. When obstructive growth occurred, after protein precipitation, wells were swabbed and washed as for albumen hydrolysis. Positive OTU's were scored for their clarification of serum.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.mediterranaei* UQM 3076.

3.8.2.2.6.24 Tyrosine Hydrolysis.

Multiwell trays of SENA were overlaid with tyrosine agar (3.4.4.29) were inoculated and incubated for up to 14 d. Strains which cleared the layer of tyrosine were scored as positive.

Positive: *V.natriegens* UQM 2782; *V.parahaemolyticus* UQM 2201. **Negative:** *V.anguillarum* UQM2771.

3.8.2.2.6.25 Pigment Production from Tyrosine.

The trays from above were observed for the formation of a brown melanin layer.

Positive: *V.natriegens* UQM 2782. **Negative:** *V.parahaemolyticus* UQM 2201.

3.8.2.2.6.26 Xanthine Hydrolysis.

Multiwell trays of SENA were overlaid with xanthine agar (3.4.4.30) inoculated and incubated for up to 14 d. Strains which cleared the xanthine layer were scored as positive.

Positive: *V.orientalis* UQM 2921. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.7.0 Congo Red as an Indicator of Virulence and Nitrogen Fixation.

3.8.2.2.7.1 Experimental Design.

Congo Red medium (3.4.4.31) was incubated 24 h and colony colour was recorded. Red colonies according to the criteria of Payne and Finkelstein (1977) are more likely to be virulent. White colonies according to Harrigan and McCance (1966) are indicative of nitrogen fixation (See Appendix 6).

3.8.2.2.7.2 Results and Discussion.

This procedure was found with *Vibrio* spp. to produce a large number of orange colonies and was difficult to interpret, and inconsistent results occurred between duplicates.

3.8.2.2.7.3 Colonies Red on Congo Red Agar (24 h)

Positive: *V.damsela* UQM 2853. **Negative:** *V.anguillarum* UQM 2843; *V.anguillarum* UQM 2771.

3.8.2.2.7.4 Colonies White on Congo Red Agar (24 h).

Positive: *V.anguillarum* UQM 2843. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.7.5 Decolourization of Congo Red. (Appendix 6).

Positive: *V.diazotrophicus* UQM 2780. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.8 Temperature, Salinity and pH Tolerance.

3.8.2.2.8.01-5. Growth at 4, 15, 30, 37, & 42°C.

Multiwell trays of SENA were inoculated, sealed in plastic bags and incubated at 15, 30, 37, & 42°C.; 24 h or at 4°C. 7d.

3.8.2.2.8.01 Growth at 4°C.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.natriegens* UQM 2782.

3.8.2.2.8.02 Growth 15°C.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.hollisae* UQM 2852.

3.8.2.2.8.03 Growth 30°C.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.anguillarum* UQM 3243.

3.8.2.2.8.04 Growth 37°C.

Positive: *V.parahaemolyticus* UQM 2776. **Negative:** *V.costicola* UQM 2888.

3.8.2.2.8.05 Growth 42°C.

Positive: *V.parahaemolyticus* UQM 2776. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.8.06-11 Growth 0 - 10 % Sodium Chloride.

Salt medium (3.4.4.32) was prepared with 0, 0.5, 1, 3, 6, and 10 % sodium chloride sterilized and dispensed into multiwell trays, inoculated and incubated 24 h.

3.8.2.2.8.06 Growth in the presence of 0 % NaCl.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.damsela* UQM 2853.

3.8.2.2.8.07 Growth in the presence of 0.5 % NaCl.

Positive: *V.damsela* UQM 2853. **Negative:** *V.nereis* UQM 2783.

3.8.2.2.8.08 Growth in the presence of 1.0% NaCl.

Positive: *V.nereis* UQM 2783. **Negative:** *V.orientalis* UQM 3276.

3.8.2.2.8.09 Growth in the presence of 3.0% NaCl.

Positive: *V.nereis* UQM 2783. **Negative:** *Vibrio* sp. Be 14.

3.8.2.2.8.10 Growth 6% NaCl.

Positive: *V.nereis* UQM 2783. **Negative:** *V.vulnificus* UQM 3032.

3.8.2.2.8.11 Growth 10% NaCl.

Positive: *V.gazogenes* UQM 2840. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.8.12 Growth pH 10. Multiwell trays of SENA buffered with valine to pH 10 (3.4.4.33) were inoculated and incubated 24 h. Growth was scored as positive.

Positive: *V.natriegens* UQM 2782. **Negative:** *V.nereis* UQM 2783.

3.8.2.2.8.13 Growth pH 4.5.

Multiwell trays of SENA buffered with succinate (3.4.4.34) to pH 4.5 were inoculated and incubated 24 h. Growth was scored as positive.

Positive: *V.natriegens* UQM 879. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.9 Tolerance to Antibiotics and Miscellaneous Organics.

Multi-well trays of SENA was supplemented with inhibitors to working strengths as specified in Table 3.1 were inoculated and incubated 24 h. OTU's which showed growth were scored as positive except were otherwise stated.

3.8.2.2.9.01 Growth Novobiocin, 10 µg/ml.

Positive: *V.zobellii* UQM 3028. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.9.02 Growth Penicillin, 15 units/ml.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.vulnificus* UQM 2744.

3.8.2.2.9.03 Growth Polymyxin, 5 µg/ml.

Positive: *V.damsela* UQM 2853. **Negative:** *V.hollisae* UQM 2852.

3.8.2.2.9.04 Growth Streptomycin, 10 µg/ml.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.ordalii* UQM 2890.

3.8.2.2.9.05 Growth Sulphamethoxazole, 5 µg/ml.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.zobellii* UQM 3029.

3.8.2.2.9.06 Growth Tetracycline, 10 µg/ml.

Positive: *V.ordalii* UQM 2906. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.9.07 Growth Trimethoprim. 10 µg/ml

Positive: *V.parahaemolyticus* UQM 2201. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.9.08 Susceptibility to 10 µg/ml 0/129 phosphate.

Growth was scored as negative.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.parahaemolyticus* UQM 2125.

3.8.2.2.9.09 Susceptibility to 150 µg/ml 0/129 phosphate.

Growth was scored as negative.

Positive: *V.parahaemolyticus* UQM 2125. **Negative:** *V.harveyi* UQM 2839.

3.8.2.2.9.10 Growth Basic Fuchsin, 0.025%.

Positive: *V.parahaemolyticus* UQM 2125. **Negative:** *V.gazogenes* UQM 2840.

3.8.2.2.9.10 Growth Brilliant Green, 0.005%.

Positive: *V.harveyi* UQM 2839. **Negative:** *V.parahaemolyticus* UQM 2200.

3.8.2.2.9.11 Growth Dichlorophene, 0.005%.

Positive: *V.parahaemolyticus* UQM 2200. **Negative:** *V.gazogenes* UQM 2740.

3.8.2.2.9.12 Growth EDDA 10 µM/ml.

Positive: *V.parahaemolyticus* UQM 2776. **Negative:** *V.harveyi* UQM 2849.

3.8.2.2.9.13 Growth Fast Yellow, 0.1 %.

Positive: *V.parahaemolyticus* UQM 2201. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.9.14 Growth Haloquinol, 0.0005%.

Positive: *Photobacterium* sp. UQM 3269. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.9.15 Growth on 8-hydroxyquinoline, 0.0005 %.

Positive: *V.harveyi* UQM 2839. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.9.16 Growth on Methyl Violet, 0.005%.

Positive: *V.parahaemolyticus* UQM 2200. **Negative:** *V.nereis* UQM 2783.

3.8.2.2.9.17 Growth on Pyronin-Y, 0.002%

Positive: *V.ordalii* UQM 2906. **Negative:** *V.nereis* UQM 2783.

3.8.2.2.9.18 Growth 0.2%(w/v) Sodium Dodecylsulphate (SDS).

Positive: *V.anguillarum* UQM 2843; *V.natriegens* UQM 2782. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.9.19 Growth Thionine, 10 µg/ml.

Positive: *V.parahaemolyticus* UQM 2200; *V.cholerae* UQM 2731. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.9.20 Reduction of Thionine.

Trays from above were scored for OTU's reduction of thionine. Score yellow wells positive.

Positive: *V.parahaemolyticus* UQM 2200. **Negative:** *V.cholerae* UQM 2731.

3.8.2.2.9.21 Growth 0.001 % TTC.

Strains which produced red colonies were scored as positive.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.metschnikovii* UQM 211.

3.8.2.2.10 Acid Production from Carbohydrates.

Tests 3.8.2.2.10.01-11 were undertaken by the addition of carbohydrates to Fermentation medium (3.4.4.37) to a final concentration of 1 % in marine oxidation fermentation medium.

3.8.2.2.10.01 Acid from Xylose.

Positive: *V.gazogenes* UQM 2840. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.10.02 Acid from Arabinose.

Positive: *V.gazogenes* UQM 2840. **Negative:** *V.nereis* UQM 2783.

3.8.2.2.10.03 Acid from Mannose.

Positive: *V.gazogenes* UQM 2840. **Negative:** *V.nigripulchritudo* ATCC 27043.

3.8.2.2.10.04 Acid from Sucrose.

Positive: *V.gazogenes* UQM 2840. **Negative:** *V.vulnificus* UQM 2778.

3.8.2.2.10.05 Acid from Maltose.

Positive: *V.gazogenes* UQM 2840.

3.8.2.2.10.06 Acid from Cellobiose.

Positive: *V.gazogenes* UQM 2840. **Negative:** *V.vulnificus* UQM 2740.

3.8.2.2.10.07 Acid from Salicin.

Positive: *V.natriegens* UQM 2782. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.10.08 Acid from Mannitol.

Positive: *V.gazogenes* UQM 2840 **Negative:** *V.natriegens* UQM 2782.

3.8.2.2.10.09 Acid from Sorbitol.

Positive: *V.anguillarum* UQM 2843 **Negative:** *V.gazogenes* UQM 2840.

3.8.2.2.10.10 Acid from Inositol.

Negative: *V.gazogenes* UQM 2840.

3.8.2.2.10.11 Acid from Dulcitol.

Negative: *V.gazogenes* UQM 2840.

3.8.2.2.11 Nutritional Versatility.

Tests for the separate utilization of single carbon sources were performed upon the medium of Lee *et al.*, (1981), (3.4.4.38). Stock solutions of carbon sources (Table 3.3) were added to make a final concentration of 0.1 %. The media were dispensed to multiwell trays, inoculated, and incubated 14 d except for poly-β-hydroxybutyrate trays, which were incubated 28 d. Determination of growth was by comparison between positive- and negative-controls for both OTU's and attributes (Plate 3.7, bottom). In Batch [6.] some OTU's were able to produce weak to moderate growth on negative-control trays of carbon assimilation medium because of contamination from a derelict departmental distillation apparatus. Purified water for subsequent batches was prepared by reverse osmosis.

3.8.2.2.11.1 Utilization of Aliphatic Amino-acids.

3.8.2.2.11.1.01 Separate Utilization of Glycine.

Positive: *V.parahaemolyticus* UQM 2200. **Negative:** *V.vulnificus* UQM 2740.

3.8.2.2.11.1.02 Separate Utilization of l-α-Alanine.

Positive: *V.parahaemolyticus* UQM 2201. **Negative:** *V.cholerae* UQM 2731.

3.8.2.2.11.1.03 Separate Utilization of d-α-Alanine.

Positive: *V.natriegens* UQM 2782. **Negative:** *V.ordalii* UQM 2906.

3.8.2.2.11.1.04 Separate Utilization of dl-β-Alanine.

Positive: *V.natriegens* UQM 2782. **Negative:** *V.nereis* UQM 2783.

3.8.2.2.11.1.05 Separate Utilization of Serine.

Positive: *V.parahaemolyticus* UQM 2200. **Negative:** *V.carchariae* UQM 2849.

3.8.2.2.11.1.06 Separate Utilization of l-Leucine.

Positive: *V.parahaemolyticus* UQM 2200. **Negative:** *V.cholerae* UQM 2731.

3.8.2.2.11.1.07 Separate Utilization of Valine.

Positive: *V.natriegens* UQM 2782. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.11.1.08 Separate Utilization of l+Glutamic acid.

Positive: *V.natriegens* UQM 2782. **Negative:** *V.damsela* UQM 2853.

3.8.2.2.11.1.09 Separate Utilization of l-Lysine.

Positive: *V.parahaemolyticus* UQM 2776. **Negative:** *V.alginolyticus* UQM 2770.

3.8.2.2.11.1.10 Separate Utilization of l-Arginine.

Positive: *V.natriegens* UQM 2782. **Negative:** *V.ordalii* UQM 2906.

3.8.2.2.11.1.11 Separate Utilization of l-Ornithine.

Positive: *V.parahaemolyticus* UQM 2776. **Negative:** *V.alginolyticus* UQM 2770.

3.8.2.2.11.1.12 Separate Utilization of l-Citrulline.

Positive: *V.zobellii* UQM 3028. **Negative:** *V.gazogenes* UQM 2840.

3.8.2.2.11.1.13 Separate Utilization of ɣ-Amino-butyrate.

Positive: *V.parahaemolyticus* UQM 2200. **Negative:** *V.cholerae* UQM 2732.

3.8.2.2.11.1.14 Separate Utilization of δ-aminovalerate.

Positive: *V.parahaemolyticus* UQM 2200. **Negative:** *V.cholerae* UQM 2732.

3.8.2.2.11.1.15 Separate Utilization of Norvaline.

Positive: *V.natriegens* UQM 2782. **Negative:** *V.zobellii* UQM 3028.

3.8.2.2.11.1.16 Separate Utilization of l-Proline.

Positive: *V.parahaemolyticus* UQM 2776. **Negative:** *V.cholerae* UQM 2732.

3.8.2.2.11.2 Utilization of Amines.

3.8.2.2.11.2.01 Separate Utilization of Putrescine.

Positive: *V.parahaemolyticus* UQM 2200. **Negative:** *V.cholerae* UQM 2732.

3.8.2.2.11.2.02 Separate Utilization of Sarcosine.

Positive: *V.parahaemolyticus* UQM 2200. **Negative:** *V.parahaemolyticus* UQM 2201.

3.8.2.2.11.2.03 Separate Utilization of l-Glutamine.

Positive: *V.hollisae* UQM 2852. **Negative:** *V.ordalii* UQM 2906.

3.8.2.2.11.2.04 Separate Utilization of d-Glucosamine.

Positive: *V.carchariae* UQM 2849. **Negative:** *V.cholerae* UQM 2731.

3.8.2.2.11.2.05 Separate Utilization of *n*-acetyl-glucosamine.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.cholerae* UQM 2732.

3.8.2.2.11.3 Utilization of Carbohydrates and Sugar Derivatives.

3.8.2.2.11.3.01 Separate Utilization of d-Ribose.

Positive: *V.parahaemolyticus* UQM 2200. **Negative:** *V.cholerae* UQM 2732.

3.8.2.2.11.3.02 Separate Utilization of Xylose.

Positive: *V.gazogenes* UQM 2840. **Negative:** *V.nereis* UQM 2783.

3.8.2.2.11.3.03 Separate Utilization of l-Arabinose.

Positive: *V.zobellii* UQM 3028. **Negative:** *V.vulnificus* UQM 3032.

3.8.2.2.11.3.04 Separate Utilization of l-Rhamnose.

Positive: *V.natriegens* UQM 2782. **Negative:** *V.nereis* UQM 2783.

3.8.2.2.11.3.05 Separate Utilization of Glucose.

Positive: *V.parahaemolyticus* UQM 2200. **Negative:** *V.cholerae* UQM 2731.

3.8.2.2.11.3.06 Separate Utilization of d-Mannose.

Positive: *V.hollisae* UQM 2852. **Negative:** *V.zobellii* UQM 3028.

3.8.2.2.11.3.07 Separate Utilization of d-Galactose.

Positive: *V.hollisae* UQM 2852. **Negative:** *V.damsela* UQM 2853.

3.8.2.2.11.3.08 Separate Utilization of Sucrose.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.cholerae* UQM 2732.

3.8.2.2.11.3.09 Separate Utilization of Trehalose.

Positive: *V.parahaemolyticus* UQM 2200. **Negative:** *V.cholerae* UQM 2732.

3.8.2.2.11.3.10 Separate Utilization of Maltose.

Positive: *V.gazogenes* UQM 2840 **Negative:** *V.cholerae* UQM 2732.

3.8.2.2.11.3.11 Separate Utilization of Cellobiose.

Positive: *V.natriegens* UQM 2782. **Negative:** *V.nereis* UQM 2783.

3.8.2.2.11.3.12 Separate Utilization of Lactose.

Positive: *V.nereis* UQM 2783. **Negative:** *V.gazogenes* UQM 2840.

3.8.2.2.11.3.13 Separate Utilization of Raffinose.

Positive: *V.natriegens* UQM 2782. **Negative:** *V.fluvialis* UQM 2774.

3.8.2.2.11.3.14 Separate Utilization of Galactarate.

Positive: *Vibrio hollisae*-like UQM 3282. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.11.3.15 Separate Utilization of Galacturonic acid.

Positive: *V.fluvialis* UQM 3239. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.11.3.16 Separate Utilization of Gluconate.

Positive: *V.natriegens* UQM 2782. **Negative:** *V.gazogenes* UQM 2840.

3.8.2.2.11.3.17 Separate Utilization of Glucuronic acid.

Positive: *(V.tubiashii)* UQM 2923. **Negative:** *V.anguillarum* UQM 2843.

3.8.2.2.11.3.18 Separate Utilization of Salicin.

Positive: *V.gazogenes* UQM 2840. **Negative:** *V.zobellii* UQM 3028.

3.8.2.2.11.3.19 Separate Utilization of Starch.

Positive: *V.gazogenes* UQM 2840. **Negative:** *V.cholerae* UQM 2732.

3.8.2.2.11.3.20 Separate Utilization of Dextrin.

Positive: *V.anguillarum* UQM 2771. **Negative:** *V.cholerae* UQM 2742.

3.8.2.2.11.3.21 Separate Utilization of Poly-galacturonic acid.

Positive: *V.fluvialis* UQM 3241. **Negative:** *V.anguillarum* UQM 2771.

3.8.2.2.11.3.22 Separate Utilization of Inulin.

Positive: *V.gazogenes* UQM 2842. **Negative:** *V.gazogenes* UQM 2840.

3.8.2.2.11.3.23 Separate Utilization of Pullulan.

Positive: *V.gazogenes* UQM 2842. **Negative:** *V.gazogenes* UQM 2840.

3.8.2.2.11.4 Utilization of Aliphatic Organic Acids.

3.8.2.2.11.4.01 Separate Utilization of Formate.

Positive: *V.metschnikovii* UQM 211. **Negative:** *V.natriegens* UQM 2782.

3.8.2.2.11.4.02 Separate Utilization of Acetate.

Positive: *V.gazogenes* UQM 2842. **Negative:** *V.gazogenes* UQM 2840.

3.8.2.2.11.4.03 Separate Utilization of Propionate.

Positive: *V.alginolyticus* UQM 3254. **Negative:** *V.natriegens* UQM 2782.

3.8.2.2.11.4.04 Separate Utilization of n-Butyric acid.

Negative: *V.alginolyticus* UQM 2770.

3.8.2.2.11.4.05 Separate Utilization of Hexanoate.

Negative: *V.alginolyticus* UQM 2770.

3.8.2.2.11.4.06 Separate Utilization of Heptanoate.

Negative: *V.alginolyticus* UQM 2770.

3.8.2.2.11.4.07 Separate Utilization of Caprylate.

Negative: *V.alginolyticus* UQM 2770.

3.8.2.2.11.4.08 Separate Utilization of Nonanionate.

Positive: *V.metschnikovii* UQM 211. **Negative:** *V.alginolyticus* UQM 2770.

3.8.2.2.11.4.09 Separate Utilization of Caprate.

Positive: *V.metschnikovii* UQM 211. **Negative:** *V.alginolyticus* UQM 2770.

3.8.2.2.11.5 Utilization of Dicarboxylic Acids.

3.8.2.2.11.5.01 Separate Utilization of Malonate.

Positive: *V.fluvialis* UQM 3262. **Negative:** *V.natriegens* UQM 2782.

3.8.2.2.11.5.02 Separate Utilization of Succinate.

Positive: *V.parahaemolyticus* UQM 2200. **Negative:** *V.cholerae* UQM 2732.

3.8.2.2.11.5.03 Separate Utilization of Maleate.

Positive: *V.parahaemolyticus* UQM 2200. **Negative:** *V.anguillarum* UQM 2843.

3.8.2.2.11.5.04 Separate Utilization of Adipic acid.

Negative: *V.natriegens* UQM 2782.

3.8.2.2.11.6 Utilization of Hydroxy-acids.

3.8.2.2.11.6.01 Separate Utilization of Malate.

Positive: *V.natriegens* UQM 2782. **Negative:** *V.nereis* UQM 2783.

3.8.2.2.11.6.02 Separate Utilization of Tartarate.

Positive: *V.costicola* UQM 2888. **Negative:** *V.natriegens* UQM 2782.

3.8.2.2.11.6.03 Growth DL-3-Hydroxy Butyrate.

Positive: *V.natriegens* UQM 2782. **Negative:** UQM 2771.

3.8.2.2.11.6.04 Separate Utilization of 6-Hydroxycaproate.

Positive: *V.harveyi* Stn 1710. **Negative:** *V.alginolyticus* UQM 2770.

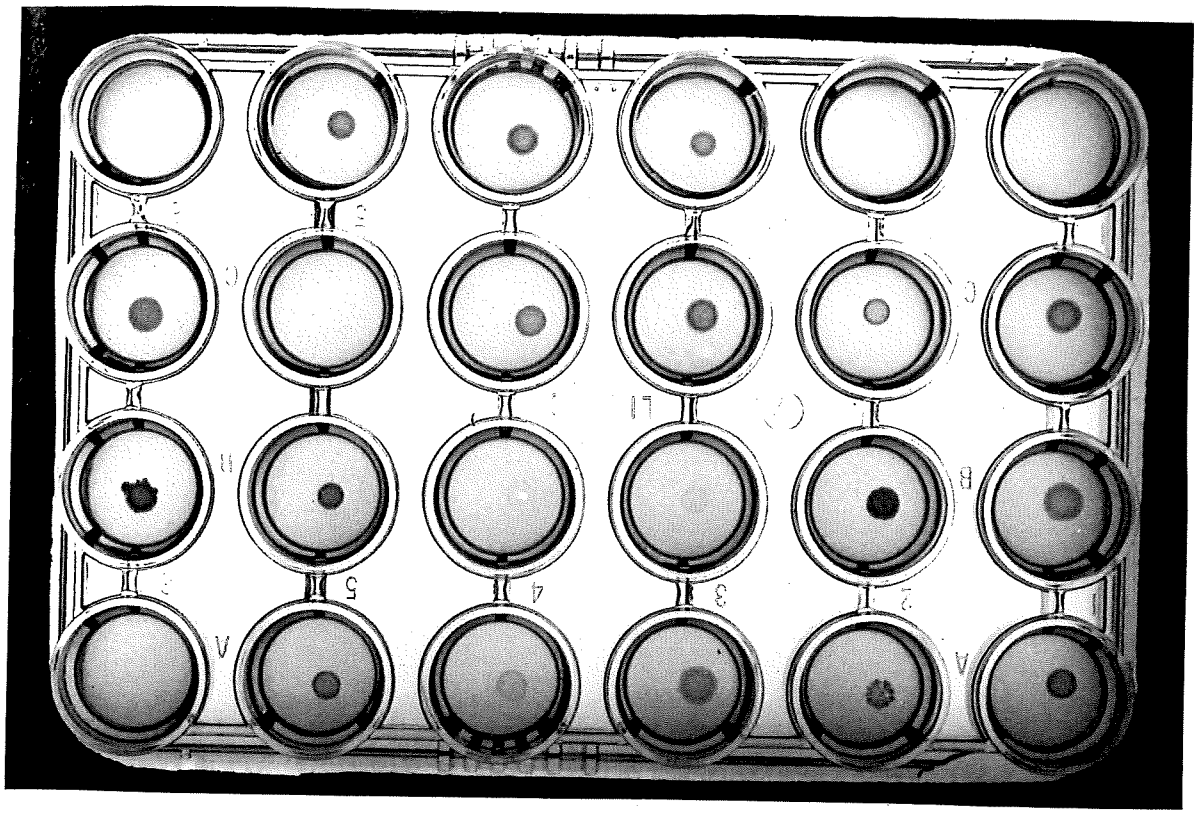
3.8.2.2.11.6.05 Separate Utilization of Lactate.

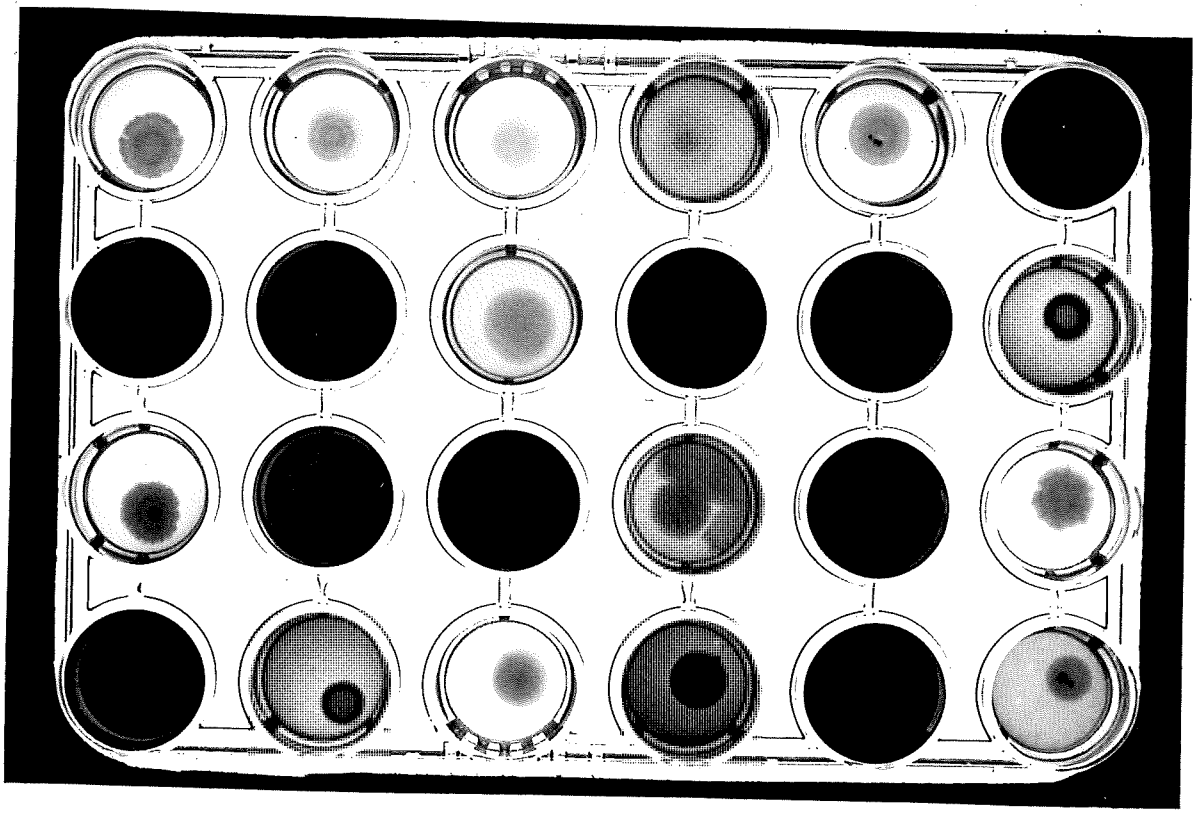
Positive: *V.natriegens* UQM 2782. **Negative:** *V.nigripulchritudo* ATCC 27043.

3.8.2.2.11.6.06 Separate Utilization of dl-glyceric acid.

Positive: *V.hollisae* UQM 2852. **Negative:** *V.pelagius* UQM 2785.

Plate 3.7: *Top,* Positive (Dark), Negative and Intermediate Hydrolysis of Aesculin; *Bottom* Presence and Absence of Growth on Carbon assimilation Medium.





3.8.2.2.11.6.07 Separate Utilization of Poly-β-Hydroxybutyrate.

Positive: *V.fluvialis* UQM 3240. Negative: *V.anguillarum* UQM 2771.

3.8.2.2.11.7 Utilization of Acids from the Kreb's Cycle

3.8.2.2.11.7.01 Separate Utilization of Citrate.

Positive: *V.parahaemolyticus* UQM 2776. Negative: *V.cholerae* UQM 2731.

3.8.2.2.11.7.02 Separate Utilization of α-Ketoglutarate.

Positive: *V.gazogenes* UQM 2840. Negative: *V.zobellii* UQM 3028.

3.8.2.2.11.7.03 Separate Utilization of Pyruvate.

Positive: *V.pelagius* UQM 2785. Negative: *V.cholerae* UQM 2731.

3.8.2.2.11.8 Utilization of Poly-alcohols.

3.8.2.2.11.8.01 Separate Utilization of Erythritol.

Positive: *V.natriegens* UQM 2782. Negative: *V.gazogenes* UQM 2840.

3.8.2.2.11.8.02 Separate Utilization of Dulcitol.

Positive: *Photobacterium* sp. UQM 3244. Negative: *V.nereis* UQM 2783.

3.8.2.2.11.8.03 Separate Utilization of Mannitol.

Positive: *V.parahaemolyticus* UQM 2200. Negative: *V.cholerae* UQM 2731.

3.8.2.2.11.8.04 Separate Utilization of Sorbitol.

Positive: *V.gazogenes* UQM 2840. Negative: *V.gazogenes* UQM 2842.

3.8.2.2.11.8.05 Separate Utilization of Inositol.

Positive: *Photobacterium* sp. UQM 3244. Negative: *V.ordalii* UQM 2906.

3.8.2.2.11.8.06 Separate Utilization of Ethanol.

Positive: *V.zobellii* UQM 3028. Negative: *V.gazogenes* UQM 2840.

3.8.2.2.11.9 Utilization of Aromatic Organic Acids and Derivatives.

3.8.2.2.11.9.01 Separate Utilization of Benzoate.

Positive: *V.alginolyticus* UQM 2770. Negative: *V.natriegens* UQM 2782.

3.8.2.2.11.9.02 Separate Utilization of Hydroxybenzoate.

Positive: *V.fluvialis* UQM 3375. Negative: *V.anguillarum* UQM 2771.

3.8.2.2.11.9.03 Separate Utilization of Phenylacetic acid.

Positive: *Photobacterium* sp. Stn 8061. Negative: *V.natriegens* UQM 2782.

3.8.2.3 Scoring of Results and Standardization of Datasets.

Positive and negative data were coded as 1 or 0 respectively, missing or ambiguous data were scored as 9. Duplicate OTU's within- and across- batches were examined for consistency. If results were discrepant for an attribute all OTU's in that batch were scored as ND, (9), for that attribute (See 3.8.5.1). Where discrepancies for that attribute existed between batches, a decision was made by cross-correlation with other replicated OTU's as to which batch was least consistent for that attribute, and all OTU's in this batch, for that attribute were coded as if ND. If such evaluations could not be readily made for characters unstandardized results for all batches were presented for analysis (3.8.3.1, 3.8.4.1,3.8.5.1). Subsequent to this damping of procedural noise, (experimental error) cluster analyses for resolution between OTU's and replicate correlation was performed (3.8.5). This data is contained in the files VIB01-8.DAT and VIB05A-8A.DAT and a batch-wise OTU-key, KEY.DAT, are stored on magnetic media in Appendix 12 (Disk_2, APNDICES\APNDX_12).

3.8.2.4 Computer Analysis.

Analysis of phenetic data was performed on an International Business Machines (IBM) *3083* mainframe computer system, (4 megabyte virtual machine), operated on site at the University of Queensland and an IBM compatible personal computer *(PC XT)*. Two statistical analysis suites (Appendix 16.) were operated on the 3083, D.Wishart's *Clustan*[r.] release 3.2 (Clustan Ltd.) and *SAS*[r.] release 5.08 (SAS Inc.). The personal computer was used also to operate specially devised Fortran 77 accessory programs (Appendix 11.), before or independent of mainframe analyses, and also to operate three numerical analysis suites (Appendix 16.), *Microcluster*[r.] version 6.3, (B.Edmondston); and *Paup*[r.] version 2.4.1 (D.Swofford); and the STS Corporation program *Statgraph*[r.] version 2.5. *Sun-Ray* glyphs generated by the later program were depicted by a generic Hewlett Packard emulating plotter.

3.8.3.0 Characterization and Evaluation of OTU's in the Proposed *Rosetta*.

3.8.3.1 Experimental Design.

The first Batch [1.], collated phenetic records from 48 OTU's or, when replicates are discounted, 46 strains. This batch was composed of formal *Vibrionaceae* species and other taxonomically or ecologically peculiar provincial- environmental or fish-associated strains. After a series of numerical analyses the taxonomic positions of all strains were comprehensively and comparatively evaluated and there-after Batch [1.] was referred to as the *Rosetta* because through concurrent numerical analyses with other batches it could be used to help unlock the identities of unidentified OTU's from those other batches.

The strains selected to serve in the diagnostic *Rosetta* comprised 26 type or reference cultures including 8 tentatively identified provincial isolates. The remaining 20 (provincial) isolates were representative of the diversity of sampled niches ranging from sediment to fish, to their isopod-crustacean parasites (Table 3.7.2); and other strains which became apparent as taxonomically distinct in presumptive screening e.g. by possessing such rare attributes as red non-diffusible pigmentation (Stn 180), and luminescence Stn 1800. Some taxon-redundancy was allowed in the *Rosetta* because an indication would then be given in later diagnostic analyses about the extent of cross-batch miscibility. This redundancy functions also as a precaution to the possibility that some of the latterly fused OTU's in hierarchies from the *Rosetta* might be dispersed differently i.e. into new taxa, in the presence of other, unknown strains.

The phenetically characterized Batch [1.] OTU's were subjected to a series of component analyses and the resultant hierarchies were comparatively evaluated according to their stability across protocols, i.e. groups or single OTU's respectively always present together or alone were further examined by comparison of their attribute profiles with other similarly deduced phena and with type and reference cultures, and by comparison with the literature. In order of priority Baumann, Furniss and Lee (1984),

Reichelt and Baumann (1973) and Popoff and Veron (1976) were used to test taxonomic assignments. In some cases assignments of provincial OTU's to taxa were aided by supplementary procedures i.e. by examination of polar flagellation by electron microscopy (Appendix 7.) and/or by DNA based examinations (Appendix 9.)

Three hierarchical methods, Average linkage *(SAS 5.08),* Scalar data assumed, Error Sum of Squares (ESS), *Clustan 3.2,* and Consensus Parsimony (CP), *(Paup 2.4.1* with *Contree 1/3/86),* together with euclidean and Jaccard similarity matrices were used to make cumulative diagnosis of clusters into phena, rather than to arbitrarily truncate these at externally defined similarities (2.9.3.6.2).

The latter two programs as implemented were not suited to the analysis of large numbers of OTU's, *(Clustan 3.2* was only partially implemented, and *Paup* was computationally intense and available only for personal computers). Similarity matrices (A11.3) were constructed with Jaccard and euclidean (simple matching) similarity criteria.

The first two hierarchical procedures from above and the similarity matrices each used different protocols for assimilation of incomplete character records. The first, adopted for the average linkage analysis, assumed scaler data and assigned missing character data to row averages; this could only be done manually and was laborious; the second an automatic facility only available in the ESS analysis, assigned and reassigned missing characters to within-cluster, means. The third, used to construct similarity matrices, excluded all incomplete character data from paired comparisons (See 2.9.3.5). Because two duplicated OTU's were installed in the reference dataset it was possible to observe the effects of these different treatments upon measured similarities.

The protocol for treatment of missing data by the parsimony procedure is unpublished, however, because there was only program facility for concurrent assay of 46 OTU's; duplicated OTU's were excluded from analysis.

and *Paup's* operation upon missing data and its effect on grouping were not considered further. The 100 derived equally parsimonious trees were processed into a "Consensus Tree" which only indicated overlapping hierarchical structure.

The *SAS* protocol expressed similarities in terms of the proportions of total variance contributed by successive eigen-vectors. Analysis by *Clustan* is upon distance rather than similarity values but these were converted to similarity for consistency with microbiological literature. Consensus parsimony analysis only compares hierarchical topology and not relatedness and consequently the vertical scale is indicative only of the number of clusters.

The reference dataset comprised OTU's with origins as specified in Tables 3.4-5. The OTU's UQM 2771 and Stn 180, (UQM 2840), were analyzed in duplicate. Average linkage, ESS, and CP, analyses of transposed (A11.1) data are presented in Figure 3.4 a-c. Command programs for ESS and CP protocols are in A11.7 and A11.8.1 respectively. Phena within dendrograms have been flagged by an alphanumeric code originated from the average linkage analysis, and delimited by the distribution of type cultures, and stability of sub-phena or single OTU's between procedures.

OTU character records were sorted in accord with the phena diagnosed above (A11.2) on an IBM-compatible personal computer and similarity matrices for euclidean (simple matching) $(S_{e.})$, Table 3.11.1, and Jaccard similarity criteria, $(S_{j.})$, Table 3.11.2, were calculated using programs A11.3-4 and phenon attribute frequencies determined with program A11.5.

3.8.3.2 Results.

Following comparison of duplicate OTU's and prior to numerical analysis all Batch [1.] OTU's were coded as missing for the following variable characters: Congo red, sulphide production, no serum hydrolysis, no chitin hydrolysis, separate utilization of succinate, pyruvate and erythritol. For duplicate OTU's of the red pigmented *Vibrio* sp., Stn 180, attributes from

181 characters were coded; there were 2 unbalanced missing characters and 5 remaining discrepancies. For *V.anguillarum* UQM 2771, attributes from 181 characters were coded with no unbalanced missing values and 13 remaining discrepancies. Upon examination of this data from similarity matrices the corresponding $S_{e.}$ & $S_{j.}$ coefficients were 3 and 6%, and 7 and 14% for Stn 180 and UQM 2771 respectively. By average linkage analysis Stn 180 and UQM 2771 represented 1.25% and 2.5% of the total variance between OTU's. By ESS, the estimated error for each pair of duplicate OTU's was 3%. Under all hierarchical analyses these OTU's formed primary associations.

Attributes which varied between duplicate OTU's of Stn 180, were growth in the presence of methyl violet, and separate utilization of ɣ-amino-butyrate, δ-aminovalerate, l-proline, glucose, and galactarate. OTU's of *V.anguillarum* UQM 2771 were variable for the attributes: Growth in the presence of basic fuchsin, and separate utilization of mannose, gluconate, dl-glyceric acid and citrate.

Apart from duplicated OTU's, other paired OTU's were placed consistently across analyses, these were *V.nigripulchritudo* UQM 2784 and *V.splendidus* UQM 2786; Stn 3230 and 7800; and *Aeromonas hydrophila* UQM 2838 and Stn 7770. *V.alginolyticus* UQM 2770 and *V.parahaemolyticus* UQM 2776 were fused by ESS and CP, but not by average linkage procedure.

The OTU's *V.metschnikovii,* UQM 211; *A.hydrophila,* UQM 2769; Stn 180, Stn 1800 and Stn 7650 were allocated to different clusters in each analysis. The highest $S_{e.}$ and $S_{j.}$ values of UQM 211 were with UQM 2771; consistent with placement by the average linkage procedure. The highest $S_{e.}$ for UQM 2769 was shared with several OTU's, but the highest relatedness of this phenetically weak strain by $S_{j.}$ was only with Stn 3230 and Stn 7770.

The duplicate OTU's from Stn 180 were not markedly similar to any of the OTU's by either $S_{e.}$ or $S_{j.}$ criteria. By average linkage the OTU Stn 1800 associated first with Stn 180. By ESS this OTU was intermediate between the OTU's Stn 3270 and 7800, and 7770, and by CP it was placed between

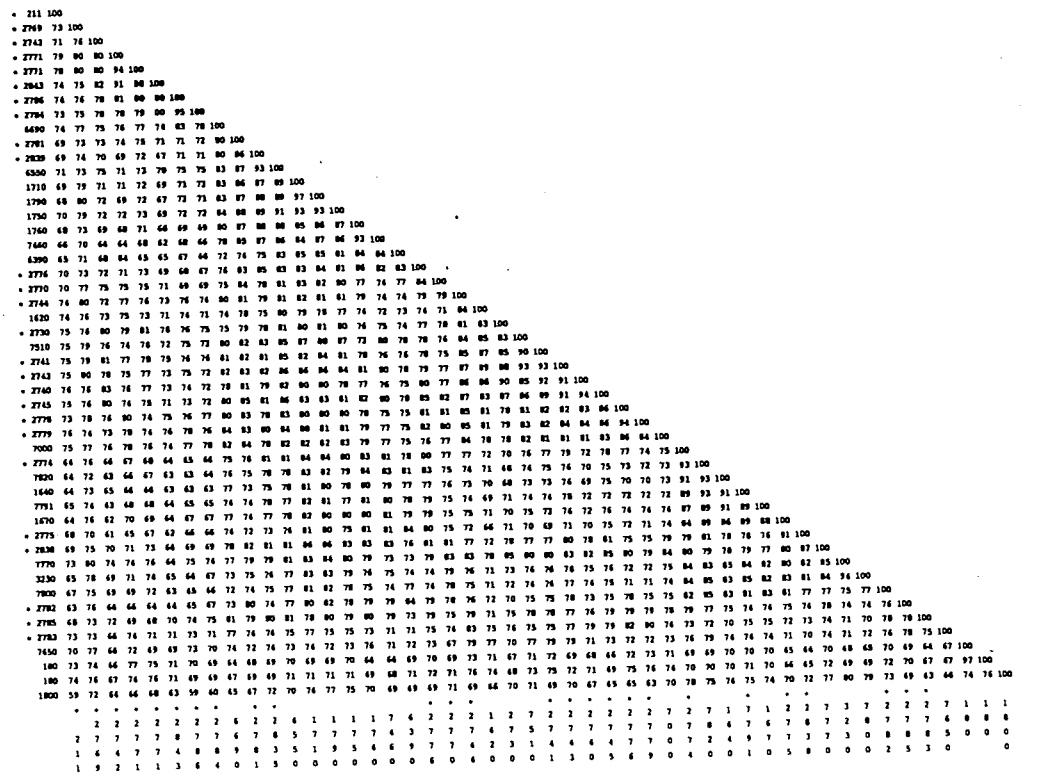


Table 3.11.1: Euclidean Similarity Matrix for Reference OTU's.

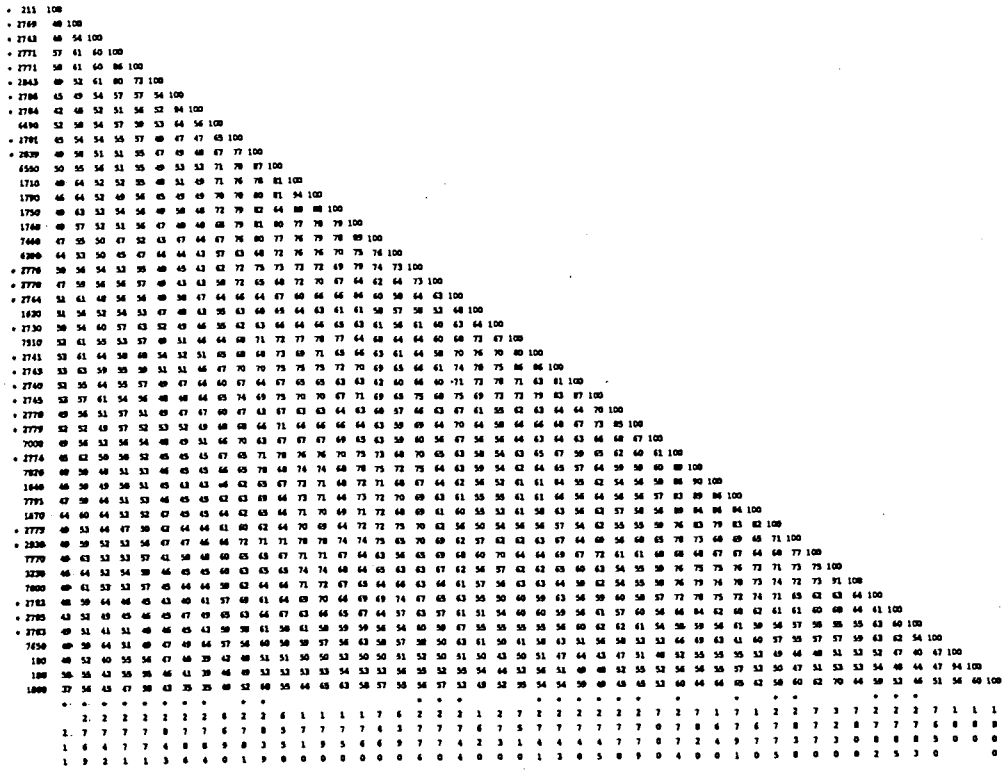
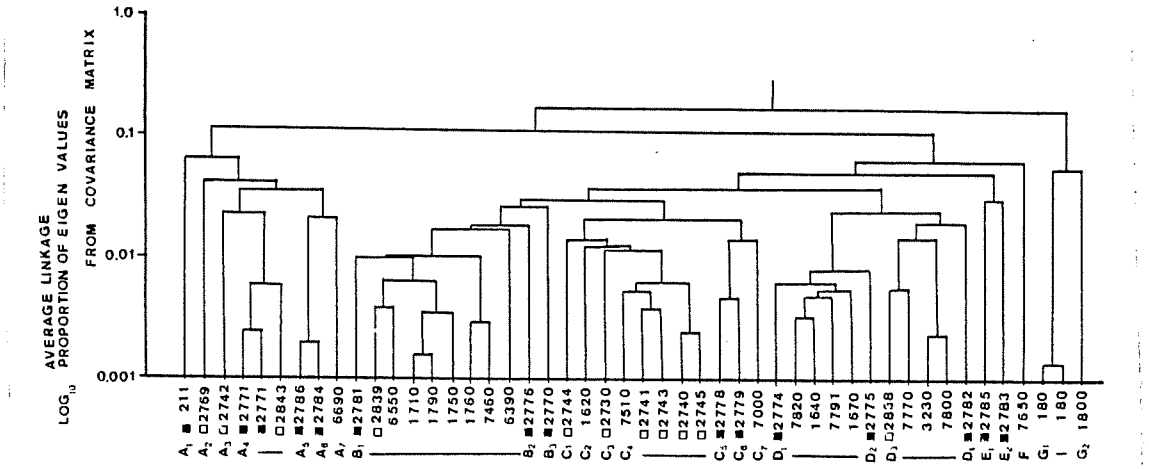
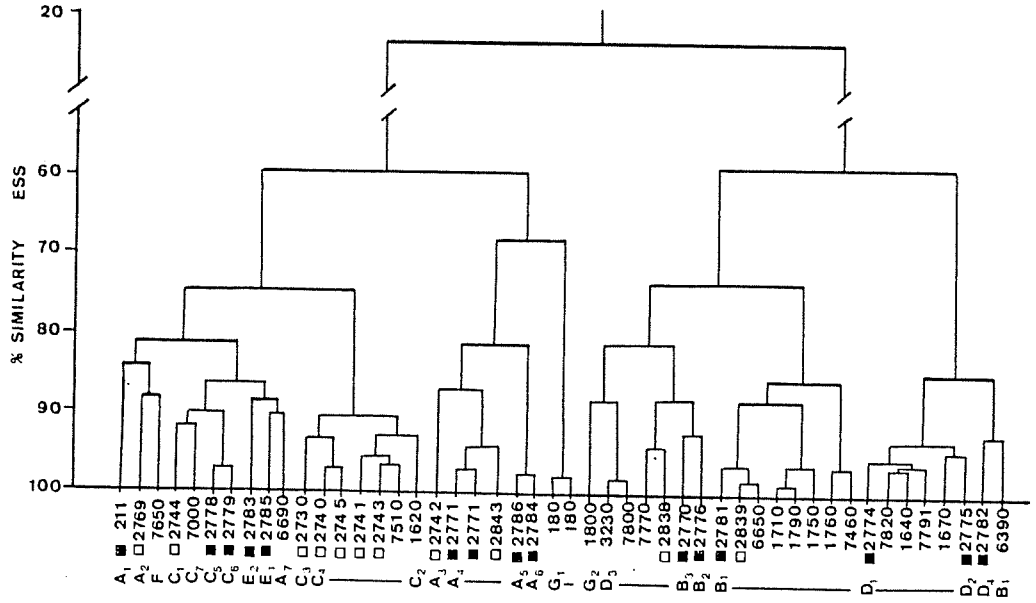


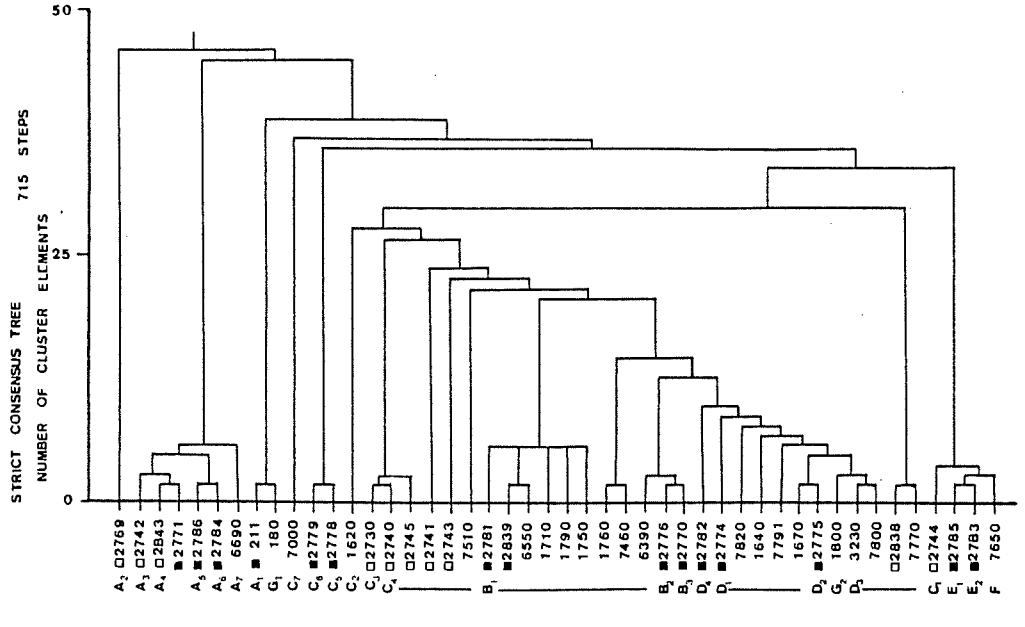
Table 3.11.2: Jaccard Similarity Matrix for Reference OTU's.



(a)



(b)



(c)

Figure 3.4 (a-c): Average Linkage, ESS and CP Dendrograms for Reference OTU's. Type Cultures ■, Reference Cultures □; Unflagged numbers correspond to provincial OTU's, which in the text have a Stn prefix.

doublets for OTU's Stn 1670 and UQM 2775, and Stn 3230 and 7800. The highest $S_{e.}$ and $S_{j.}$ for Stn 1800 were respectively with UQM 2775 and Stn 3230 which was consistent with placement by CP analysis.

The cluster [A3,A4] containing OTU's *V.cholerae* UQM 2742 and *V.anguillarum* UQM 2771 and UQM 2843, was resolved by all protocols. By ESS these were next linked to the doublet formed by UQM 2784 *V.nigripulchritudo* and UQM 2786 *V.splendidus.* By average linkage and CP methods this cluster was interrupted by prior fusion with the OTU Stn 6690.

The average linkage and ESS regimes placed the OTU's UQM 2781, UQM 2839, Stn 6550, 1710, 1790, 1750, 1760 and 7460 into the phenon B1. The OTU Stn 6390 placed at the edge of this cluster by average linkage, also showed both high $S_{e.}$ (84%); and $S_{j.}$ (74%); similarities to the OTU UQM 2782 *V.natriegens;* this was recognized by ESS and CP analyses. The CP analysis placed this OTU as an intermediate between *V.natriegens* UQM 2782 and the variably clustered OTU's from phenon B1.

The cluster [C2-4] formed around the OTU's Stn 1620 and *V.cholerae* UQM 2730 and with OTU's UQM 2740, UQM 2745, UQM 2743 and Stn 7510 was presented in both average linkage and ESS analyses. The consensus parsimony procedure did not separately resolve this cluster but the same OTU's were present as intermediate components to its hierarchy. The average linkage regimen linked UQM 2744, (phenon C1), to this cluster and then to the triplet formed by the fusion of Stn 7000 with *V.vulnificus* UQM 2778 and *V.campbellii* UQM 2779. By ESS UQM 2744 was linked to the OTU Stn 7000 and this doublet fused to the cluster formed by UQM 2778 and UQM 2779. This sub-group was separated from the formally described groups by prior fusions with the OTU's *V.nereis* UQM 2783, *V.pelagius* UQM 2785, and the OTU Stn 6690, UQM 211 *V.metschnikovii,* and *A.hydrophila* UQM 2769 and Stn 7650.

The cluster [D1-2], comprising the OTU's *V.fluvialis* UQM 2774, Stn 7820, 1640, 7791, 1670 and *V.furnissii* UQM 2775 was resolved by average linkage and ESS protocols, but presented in differing hierarchies. CP analysis

placed these OTU's as intermediates to a larger cluster. Provincial OTU's had marginally greater $S_{e.}$ coefficients for *V.furnissii* UQM 2775 than *V.fluvialis* UQM 2774, but this was substantially reversed by examination of the corresponding $S_{j.}$ values. Examination of euclidean and Jaccard similarity coefficients supported the distribution produced by the average linkage approach.

3.8.3.2.1 **Review of Phena.**

Attribute percent frequencies from stable phena from sub-divided clusters, type cultures and unassociated OTU's are presented in Tables 3.8.3.3-4. Fourteen of the twenty-six phena dissected from clusters were measured as arginine dihydrolase (ADH) positive. Four were decarboxylase negative, and of the remaining eight, four phena were ornithine- as well as lysine-decarboxylating. All OTU's were Gram-negative, fermentative, produced catalase and grew in 1 and 3 % saline and at 15 and 30°C. None produced a brown or yellow-orange cellular pigment, or could hydrolyze xanthine, grow in the presence of haloquinol, or separately assimilate butyric, hexanoic, heptanoic or caprylic acids, or the esters benzoate and phenylacetate or inositol. Provincial OTU's and reference cultures after analysis and cluster diagnosis were assigned to the phena containing the type cultures of *V.anguillarum, V.harveyi,* and *V.fluvialis.*

For all OTU's, thresholds for scoring separate growth appeared greater than published by Baumann *et al.,* (1984) for the carbon sources valine, proline, n-acetylglucosamine, ribose, glucose, sucrose, lactose, and aliphatic organic acids, or their salts, of chain length greater than two, and less for arginine and citrulline.

V.metschnikovii UQM 211 further differed from Baumann *et al.* by its failure to separately utilize l-arginine, d-alanine and ribose and by its utilization of rhamnose. The type cultures of *V.anguillarum* UQM 2771 further differed from Baumann *et al.* by production of β-galactosidase, growth in the absence of salt, and failure to separately utilize l- or d-α-

alanine, citrulline, serine, l-proline, sarcosine, mannose, maltose, cellobiose, malate or dl-glyceric acid.

The provincial isolate of *V.anguillarum,* UQM 2843 differed from the duplicates of UQM 2771 by possession of different colony and broth growth characteristics, production of ornithine decarboxylase, growth in the presence of 0.2% sodium dodecylsulphate (SDS), and by separate utilization of glycine, proline, l-arabinose, α-ketoglutarate; and by its failure to utilize n-acetylglucosamine, sucrose, maltose, starch, dextrin, lactate, and mannitol. The presence of sheathed polar flagella on UQM 2843 was verified by electron microscopy Plate (A7.1b). The G + C value was 0.2 mole % greater than the type culture (Appendix 9.) i.e. not significantly different.

The reference culture of *V.anguillarum* UQM 2744, (Phenon C1), differed from Phenon A3 by production of opaque rather than translucent colonies on SENA medium, by the absence of a pellicle in broth growth, by its failure to produce acetoin, oxidize gluconate, produce β-galactosidase, grow at 4°C. or 37°C., grow in 0% or 6% sodium chloride, sensitivity to penicillin, absence of growth on EDDA, basic fuchsin, methyl violet, failure to produce acid from arabinose, and to separately utilize arabinose, mannose or sorbitol.

Attributes possessed by this OTU not observed in *V.anguillarum* UQM 2771 were: Acidification of MRVP medium, hydrolysis of arbutin, growth in the presence of 50 i.u./ml polymyxin, hydrolysis of tyrosine to form a brown pigment, growth on, and production of esterase for SDS, production of acid from salicin, the separate utilization of l- and d-α-alanine, serine, l-proline, d-glucosamine, ribose, d-galactose, trehalose, cellobiose, salicin, and α-ketoglutarate. This OTU was confirmed for sheathed polar flagella, by electron microscopy (Plate A7.1(a)).

The unassociated OTU Stn 6690 from phenon A7, could be differentiated from other OTU's from Phenon A by its requirement for at least 1 % saline, and by failure to grow in the presence of polymyxin, 10 ug/ml 0/129 phosphate.

Table 3.12.1: **Attribute Percentages for *Vibrionaceae* Type and Reference Cultures.**

Phenon:	A1	A2	A3	A4	A5	A6	A7	B1	B2	B3	C1	C2	C3
Accession Number:	UQM	UQM	UQM	*V.*	UQM	UQM	Stn	*V.*	UQM	UQM	UQM	UQM	UQM
	211	2769	2742	*angu*	2786	2784	6690	*harv*	2776	2770	2744	3032	2730
Attribute.	n=1	n=1	n=1	n=3	n=1	n=1	n=1	n=9	n=1	n=1	n=1	n=1	n=1
Colony diameter > 3 mm.	0	0	0	0	0	0	100	11	0	0	0	0	0
Colony mucoid.	0	0	0	33	0	0	0	11	0	0	0	0	0
Colony matt.	0	0	0	0	0	0	0	0	0	0	0	0	0
Colony opaque.	0	0	0	0	0	0	0	11	0	0	100	0	0
Colony entire.	100	100	100	100	100	100	100	88	100	0	100	100	100
Colony convex.	100	100	100	100	100	100	100	100	100	0	100	100	100
Swarming growth.	0	0	0	0	0	0	0	11	0	100	0	100	0
Luminous growth.	0	0	0	0	0	0	0	11	0	0	0	100	0
Red pigment.	0	0	0	0	0	0	0	0	0	0	0	0	0
Black pigment.	0	0	0	0	0	100	0	0	0	0	0	0	0
Length > 2 times width.	100	0	100	100	100	100	100	33	100	100	100	100	0
Strong catalase.	0	0	100	0	0	0	100	11	0	0	0	0	0
Oxidase.	0	100	100	100	100	100	100	100	100	100	100	100	100
Oxidase (Toluene).	0	100	100	100	100	100	100	100	100	100	100	100	100
Broth uniform turbidity.	100	100	100	100	100	100	100	100	100	100	100	100	100
Broth with sediment.	0	100	100	66	100	100	100	100	100	100	100	100	100
Broth sediment mucoid.	0	0	0	0	100	0	100	11	100	0	0	0	0
Broth growth mod. to heavy.	0	100	100	33	100	100	100	88	100	100	100	0	100
Pellicle.	0	0	100	100	0	0	0	22	100	100	0	0	0
Indole.	100	0	100	100	0	0	100	66	0	0	0	0	100
Weak indole.	100	100	100	100	100	0	100	100	100	100	100	100	100
Motile.	100	100	100	100	100	100	100	100	100	100	100	100	100
Polar Flagellation.	100	100	100	100	100	100	100	100	100	100	100	100	100
5 day pH > 7.05.	0	100	100	100	0	0	0	0	0	100	0	0	0
5 day pH > 5.15.	100	100	100	100	0	0	0	0	100	100	100	100	100
Acetoin production.	100	0	100	100	0	0	0	0	0	100	0	0	0
Nitrate reduction.	0	0	100	100	100	100	100	100	100	100	100	100	100
Nitrite reduction.	0	100	0	0	0	0	100	66	100	100	0	0	0
Cholera-Red.	100	100	100	0	0	0	0	0	0	0	0	0	0
Gluconate oxidation.	0	100	100	100	0	0	0	0	0	100	0	0	0
Arginine dihydrolase.	100	100	0	100	100	100	100	33	0	0	100	0	0
Lysine decarboxylase.	100	0	100	0	0	0	0	100	100	100	0	100	100
Ornithine decarboxylase.	0	0	100	33	0	0	0	100	100	0	0	100	100
Gas from glucose.	0	0	0	0	0	0	0	0	0	0	0	0	0
ONPG hydrolysis.	100	100	100	100	100	100	100	88	0	0	0	100	100
Urea hydrolysis.	0	0	0	0	0	0	0	22	100	0	0	0	0
Aesculin hydrolysis.	0	100	0	0	0	0	100	33	0	0	0	0	0
Weak aesculin hydrolysis.	ND	100	0	0	100	100	100	88	0	0	0	100	0
Agar hydrolysis.	0	0	0	0	0	0	0	0	0	0	0	0	0
Egg Albumen hydrolysis.	0	0	0	100	0	0	0	0	0	0	0	0	0
Arbutin hydrolysis.	0	100	0	0	0	0	0	66	0	0	100	100	0
Aryl-sulphatase.	0	0	0	0	0	0	0	55	0	0	0	0	100
Casein hydrolysis.	100	100	0	100	100	100	100	100	100	100	100	100	100
Chitin hydrolysis.	100	100	100	100	100	100	100	100	ND	100	100	100	100
DNA hydrolysis.	100	100	100	100	100	100	100	100	100	100	100	100	100
Gelatin hydrolysis.	100	100	100	100	100	100	100	100	100	100	100	100	100
Lecithinase.	100	100	100	100	0	0	100	100/5	100	ND	100	100	100
Lipase.	100	100	100	100	100	100	100	100	100	100	100	100	100
Esterase (SDS).	0	0	100	0	0	0	100	88	0	100	100	100	100
Egg proteolysis.	100	100	100	100	100	100	100	100	100	100	100	100	0
Phosphatase.	100	100	100	100	100	100	100	100	100	100	100	100	ND
Starch hydrolysis.	100	100	100	100	100	100	100	100	100	100	100	100	0
Sudanophilic inclusions.	0	0	100	67	0	0	0	0	0	100	0	100	0
Sulphide production.	0	0	0	0	0	0	0	0/5	0	0	0	ND	0
Serum hydrolysis.	ND	ND	100	100	100	100	100	100/6	100	ND	ND	ND	100
Tyrosine hydrolysis.	0	0	0	0	100	0	100	100	100	100	100	0	0

Table 3.12.1; (Cont.): Attribute Percentages for *Vibrionaceae* Type and Reference Cultures.

Phenon:	A1	A2	A3	A4	A5	A6	A7	B1	B2	B3	C1	C2	C3
Accession Number:	UQM	UQM	UQM	*V.*	UQM	UQM	Stn	*V.*	UQM	UQM	UQM	UQM	UQM
	211	2769	2742	*angu*	2786	2784	6690	*harv*	2776	2770	2744	3032	2730
Attribute.	n=1	n=1	n=1	n=3	n=1	n=1	n=1	n=9	n=1	n=1	n=1	n=1	n=1
Tyrosine pigment.	100	0	0	0	100	100	100	100	100	100	100	0	0
Growth 4°C.	0	0	0	100	0	0	0	11	0	0	0	0	0
Growth37°C.	100	100	100	100	0	0	0	100	100	100	0	100	100
Growth42°C.	100	0	100	0	0	0	0	33	100	100	0	100	100
Growth 0 % NaCl.	0	100	100	100	100	0	100	11	0	0	0	100	100
Growth 0.5 % NaCl.	100	100	100	100	0	0	100	100	100	100	100	100	100
Growth 6.0 % NaCl.	100	0	0	100	0	0	100	100	100	100	0	0	0
Growth 10 % NaCl.	0	0	0	0	0	0	0	0	0	0	0	0	0
GrowthpH 10.	100	0	100	100	100	100	100	88	0	0	0	100	100
GrowthpH 4.5.	0	0	0	0	0	0	0	0	0	0	0	0	0
GrowthNovobiocin.	0	0	0	0	0	0	0	0	0	0	0	0	0
GrowthPenicillin.	0	100	100	100	100	100	100	100	100	100	0	100	100
GrowthPolymyxin.	0	0	100	0	0	0	0	100	100	100	100	100	100
GrowthStreptomycin.	100	100	100	100	100	100	100	100	100	100	100	100	100
GrowthSulphamethoxazole	100	100	100	100	100	100	100	100	100	100	100	100	100
GrowthTetracycline.	0	0	0	0	0	0	0	0	0	0	0	0	0
GrowthTrimethoprim.	0	100	100	0	0	0	0	100	100	100	0	0	100
No growth10µg/ml0/129.	100	0	0	100	100	0	100	0	0	0	100	100	100
No growth150µg/ml0/129.	100	0	100	100	100	100	100	88	100	100	100	100	100
GrowthBasic fuchsin.	0	100	100	33	100	100	100	88	100	100	0	0	100
GrowthBrilliantgreen.	0	0	0	0	0	0	0	44	0	0	0	0	0
GrowthDichlorophene.	0	100	100	0	100	100	100	100	100	100	0	0	0
GrowthEDDA.	0	0	100	100	100	100	100	100	100	0	0	0	0
Growthon Fast yellow.	0	0	100	100	100	100	100	100	100	100	100	100	100
Growth8-Hydroxyquinoline	0	0	0	0	0	0	0	0	0	0	0	0	0
GrowthMethylviolet.	0	100	100	100	100	100	0	88	100	100	0	0	100
GrowthPyronin-Y.	0	0	0	0	0	0	0	0	0	100	0	0	0
Growth0.2% SDS.	0	100	100	33	0	0	100	100	100	100	100	100	100
GrowthThionine.	0	0	0	0	0	0	100	33	0	0	0	0	0
GrowthT.T.C.	0	100	100	100	100	100	100	100	100	100	100	100	100
Acid from Xylose.	0	0	0	0	0	0	0	0/4	ND	100	0	0	0
Acid from Arabinose.	0	100	0	100	0	0	0	0/7	ND	100	0	0	0
Acid from Mannose.	100	100	0	100	0	0	100	100/4	ND	100	100	100	100
Acid from Sucrose.	100	100	100	100	0	0	0	50/4	ND	100	100	0	100
Acid from Maltose.	100	100	100	100	100	100	100	100/4	ND	100	100	100	100
Acid from Cellobiose.	0	100	0	100	100	100	100	100/4	ND	100	100	100	100
Acid from Salicin.	0	100	0	0	0	0	0	100/4	ND	0	100	100	0
Acid from Mannitol.	100	100	100	100	100	100	100	100/4	ND	100	100	100	100
Acid from Sorbitol.	0	0	0	0	0	0	0	100/4	ND	100	0	0	0
Acid from Inositol.	100	0	0	0	0	0	0	0/4	ND	0	0	0	0
Acid from Dulcitol.	0	0	0	0	0	0	0	0/4	ND	0	0	100	0
GrowthGlycine.	0	0	0	0	0	100	100	100	100	100	0	0	0
Growthl-α-Alanine.	ND	0	100	0	0	0	100	100	100	100	100	0	0
Growthd-α-Alanine.	0	0	0	0	0	0	100	88	100	100	100	0	100
Growthdl-β-Alanine.	0	0	0	0	0	0	0	0/4	0	ND	0	0	0
GrowthSerine.	ND	100	0	0	100	100	100	88	0	100	100	0	0
Growthl-Leucine.	0	0	0	0	0	0	0	11	0	100	0	0	0
GrowthValine.	0	0	0	0	0	0	0	11	0	0	0	100	0
Growthl+Glutamicacid.	100	100	0	100	100	100	100	100	100	100	100	100	100
Growthl-Lysine.	100	0	0	0	0	0	0	33	100	0	0	100	0
Growthl-Arginine.	0	100	0	100	0	0	100	100	100	100	100	0	0
Growthl-Ornithine.	100	0	0	0	0	0	100	55	100	0	0	0	0
Growthl-Citrulline.	0	100	100	66	0	0	100	22	100	0	100	100	0
Growthγ-Amino-butyrate.	0	0	0	0	0	0	0	11	0	0	0	0	0
Growthδ-Amino-valerate.	ND	0	0	0	0	0	0	11	0	0	0	0	0
Growthl-Proline.	0	100	100	0	0	0	100	100	100	100	100	100	0

Table 3.12.1; (Cont.): Attribute Percentages for *Vibrionaceae* Type and Reference Cultures.

Phenon:	A1	A2	A3	A4	A5	A6	A7	B1	B2	B3	C1	C2	C3
Accession Number:	UQM	UQM	UQM	*V.*	UQM	UQM	Stn	*V.*	UQM	UQM	UQM	UQM	UQM
	211	2769	2742	*angu*	2786	2784	6690	*harv*	2776	2770	2744	3032	2730
Attribute.	n=1	n=1	n=1	n=3	n=1	n=1	n=1	n=9	n=1	n=1	n=1	n=1	n=1
Growth Putrescine.	0	0	0	0	0	0	0	11	100	0	0	0	0
Growth Sarcosine.	0	0	0	0	0	0	0	33	0	0	0	0	0
Growth l-Glutamine.	100	100	100	100	0	0	100	100	100	100	100	0	100
Growth d-Glucosamine.	0	0	0	0	0	0	0	33	100	0	100	100	100
Growth n-Acetylglucosamine.	ND	100	0	66	0	0	0	77	100	100	100	100	100
Growth d-Ribose.	0	0	0	0	0	0	0	100	100	100	100	100	100
Growth Xylose.	0	0	0	0	0	0	0	11	0	0	0	0	0
Growth l-Arabinose.	0	100	0	33	0	0	0	33	100	0	0	0	0
Growth l-Rhamnose.	100	0	0	0	0	0	0	33	100	0	0	0	0
Growth Glucose.	ND	100	0	33	0	0	100	100	100	100	100	100	100
Growth d-Mannose.	100	0	0	33	100	100	100	100	100	0	100	100	100
Growth d-Galactose.	0	100	0	0	0	0	0	88	100	0	100	100	0
Growth Sucrose.	100	100	0	66	0	0	100	77	100	100	100	0	0
Growth Trehalose.	ND	100	0	0	0	0	0	100	100	100	100	100	100
Growth Maltose.	100	100	100	66	100	0	100	100	100	100	100	100	100
Growth Cellobiose.	ND	100	0	0	0	0	100	88	0	0	100	100	0
Growth Lactose.	0	0	0	0	0	0	0	0	0	0	0	100	0
Growth Raffinose.	0	0	0	0	0	0	0	0	100	0	0	0	0
Growth Galactarate.	0	0	0	0	0	0	0	55	0	0	0	0	0
Growth Gluconate.	100	0	0	33	0	0	100	88	100	100	100	100	100
Growth Glucuronic acid.	0	0	0	0	0	0	0	88	100	0	0	0	0
Growth Salicin.	0	0	0	0	0	0	0	44	0	0	100	100	0
Growth Starch.	100	100	100	66	100	100	100	100	100	100	100	100	0
Growth Dextrin.	100	100	0	66	0	0	100	100	100	100	100	100	100
Growth Inulin.	ND	0	0	0	0	0	0	0	0	0	0	0	0
Growth Pullulan.	100	100	0	0	0	0	100	100	100	100	0	100	0
Growth Formate.	100	0	0	0	0	0	0	0	0	0	0	0	0
Growth Acetate.	100	0	0	0	0	0	0	11	100	100	0	0	0
Growth Propionate.	0	0	0	0	0	0	0	0	0	0	0	0	0
Growth Nonanionic acid.	100	0	0	0	0	0	0	0	0	0	0	0	0
Growth Caprate.	100	0	0	0	0	0	0	0	0	0	0	0	0
Growth Malonate.	0	0	0	0	0	0	0	33	0	0	0	0	0
Growth Succinate.	100	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Growth Maleate.	0	0	0	0	0	0	0	0/4	0	0	0	0	0
Growth Adipic acid.	0	0	0	0	0	0	0	44	0	0	0	0	0
Growth Malate.	ND	0	0	0	0	0	100	100	100	100	0	0	100
Growth Tartarate.	0	0	0	0	0	0	0	0/6	ND	0	0	0	0
Growth dl-3-OH-butyrate.	0	0	0	0	0	0	0	0	0	0	0	0	0
Growth 6-Hydroxy Caproate.	0	0	0	0	0	0	0	11	0	0	0	0	0
Growth Lactate.	ND	100	0	66	0	0	100	100	100	100	100	100	100
Growth Poly-β-OH-butyrate.	0	0	0	0	0	0	0	0	0	0	0	0	0
Growthdl-Glyceric acid.	ND	100	100	33	0	100	0	55	100	100	100	0	100
GrowthCitrate.	ND	0	0	33	0	0	0	100	100	100	100	100	100
Growthα-Ketoglutarate.	0	0	100	33	0	0	100	100	100	100	100	100	100
GrowthPyruvate.	ND	ND	ND	ND	ND	ND	100	100	100	100	ND	ND	ND
GrowthErythritol.	0	0	0	0	0	0	0	0/4	0	0	0	0	0
GrowthDulcitol.	0	0	0	0	0	0	0	0/6	0	0	0	0	0
GrowthMannitol.	100	100	0	66	0	0	0	100	100	100	100	100	100
GrowthSorbitol.	0	0	0	100	0	0	0	44	100	0	0	0	0
GrowthHydroxybenzoate.	0	0	0	0	0	0	0	44	0	0	0	0	0

Table 3.12.2: **Attribute Percentages for *Vibrionaceae* Type and Reference Cultures.**

Phenon:	C4	C5	C6	C7	D1	D2	D3	D4	E1	E2	F	G1	G2
Accession Number:	*V.*	UQM	UQM	Stn	*V.*	UQM	*A.*	UQM	UQM	UQM	Stn	Stn	Stn
	vuln	2778	2779	7000	*fluv*	2775	*hydr*	2782	2785	2783	7650	180	1800
Attribute.	n=5	n=1	n=1	n=1	n=5	n=1	n=4	n=1	n=1	n=1	n=1	n=2	n=1
Colony diameter > 3 mm.	0	0	0	0	0	0	0	0	0	0	0	0	0
Colony mucoid.	0	0	0	0	0	0	0	0	0	0	0	100	0
Colony matt.	0	0	0	0	0	0	0	0	0	0	0	0	0
Colony opaque.	40	0	0	0	0	0	50	100	100	100	100	100	100
Colony entire.	100	100	100	100	100	100	100	100	100	100	100	100	100
Colony convex.	100	100	100	100	100	100	100	100	100	100	100	100	100
Swarming growth.	0	0	0	0	0	0	0	100	0	100	100	0	0
Luminous growth.	0	0	0	0	0	0	0	0	0	0	0	0	0
Red pigment.	0	0	0	0	0	0	0	0	0	0	0	100	0
Black pigment.	0	0	0	0	0	0	0	0	0	0	0	0	0
Length > 2 times width.	40	100	100	100	60	100	75	0	100	0	100	100	0
Strong catalase.	0	0	0	100	20	0	0	0	0	0	0	0	100
Oxidase.	100	100	100	100	100	100	100	100	100	100	100	0	0
Oxidase (Toluene).	100	100	100	100	100	100	100	100	100	100	100	0	0
Broth uniform turbidity.	100	100	100	100	100	100	100	100	100	100	100	100	100
Broth with sediment.	100	100	100	100	100	100	100	100	100	100	100	0	100
Broth sediment mucoid.	0	0	100	0	0	0	0	0	0	0	0	100	0
Broth growth mod. to heavy	80	0	0	0	80	100	100	100	100	100	100	0	0
Pellicle.	0	0	0	0	0	0	25	0	0	0	0	0	0
Indole.	80	0	0	0	0	0	25	0	0	0	0	0	0
Weak indole.	100	0	0	0	0	0	25	0	0	100	0	0	0
Motile.	100	0	100	100	80	100	100	100	100	100	100	100	100
Polar Flagellation.	100	ND	100	100	100/4	100	100	100	100	100	100	100	0
5 day pH > 7.05.	0	0	0	0	40	0	50	0	0	0	0	100	100
5 day pH > 5.15.	100	100	100	0	100	0	75	100	0	100	100	100	100
Acetoin production.	0	0	0	0	60	0	50	0	0	0	0	100	100
Nitrate reduction.	100	100	100	100	100	100	100	100	100	100	100	0	100
Nitrite reduction.	0	0	0	0	80	100	50	100	100	0	0	0	100
Cholera-Red.	0	0	0	0	0	0	0	0	0	0	0	0	0
Gluconate oxidation.	0	0	0	0	20	0	50	0	0	0	0	0	100
Arginine dihydrolase.	0	0	0	100	100	100	100	0	0	100	100	0	100
Lysine decarboxylase.	100	0	100	0	0	0	100	0	0	0	0	0	0
Ornithine decarboxylase.	100	0	0	0	0	0	0	0	0	0	0	0	100
Gas from glucose.	0	0	0	0	0	0	75	0	0	0	0	0	0
ONPG hydrolysis.	60	0	0	100	100	100	100	0	100	0	100	100	100
Urea hydrolysis.	0	0	0	0	0	0	0	0	0	0	0	0	0
Aesculin hydrolysis.	60	0	0	0	100	0	50	100	0	ND	100	0	100
Weak aesculin hydrolysis.	60	0	0	0	100	0	50	100	100	ND	100	100	100
Agar hydrolysis.	0	0	0	0	0	0	0	0	0	0	100	0	0
Egg Albumen hydrolysis.	20	0	0	0	100	100	50	0	0	0	0	100	0
Arbutin hydrolysis.	60	0	0	0	80	0	50	100	0	0	100	100	100
Aryl-sulphatase.	0	0	0	0	0	0	0	0	100	0	0	0	0
Casein hydrolysis.	100	100	100	100	60	100	100	100	0	100	0	100	0
Chitin hydrolysis.	100	100	100	100	100	100	100	100	100	100	100	ND	ND
DNA hydrolysis.	100	100	100	100	100	100	100	100	100	100	100	100	100
Gelatin hydrolysis.	100	100	100	100	100	100	100	0	0	100	0	100	0
Lecithinase.	100	100	100	100	100	100	100	0	100	0	100	0	0
Lipase.	100	100	100	100	100	100	100	100	100	100	100	100	100
Esterase (SDS).	100	0	0	100	60	0	75	100	100	0	100	0	0
Egg proteolysis.	100	100	100	100	100	100	100	100	100	0	100	100	100
Phosphatase.	100	100	100	100	100/4	ND	100/3	ND	ND	ND	100	100/1	ND
Starch hydrolysis.	100	100	100	100	100	0	75	100	0	0	100	100	0
Sudanophilic Inclusions.	40	0	0	0	0	0	0	0	0	100	100	100	0
Sulphide production.	0	0	0	ND	ND	ND	0/1	0	0	0	0	0	ND
Serum hydrolysis.	100	ND	ND	ND	100/4	100	100/2	ND	ND	100	ND	100/1	100
Tyrosine hydrolysis.	60	100	100	0	100	100	100	100	100	100	100	0	0

Table 3.12.2; (Cont.): Attribute Percentages for *Vibrionaceae* Type and Reference Cultures.

Phenon:	C4	C5	C6	C7	D1	D2	D3	D4	E1	E2	F	G1	G2
Accession Number:	*V.*	UQM	UQM	Stn	*V.*	UQM	*A.*	UQM	UQM	UQM	Stn	Stn	Stn
	vuln	2778	2779	7000	*fluv*	2775	*hydr*	2782	2785	2783	7650	180	1800
Attribute.	n=5	n=1	n=1	n=1	n=5	n=1	n=4	n=1	n=1	n=1	n=1	n=2	n=1
Tyrosine pigment.	80	0	100	0	100	100	25	100	100	100	100	0	0
Growth 4°C.	0	0	0	100	0	0	25	0	0	0	0	0	100
Growth37°C.	100	100	100	0	100	100	100	100	100	100	100	100	100
Growth42°C.	100	0	0	0	80	100	100	100	0	0	0	100	100
Growth 0 % NaCl.	100	0	0	0	100	100	100	0	0	0	100	0	100
Growth 0.5 % NaCl.	100	100	100	100	100	100	100	100	100	0	100	100	100
Growth 6.0 % NaCl.	0	100	100	100	100	100	75	100	100	100	0	100	100
Growth 10 % NaCl.	0	0	100	0	20	0	0	0	0	0	0	100	0
GrowthpH 10.	80	0	0	100	100	100	100	100	100	100	100	0	100
GrowthpH 4.5.	0	0	0	0	0	0	0	0	0	0	0	0	0
GrowthNovobiocin.	0	0	0	0	60	100	100	0	0	0	0	0	100
GrowthPenicillin.	80	100	100	0	100	100	100	100	100	0	0	100	100
GrowthPolymyxin.	100	100	100	100	0	0	50	0	0	0	0	0	100
GrowthStreptomycin.	100	100	100	100	100	100	100	100	100	100	100	100	100
GrowthSulphamethoxazole.	100	100	100	100	100	100	100	100	100	100	100	100	100
GrowthTetracycline.	0	0	0	0	0	0	0	0	0	0	0	0	100
GrowthTrimethoprim.	100	100	0	100	60	100	100	100	100	100	100	0	100
No growth10µg/ml0/129.	60	0	100	0	0	0	0	0	0	100	100	0	0
No growth150µg/ml0/129.	100	100	100	100	100	100	0	100	100	100	100	100	0
GrowthBasic fuchsin.	0	0	0	0	100	100	100	0	0	0	0	0	100
GrowthBrilliantgreen.	0	0	0	0	100	100	75	100	0	100	0	0	100
GrowthDichlorophene.	0	0	0	100	100	100	100	100	100	0	100	100	100
GrowthEDDA.	40	100	100	100	40	0	50	100	100	0	0	100	100
Growthon Fast yellow.	100	100	100	100	100	100	100	100	0	100	100	0	100
Growth8-Hydroxyquinoline.	0	0	0	0	0	0	25	0	0	0	0	0	0
GrowthMethylviolet.	0	0	0	0	100	100	100	100	0	0	100	50	100
GrowthPyronin-Y.	0	0	0	0	0	0	100	0	0	0	0	0	100
Growth0.2% SDS.	100	100	100	100	100	100	100	100	100	0	100	0	100
GrowthThionine.	0	0	0	0	100	100	50	0	0	0	0	0	100
GrowthT.T.C.	100	100	100	100	100	100	100	100	100	100	100	100	100
Acid from Xylose.	0	0	0	0	ND	ND	0/2	0	100	0	0	100	100
Acid from Arabinose.	0	0	0	0	ND	ND	0/2	100	0	0	0	100	100
Acid from Mannose.	100	0	100	100	ND	ND	100/2	0	100	100	100	100	100
Acid from Sucrose.	0	0	0	0	ND	ND	100/2	100	100	100	100	100	100
Acid from Maltose.	100	100	100	100	ND	ND	100/2	100	100	100	100	100	100
Acid from Cellobiose.	60	100	100	0	ND	ND	0/2	100	100	ND	100	100	100
Acid from Salicin.	20	0	0	0	ND	ND	100/2	100	100	100	100	100	100
Acid from Mannitol.	100	100	100	0	ND	ND	100/2	0	100	100	100	100	100
Acid from Sorbitol.	20	0	0	0	ND	ND	100/2	0	100	0	100	0	100
Acid from Inositol.	0	0	0	0	0/2	ND	0/2	0	0	0	100	0	0
Acid from Dulcitol.	0	0	0	0	ND	ND	0/2	0	0	0	0	0	0
GrowthGlycine.	0	100	100	0	100	100	75	100	100	100	0	0	0
Growthl-α-Alanine.	100	100	100	100	100	100	100	100	100	100	0	100	100
Growthd-α-Alanine.	40	100	100	0	100	100	100	100	100	100	0	0	100
Growthβ-Alanine.	0/3	0	0	0	0/1	ND	0	0	0	0	0	0	0
GrowthSerine.	20	100	100	100	100	100	100	100	100	100	100	0	100
Growthl-Leucine.	0	0	0	0	80	0	0	0	0	100	0	0	0
GrowthValine.	0	0	0	0	40	100	25	100	0	0	100	0	0
Growthl+Glutamicacid.	100	100	100	100	100	100	100	100	100	100	100	100	100
Growthl-Lysine.	0	100	100	0	40	0	0	0	100	100	100	0	0
Growthl-Arginine.	60	100	100	100	100	100	100	100	100	100	100	0	0
Growthl-Ornithine.	20	0	0	0	100	100	0	0	100	100	100	0	0
Growthl-Citrulline.	80	100	100	0	100	0	0	100	100	100	100	0	0
Growthγ-Amino-butyrate.	0	0	0	0	100	100	75	0	100	0	0	50	0
Growthδ-Amino-valerate.	0	0	0	0	0	100	0	100	0	100	0	0	0
Growthl-Proline.	100	100	100	100	100	100	100	100	100	100	0	50	100

Table 3.12.2; (Cont.): Attribute Percentages for *Vibrionaceae* Type and Reference Cultures.

Phenon:	C4	C5	C6	C7	D1	D2	D3	D4	E1	E2	F	G1	G2
Accession Number:	*V.*	UQM	UQM	Stn	*V.*	UQM	*A.*	UQM	UQM	UQM	Stn	Stn	Stn
	vuln	2778	2779	7000	*fluv*	2775	*hydr*	2782	2785	2783	7650	180	1800
Attribute.	n=5	n=1	n=1	n=1	n=5	n=1	n=4	n=1	n=1	n=1	n=1	n=2	n=1
Growth Putrescine.	0	0	0	0	80	100	25	100	100	100	100	0	0
Growth Sarcosine.	0	0	0	0	80	100	0	100	0	0	0	0	0
Growth l-Glutamine.	100	100	100	100	100	100	100	100	100	100	0	100	100
Growth d-Glucosamine.	80	0	0	0	100	0	100	0	100	100	100	0	100
Growth n-Acetylglucosamine	60	100	0	100	100	100	100	100	0	100	100	100	100
Growth d-Ribose.	60	100	100	100	100	100	100	100	0	100	0	100	100
Growth Xylose.	0	0	0	0	20	0	0	0	0	0	0	0	100
Growth l-Arabinose.	0	0	0	0	100	100	0	100	0	0	100	100	100
Growth l-Rhamnose.	0	0	0	0	40	100	25	100	0	0	100	0	0
Growth Glucose.	100	100	100	100	100	100	100	100	0	100	0	50	100
Growth d-Mannose.	100	0	100	100	100	100	100	0	100	0	100	100	100
Growth d-Galactose.	100	0	0	100	100	100	100	100	100	0	100	100	100
Growth Sucrose.	0	0	0	100	100	100	100	100	0	100	100	100	100
Growth Trehalose.	100	100	100	100	100	100	100	100	100	100	100	100	100
Growth Maltose.	100	100	100	100	100	100	100	100	100	100	100	100	100
Growth Cellobiose.	60	100	100	100	60	0	50	100	0	0	100	100	100
Growth Lactose.	0	0	0	0	0	0	25	0	0	100	0	0	0
Growth Raffinose.	0	0	0	0	40	100	0	0	0	0	100	0	0
Growth Galactarate.	20	0	0	0	40	0	0	0	0	0	0	50	0
Growth Gluconate.	100	0	0	0	100	100	100	100	100	100	0	0	100
Growth Glucuronic acid.	20	0	0	0	100	100	0	0	ND	0	0	100	100
Growth Salicin.	20	0	0	0	100	100	50	100	0	0	100	100	100
Growth Starch.	100	100	100	100	100	100	100	100	100	100	100	100	100
Growth Dextrin.	100	100	100	100	100	100	100	100	100	100	100	100	100
Growth Inulin.	0	0	0	0	0	0	0	0	0	0	0	0	0
Growth Pullulan.	100	100	100	100	100	100	75	100	0	100	100	0	0
Growth Formate.	0	0	0	0	20	100	25	0	0	0	0	0	0
Growth Acetate.	0	0	0	0	100	100	50	100	0	0	0	100	100
Growth Propionate.	0	0	0	0	0	0	0	0	0	0	0	0	100
Growth Nonanionic acid.	0	0	0	0	0	0	0	0	0	0	0	0	0
Growth Caprate.	0	0	0	0	0	100	50	0	0	0	0	0	0
Growth Malonate.	0	0	0	0	20	100	0	0	0	0	0	0	0
Growth Succinate.	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Growth Maleate.	0	0	0	0	0	ND	0/2	0	0	0	0	0	ND
Growth Adipic acid.	0	0	0	0	0	0	0	0	0	0	100	0	0
Growth Malate.	60	0	100	100	100	100	100	100	100	0	100	0	100
Growth Tartarate.	0	0	0	0	0	ND	0	0	ND	0	0	0	0
Growth dl-3-OH-butyrate.	0	0	0	0	40	0	0	100	0	0	0	0	0
Growth 6-Hydroxy Caproate.	0	0	0	0	0	0	0	0	0	0	0	0	0
Growth Lactate.	100	100	100	100	100	100	100	100	100	100	100	100	100
Growth dl-Glyceric acid.	100	0	0	100	100	100	100	100	100	0	0	0	100
Growth Poly-β-OH-butyrate.	0	0	0	0	0	0	0	0	0	0	0	0	0
GrowthCitrate.	60	100	100	100	100	100	50	100	0	100	0	100	100
Growthα-Ketoglutarate.	100	100	100	100	100	100	25	100	100	100	100	100	100
GrowthPyruvate.	0/3	100	100	100	100	100	100	100	100	100	ND	100	100
GrowthErythritol.	0	0	0	0	ND	ND	0/1	ND	0	0	0	0	ND
GrowthDulcitol.	0/4	0	0	0	ND	ND	0/1	0	0	0	0	0	ND
GrowthMannitol.	100	100	100	0	100	100	100	100	0	0	100	100	100
GrowthSorbitol.	0	0	0	0	40	0	25	0	0	0	100	100	100
GrowthHydroxybenzoate.	0	0	0	0	100	100	50	100	0	0	0	0	0

brilliant green, or to produce acid from mannose. It further differed from phenon B1 which also contained some ADH positive OTU's by not separately utilizing the l-or d-α-alanine, l-ornithine, proline, d-glucose, sucrose, trehalose, maltose, cellobiose and citrate.

Phenon B1 fused the type culture for *V.harveyi* UQM 2781, and eight provincial OTU's. Three arginine dihydrolase positive OTU's from this phenon Stn 1710, 1750 and 1760 were also quite similar to other OTU's. This phenon was between 88 and 100 % positive for differential attributes including LDC, ODC, hydrolysis of ONPG, weak aesculin hydrolysis, casein, chitin, DNA, gelatin, lecithin, lipase, starch growth in 0.5 - 6 % saline, at pH 10, in the presence of polymyxin, trimethoprim, susceptibility to 150μg/ml 0/129 and attack of glycine, l-α-alanine, d-α-alanine, serine, l+glutamic acid, l-arginine, l-proline, l-glutamine, d-ribose, glucose, mannose, trehalose, maltose, starch, malate, α-ketoglutarate, and mannitol. None, or less than 12 % of the OTU's from this phenon were positive for the characters acetoin production, growth without saline, susceptibility to 10 μg/ml 0/129, and separate attack of ɣ-aminobutyrate, δ-aminovalerate and lactose. The 0/129 resistant OTU from this phenon, UQM 2839, was found by electron microscopy to have a sheathed polar flagella.

If all reference strains from Table 3.5 were correctly identified then the sucrose negative Phena C2-C6 contained at least four species. The OTU, Stn 1620, Phenon C2 was phenotypically most similar to *V.vulnificus* as described by Baumann *et al.*, (1984). It was exceptional by its luminescence, growth in 0% but not 6% sodium chloride, and separate utilization of citrulline (See above), mannitol and galactose, but not of l- or d-α-alanine, arginine, putrescine or acetate. The sucrose-negative *V.cholerae* UQM 2730 was able to grow at both 15 and 42°C., did not produce acetoin at 25°C., was separately able to utilize d-glucuronate, produce esterases to both egg lipid and SDS, unable to hydrolyze starch, insensitive to 10μg/ml polymyxin, and non-luminescent. The reference culture of UQM 2742 *V.cholerae* Phenon A3 apart from its separate utilization of and acid production from

sucrose grew between 15 and 42°C., produced acetoin, and was unable to utilize glucuronate, produced esterase, to egg but not SDS, hydrolyzed starch, and was insensitive to polymyxin. Additional attributes which distinguished UQM 2742 were the presence of a strong catalase reaction, presence of an alkaline pH in MRVP broth, egg proteolysis, an insensitivity to 10 μg/ml 0/129, the separate utilization of l-α-alanine, citrulline, proline, d-glucosamine and starch, but failure to hydrolyze casein, and to separately utilize d-α-alanine, n-acetylglucosamine, ribose, glucose, mannose, gluconate, malate, citrate, α-ketoglutarate or mannitol. This OTU was found by electron microscopy to have a sheathed polar flagellum but differed phenotypically from Baumann *et al.* by its resistance to 10 μg/ml 0/129 phosphate, separate utilization of l-α-alanine and citrulline, and non-utilization of arginine, ornithine, ribose, galactose, sucrose, trehalose, gluconate, malate and lactate.

Phenon C4, which contained 5 OTU's, differed from sucrose-negative *V.cholerae* UQM 2730 by the following attributes: Frequencies in phenon C4 between 20 and 80 % are in bold type - Opaque colonies, indole, β-galactosidase, aesculin, arbutin, egg proteolysis, starch hydrolysis, tyrosine hydrolysis, tyrosine pigment, growth at pH 10 and in the presence of penicillin, 10 μg/ml 0/129 phosphate, EDDA, fast yellow, methyl violet, ribose, galactose, cellobiose, starch, pullulan, galactarate, salicin, malate, citrate and sorbitol.

The OTU Stn 7000, Phenon C7, was not closely associated with any single species; phenotypically it was distinct from both *V.campbellii* UQM 2779 and *V.vulnificus* UQM 2778 by its positive reaction for the following substantive attributes; strong catalase, 5d MRVP pH less than 5.15, ADH, β-galactosidase was produced, production of SDS esterase, growth at 4°C., growth at pH 10, trimethoprim resistance, sensitivity to 10 μg/ml 0/129, dichlorophene resistance, separate utilization of galactose, sucrose, dl-glyceric acid, neither lysine or ornithine decarboxylase produced, no growth at 37°C., in 10% NaCl, or on penicillin agar, no acid from cellobiose or mannitol, no

separate utilization of glycine, d-α-alanine, lysine, citrulline or mannitol.

The anaerogenic culture of *A.hydrophila* UQM 2769, Phenon A2, phenotypically resembled *A.hydrophila* as described in Popoff and Veron (1976) but was not associated with any provincial OTU's from Phenon D3 which contained halo-tolerant, arginine dihydrolase positive, lysine decarboxylating and 0/129 resistant OTU's UQM 2838, Stn 7770, 3230 and 7800 which were also assigned to *Aeromonas hydrophila.*

Attributes possessed by all OTU's from phenon D3 but not by *A.hydrophila* UQM 2769 were for nitrate and nitrite reduction, tyrosine hydrolysis, growth at 42°C., and in 6% sodium chloride, at pH 10, in the presence of novobiocin, fast yellow, sodium dodecylsulphate, acid from sorbitol, and all were able to separately use d-alanine, arginine, glucosamine, ribose, rhamnose, mannose, gluconate, and malate. None produced cholera-red, acid from arabinose or cellobiose and no strain could separately grow on arabinose. The flagella of UQM 2838 were examined and found to lack sheaths. Phenon D3 was most prominently different from the description of *A. hydrophila* of Popoff *et al.* by its halo-tolerance and lysine decarboxylase. The phena D1 and D2, respectively containing *V.fluvialis* UQM 2774 and *V.furnissii* UQM 2775 were separable by the failure of *V.furnissii* to hydrolyze arbutin, starch, or separately utilize citrulline or d-glucosamine, in this regimen only *V.furnissii* was able to separately utilize caprate. *V.furnissii* could be distinguished from *A.hydrophila,* (Phenon D3), by attributes including sensitivity to 150μg/ml 0/129, separate utilization of ornithine, sarcosine, glucuronic acid, and malonate, and its failure to produce lysine decarboxylase, SDS esterase, grow in the presence of pyronin-Y, or to separately utilize δ-aminovalerate or d-glucosamine.

Phenon F which contained the single agar-hydrolytic OTU Stn 7650 was most similar to *V.nereis,* and differed further by its failure to produce indole, its production of β-galactosidase, arbutin hydrolysis, starch hydrolysis and

growth in 0 and 0.5% sodium chloride, its failure to grow in 6% sodium chloride, sensitivity to 0.005% brilliant green, growth in the presence of methyl violet, 0.2% SDS and production of acid from inositol and sorbitol. No comment is made regarding separate utilization of carbon sources as results may have unreliable due to the hydrolysis of the support medium.

The OTU's for Stn 180 apart from obvious pigmentation were phenotypically most similar to *V.gazogenes* as indicated by Baumann *et al.,* (1984). Apart from separate utilization of trehalose, non-utilization of organic acids, and failure to produce gas from glucose, or separately grow on serine. Additional characters measured for these OTU's not published in Baumann *et al.* included the production of acetoin and β-galactosidase and failure to hydrolyze tyrosine or xanthine. This OTU was found by electron microscopy to have sheathed polar flagella.

Attributes expressed in OTU Stn 1800 during primary screening i.e. luminescence, oxidase reaction and production of gas from glucose, were ephemeral, and not observed subsequently. Other attributes of OTU Stn 1800 unusual in the *Vibrionaceae,* were resistance to 0/129 and negative oxidase reaction.

The unassociated OTU's *V.splendidus* UQM 2786, *V.nigripulchritudo* UQM 2784, *V.pelagius* UQM 2785, *V.vulnificus* UQM 2778, *V.campbellii* UQM 2779 and *V.natriegens* UQM 2782 were unable to utilize all substrates indicated by Baumann *et al.,* 1984. The G + C base ratios (A8) for the OTU's UQM 2779 and UQM 2782 were determined respectively as 45.1 and 46.3 mole percent.

3.8.3.3 Discussion.

3.8.3.3.1 Comparative Evaluation of Phenotypic Results.

With qualifications as specified in the results above, phenotypic attributes of type and reference cultures were in reasonable agreement with the published attributes from Baumann *et al.,* (1984) although many had a reduced nutritional spectrum, particularly for their utilization of organic acids. While to an extent this may have reflected declining vigour of type

cultures as they passed between collections, other factors seemed to have been operating. The recent provincial isolate of *V.anguillarum,* UQM 2843; was not subjected to stresses as might cause a decline in culture vigour but was highly similar to the type culture, but both were deficient relative to the published species description (Baumann *et al.,* 1984). It seems then that the reduced range of substrates separately utilized by OTU's, was at least in part a result of the minimization of the cross-feeding, consortia, or gestalts present in normal petri-dish assays by the use of multiwell trays.

Stanier, Palleroni and Doudoroff (1966) by including "similar" strains for concurrent assay in petri-dishes only acknowledged cross-feeding with circularity (See 2.9.3.6.1). Stanier *et al.* also suggested that the failure to separately utilize substrate by otherwise nutritionally versatile species, despite the necessary enzyme complement, may have been due to the absence of specific permeases in particular strains. The use of multi-well trays in that study while minimizing cross-feeding might also have restricted diffusion of these shared extra-cellular permeases perhaps with the result of increased replicate variability and lesser nutritional facility. Other possibilities offered by Stanier *et al.* for non-utilization of substrates by strains of enzymically diverse species, are the requirements of individual OTU's for specific growth factors, and the possible growth retarding qualities of some substrates. The first possibility seems unlikely to have significantly influenced results here when viewed in the context of growth by such deficient OTU's on other substrates; and the second becomes less likely in the context of demonstrated viability of *Vibrio* spp. at the concentrations of fatty acids such as were collated in Baumann *et al.,* (1984).

The possibility that the difference arose as a result of a pH shift in the medium is negated by the adequacy of the phosphate buffer to counteract these organic acids and also that not only acids but also their neutral sodium salts were used as substrates. However some differences may also be explicable in the context of experimental procedure. In multiwell trays the

gaseous head space of each well is compartmentalized and so more stable than in petri dishes, consequently toxic vapors from volatile aliphatic organic acids would be expelled to a lesser extent and leave an effective concentration of substrate in these tests more closely resembling the amounts added than reported from petri-dish assays. This volatilization would also be reduced by lower incubation temperatures i.e. at 25°C. such as were used here.

Because amendment to procedure by reducing initial organic acid substrate concentrations would invalidate the *Rosetta* for comparative purposes and not provide opportunity to refute this explanation, no such procedural changes were introduced into subsequent batches.

3.8.3.3.2 Taxonomic Assignment of OTU's.

3.8.3.3.2.1 Arginine Dihydrolase (ADH) Positive OTU's.

3.8.3.3.2.1.1 *Vibrio* Species.

The allocation of the OTU UQM 2843 to *V.anguillarum,* was vindicated both phenotypically and genotypically (A9). However *V.anguillarum,* UQM 2744 and Stn 6690 (UQM 3236), and Stn 7000 were not closely associated with any phenon and did not phenotypically resemble any species portrayed by Baumann *et al.,* (1984) and consequently here are described as ADH positive unclassified *Vibrio* species. The OTU Stn 7650 most closely resembled *V.nereis* but because of possible skewing effects of its agar hydrolysis, assignment to *V.nereis* species could be premature. Here it is designated as *V.nereis*-like agar-hydrolytic *Vibrio* sp. (UQM 3245) and it is again reviewed in 3.8.3.5.1-2.

3.8.3.3.2.1.2 *Aeromonas* Species.

The OTU's UQM 2738, Stn 7770, 3230 and 7800 are designated as halo-tolerant lysine-decarboxylating *A. hydrophila.* UQM 2738 was confirmed as a non-*Vibrio* species by the absence of sheathed polar flagella.

3.8.3.3.2.1.3 *Photobacterium* sp.

The OTU Stn 1800 in the context of attributes lost since its presumptive isolation seemed most like *Photobacterium;* however due to its phenotypically atypical appearance e.g. utilization of mannitol, and its nutritional versatility, it is not consistent with previously described (Reichelt and Baumann 1973) species of *Photobacterium.* Ultimate diagnosis is reserved for later comparative analysis.

3.8.3.3.2.2 Lysine- and or Ornithine- Decarboxylating OTU's.

3.8.3.3.2.2.1 *V.harveyi*

The phenon assigned as *V. harveyi* containing the 0/129 resistant OTU UQM 2839, and Stn 6550, 1790, 6390, 7460, 6390 and the arginine dihydrolase positive OTU's 1710, 1750, 1760 might seem not to have been fully resolved by the cluster protocols; however the polythetic approach indicates that otherwise that these OTU's are most similar to traditionally classified *V.harveyi.* This may be indicative of ecological similarities between these OTU's and *V.harveyi* UQM 2781 or a greater enzymic diversity, amongst these strains of that species.

3.8.3.3.2.2.2 *Vibrio cholerae* and Similar Species.

The type culture of *V.vulnificus* UQM 2778 because it was associated more closely to *V.campbellii* UQM 2779 than to other more typical OTU's from *V.vulnificus* seems likely to have lost its vigour and so is not typically presented here. The reference culture UQM 2730 received as a sucrose-negative *Vibrio cholerae* was unassociated with the other *V. cholerae* reference culture UQM 2742 which was shown here as most similar to *V.cholerae* as described by Baumann *et al.* Because of the UQM 2730 association with other reference OTU's designated as *V.vulnificus* and its phenotypic resemblance with published description that species from Baumann *et al.,* (1984) this OTU is reassigned to that species. The luminescent OTU Stn 1620 was associated most closely with OTU's from *V.vulnificus* and is stored as *V.vulnificus* UQM 3032. *V.mimicus* (Davis *et al.,* 1981; Baumann *et al.,* 1984) is incompletely described particularly with respect to the range

of C-sources utilized. Strains assigned here as *V.vulnificus* including UQM 2730 because all posessed features both common and rarely found in *V.mimicus* consequently in the absence of a reference culture or complete phenetic profile from *V.mimicus* these strains are kept as *V.vulnificus*.

3.8.3.3.2.3 **Decarboxylase Negative OTU.**

The red pigmented OTU, Stn 180 (phenon G1), was assigned to *V.gazogenes* UQM 2840, for its high phenetic resemblance to *V.gazogenes* as described by Baumann *et al.* and for the presence of a sheathed polar flagellum.

3.8.3.3.3 **Comparative Evaluation of Analysis Protocols.**

Each of the sorting protocols in combination with methods for assimilating missing values had specific merits and problems so that no method was best in differentiating all phena. Because the methods were so different those stable phena which overlapped between analyses were subsequently shown to have been reasonably consistent internally and by comparison with similarity matrices it was possible with only one, perhaps ecological exception, to deduce significant taxa.

The CP approach resolved most of the phena depicted in Table 3.12.1-2; and by its aggregation of oxidase-negative OTU's, vindicated the use of this character as a primary differential criterion for *Vibrionaceae* classification. This type of analysis obviously has merit for determining such differential characters. However the phenon containing OTU's from *V.vulnificus* was fragmented despite high internal similarities and possibly because parsimony analysis does not use all of the data (2.9.1) consequently amongst highly similar OTU's parsimony analysis alone should not be used.

The analysis by *Clustan* was most attractive for its automatic treatment of missing character data but because this was only an option under the ESS protocol related type and reference cultures e.g. of *V.vulnificus* were segregated i.e. the analysis was too stringent, at least for this data set. The derivation of higher than expected similarities for duplicates was probably related to invalid programming in *Clustan's* application of the

concept for missing character treatments i.e. "matching" missing character records when compared seem to have been incremented onto overall similarity in the same manner as single missing characters, rather than for similarity here to have been calculated over a correspondingly reduced number of characters.

The average linkage protocol aggregated all OTU's from *V.vulnificus* and produced an otherwise robust analysis making linkages consistent with, or intermediate between, those predicted by $S_{e.}$ and $S_{j.}$ coefficients. However *Aeromonas hydrophila* was fragmented by this as well as the other procedures, but this problem was one of vigour difference between UQM 2769 and provincial OTU's and only discerned by the Jaccard similarity matrix, rather than a clustering artifact. The manual placement of missing values in this technique was time consuming and so not entirely suitable for large data sets.

3.8.3.4 Conclusion.

None of the cluster protocols alone produced an entirely satisfactory analysis, but by cumulative evaluation of the dendrograms on the basis of stability across procedures, and by further comparison of ambiguously clustered OTU's between similarity coefficients a diagnosis of phena was produced which classified all but five OTU's, Stn 1800, 6690, 7000, 7650 and the tentatively identified culture of *V.anguillarum* UQM 2744.

The differing treatments for missing character data did not affect the placements of duplicate OTU's in protocol, although none except that used to assemble similarity matrices was suitable. Procedure for analysis by *SAS* was laborious and did not recognize vigour differences between-strains while that from *Clustan 3.2* was only associated with one form of analysis and appears to require simple program adjustment to compensate for invalid treatment of "matching" missing characters.

OTU's phenetically classified here were nutritionally less versatile than published by Baumann *et al.*, (1984) but this was attributed partly to lost

culture vigour of some type cultures, the reduction through the use of multiwell trays, of cross-feeding and agar-diffusion of permeases, and also to a greater retention of toxic volatile organic compounds than would be experienced in petri dish assays and at higher incubation temperatures.

Differential attributes from derived phena were usually consistent with divisive criteria Baumann *et al.*, (1984) used to separate species; however the phenon assigned as *V.harveyi* may perhaps be equally validly seen as a polyspecific aggregate of ecologically similar species. This could be resolved through comparative molecular examination but neither of these interpretations compromises the later (3.11) ecological purpose of this study.

3.8.4.0 Evaluation of Four Cluster Protocols Sorting Two Distance Matrices.

3.8.4.1 Experimental Design.

Sorting procedure probably does not seriously influence assignments in numerical analysis of small data sets, but inherent qualities as referred to in (2.9.3.6) become apparent as more OTU's are investigated. Further, while manual insertion of row means for missing values as used for the previous analysis by *SAS*[r]. (3.8.3), may be appropriate for analysis of small data sets, this procedure is too labourious for larger analyses and computation of similarities row by row leaves no facility to recognize vigour differences between OTU's from the same species.

In acknowledgment of these precepts, square euclidean and Jaccard distance matrices from 96 OTU's were assembled by removing characters with missing attribute data and assembling similarities based upon overlapping, rather than uniformly present data, as in 3.8.3.1. These were sorted by different clustering strategies using *SAS*[r]. *(5.08)* to establish the procedure most faithful to placement of duplicates and reference cultures from the same species. The dendrogram from this analysis was used as a datum to alphabetically flag phena so that the continuity or truncation of constituent OTU's by different procedures could be most concisely described. Attributes of single provincially isolated OTU's were not tabulated.

The dataset from Batch [6.] was chosen for separate diagnosis because departmental distilled water used to prepare media contained sufficient organic contaminants to support growth in the absence of any other carbon source (3.8.2.2.11). Results for individual characters were with some difficulty interpretable by comparison between other positive and negative OTU's. Despite the use of blind duplicates it was decided that results while internally consistent were potentially severely skewed, and that any subsequent damping to permit cross-batch pooled analyses might also affect the resolution of taxa.

Apart from reference cultures, unassigned presumptive *Vibrionaceae* were included for analysis on the basis of their originating from both pathological (fish, molluscs) and non-pathological (environmental) material. A weakly fermentative OTU derived from a diseased goldfish and, tentatively assigned as a *Shewanella* sp. was examined to establish if species distribution in phenetic analysis reflected phylogeny i.e. 5S rRNA distribution, or ecology as surmised in (2.9.3.2) and suggested also in 3.8.3.

Single, average, median and complete linkage protocols, (UPGM_S-C), were used to sort the euclidean (SLE, ALE, MLE, and CLE) then Jaccard (SLJ, ALJ, MLE, and CLJ) distance matrices and produce the dendrograms (Figures 3.5.1-8 respectively, in Volume 2, pp. 495-9.). Command programs had structures as in A11.6.1-4 and all "k-linkage" list lengths were set to 8. The single linkage procedure differed from the protocol reviewed by Jardine and Sibson (1968, 2.9.3.4) by its incorporation of "trimming," here OTU's were dropped and the analysis recommenced as the number of cluster elements exceeded 5 % of unclustered OTU's. Developing phena under median linkage and sometimes also single linkage procedures were more similar upon addition of new OTU's than initially; to illustrate this in dendrograms early branch nodes were folded down before cross-branches were formed with new OTU's. Sorted similarity matrices for euclidean and Jaccard coefficients are in Tables 3.13.1-2 respectively (Volume 2, p 495).

Duplicate OTU's were included from the strains Stn 2440, 6421, 6430 and 6670. Attributes of these OTU's which were non-correspondent and for which all OTU's were coded to missing were: Positive for yellow/orange pigmentation, cell length greater than 2 times width, broth sediment mucoid, sudanophilic inclusions, growth at 42°C., no production of phosphatase, growth in the presence of fuchsin, and separate utilization of raffinose and mannitol.

Examination of streak-plates produced after inoculation of tubed and multi-

well tray tests indicated two, Stn 2290 and Stn 2800, which were mixed during inoculation of tray tests. These OTU's were removed from sorted similarity matrices and phenon attribute frequency tables (3.14.1-2).

3.8.4.2 Results.

3.8.4.2.1 Placement of Duplicate OTU's.

Except for duplicates of the OTU Stn 6421 test agreement was 88 % or above for both euclidean and Jaccard coefficients. The attributes listed in Table 3.14.3 were discrepant between OTU's. Duplicates were generally more widely dispersed by sorts of the euclidean (Figures 3.5.1,3,5,7 from Table 3.13.1) than the Jaccard (Figures 3.5.2,4,6,8 from Table 3.13.2) matrix, where sorts with most consistent concurrent placement of duplicates were from ALJ and to a lesser extent, CLJ protocols (respectively Figures 3.5.4, 3.5.8).

3.8.4.2.2 Review of Sorting Protocols.

Distributions among type and reference cultures within dendrograms (Figures 3.5.1-8) were different between similarity criteria but generally similar across sorting protocols. Phena which contained exclusively *V.parahaemolyticus* type and reference cultures UQM 2776, UQM 2200, UQM 2201, UQM 2125, UQM 2126, were constantly resolved by all protocols under both similarity criteria. However, by MLJ, (Figure 3.5.6) this phenon was much larger and contained OTU's resolved as distinct phena in other analyses.

The reference culture of *V.vulnificus* UQM 2740 and the type culture for *V.vulnificus* Biogroup II, UQM 2922, amongst all methods used to sort the euclidean distance matrix; were associated in a single phenon only by MLE (Figure 3.5.5). Sorts of the Jaccard distance matrix by MLJ and SLJ protocols orphaned the OTU UQM 2922. Phena containing both reference OTU's of *V.vulnificus* as generated by ALJ (Figure 3.5.4) and CLJ (Figure 3.5.8) linkage procedures respectively contained 5 and 6 OTU's. By ALJ the OTU was placed instead in the same phenon as *V.carchariae* UQM 2919. The S_j (Table 3.13.2) values of the discrepant OTU, Stn 3000 were examined to gauge the validity of each assignment. The affinities of this OTU were relatively

similar to otherwise widely dispersed OTU's e.g. UQM 2740, UQM 2920, and Stn 1170, 1380, 1450, 3010, 6650-70. These intermediate affinities were reflected to a greater extent in the ALJ than the CLJ analysis and consequently OTU's were flagged according to this distribution.

Table 3.14.3 : Discrepant Attributes (*), from Duplicate OTU's and Percent Similarities, as Measured by Euclidean and Jaccard Coefficients.

Attribute.	Replicated OTU.			
	Stn 2440.	Stn 6421.	Stn 6430.	Stn 6670.
Colony entire.	-	*	-	-
Colony convex.	-	*	-	-
Ornithine decarboxylase.	-	*	-	-
ONPG hydrolysis.	-	*	-	-
Aesculin hydrolysis.	-	*	-	-
Egg Albumen hydrolysis.	*	-	-	-
Tyrosine hydrolysis.	-	*	*	*
Tyrosine pigment.	-	*	-	-
Many Sudanophilic Inclusions.	-	*	-	*
Growth 10 % NaCl.	-	-	-	*
Growth Polymyxin.	-	*	-	-
Growth Pyronin-Y.	*	-	-	-
Growth Thionine.	-	-	-	*
Growth l-Arginine.	-	-	-	*
Growth l-Ornithine.	-	*	-	-
Growth l-Citrulline.	-	-	-	*
Growth δ-Amino-valerate.	-	-	-	*
GrowthNorvaline.	-	-	-	*
Growthd-Glucosamine.	-	-	-	*
Growthl-Arabinose.	-	*	-	-
Growthl-Rhamnose.	-	-	-	*
GrowthGlucose.	-	*	-	-
Growthd-Mannose.	-	*	-	-
Growthd-Galactose.	-	*	-	-
GrowthSucrose.	-	*	-	-
GrowthCellobiose.	-	*	*	-
GrowthGlucuronicacid.	-	-	*	-
GrowthAcetate.	-	*	-	*
GrowthHeptanoate.	-	*	-	-
Growth6-HydroxyCaproate.	-	-	-	*
GrowthLactate.	-	-	-	-
Growthdl-Glycericacid.	-	-	-	*
GrowthPoly-β-OH-butyrate.	-	*	-	*
Growthα-Ketoglutarate.	-	-	*	-
GrowthSorbitol.	-	-	-	*
GrowthEthanol.	-	*	-	*
	PercentSimilarity.			
Similarity(euclidean)	99	87	97	93
Similarity(Jaccard)	98	79	95	88

Most of the seventeen phena recognized by ALJ were also resolved by CLJ. OTU's in phena "J" and "K" were resolved almost consistently by all procedures except MLJ. Despite the use of trimming many OTU's sorted particularly by SLE and by SLJ were orphaned. This was also true for sorts by ALE, CLE, and MLE procedures. The OTU's associated with *V.tubiashii* UQM 2923 (ALJ Phenon F) i.e. Stn 960, 6520 and 6900 were placed separately by CLJ in a phenon with UQM 2770. The S_j values indicated a greater similarity between UQM 2923 and Stn 6520 (71 %) than with UQM 2770 (69 %), which endorsed the distribution formed by the ALJ analysis.

3.8.4.2.3 Evaluation of Phena.

Characters positive for all OTU's were catalase, oxidase, weak-oxidase, broths with uniform turbidity, broths with sediment, motile, polar flagellation, nitrate reduction, phosphatase, growth 15°C., 30°C., 0.5-3 % sodium chloride, pH 10, penicillin, streptomycin, sulphamethoxazole and fast yellow.

Characters negative for all OTU's were colonies matt, red pigment, Gram-positive, strong catalase, growth basic fuchsin, dl-β-alanine, raffinose, inulin, n-butyric-acid, malonate, hydroxybenzoate. Percent attribute frequencies for differential characters are shown in Tables 3.14.1-2.

The 17 ALJ tabulated phena (3.14.1-2) were divided first on the basis of their ADH reactions. OTU's from Phenon B, E, F, K, L and Q were exclusively ADH positive. The phena C and O contained OTU's which were variable for this character. Further subdivision of remaining phena according to decarboxylase patterns revealed four phena A, D, I and J which were entirely lysine decarboxylating and at least some OTU's, were also ornithine decarboxylating; another, G, which was only lysine decarboxylating and two phena H, and M which were decarboxylase negative.

3.8.4.2.3.1 Arginine Dihydrolase Positive Phena.

Phenon B contained two OTU's; the type culture of *V.aestuarianus* UQM 2920 and the isolate from fish mucus Stn 7450 (UQM 3376). Similarity coefficients

(Table 3.13.1-2) respectively were 86 and 68 % for $S_{e.}$ and $S_{j.}$. Stn 7650 culture had high euclidean similarities to type cultures of *V.cholerae* UQM 2773, and *V.anguillarum* UQM 2771 both 87 %, *V.tubiashii* and *V.vulnificus* UQM 2922, both 85 % and *V.orientalis* UQM 2921, 83 %. These similarities were conducted to a lesser extent to the $S_{j.}$ values, respectively, 67, 66, 66, 63 and 59%. Differential attributes common to Stn 7650 and and UQM 2920 were: pH less than 5.15, cholera-red production, ADH, ONPG hydrolysis, weak aesculin hydrolysis, egg albumen hydrolysis, growth 4°C., susceptible to 10 μg/ml 0/129, dichlorophene, ribose, starch, pullulan, succinate and citrate. Differential characters negative for both OTU's were: Pellicle production, nitrite reduction, gluconate oxidation, LDC, ODC, gas from glucose, urea hydrolysis, aesculin hydrolysis, arbutin hydrolysis, aryl-sulphatase, DNA hydrolysis, sulphide production, tyrosine hydrolysis, xanthine hydrolysis, 0 % and 10 % sodium chloride, at pH 4.5, novobiocin, brilliant green, EDDA, 8- hydroxy-quinoline, pyronin-Y, SDS, glycine, d-α-alanine, serine, leucine, valine, lysine, ornithine, citrulline, γ-amino-butyrate, δ-aminovalerate, norvaline, proline, putrescine, sarcosine, glutamine, n-acetylglucosamine, ribose, xylose, arabinose, rhamnose, mannose, trehalose, gluconate, salicin, formate, acetate, propionate, hexanoate, caprylate, nonanionate, caprate, succinate, adipic acid, tartarate, dl-3-hydroxybutyrate, 6-hydroxycaproate, lactate, dl-glyceric acid, poly-β-hydroxybutyrate, pyruvate, erythritol, sorbitol, inositol, ethanol, benzoate and phenylacetic-acid.

OTU's in the ADH positive phena E and F, respectively containing the type cultures for *V.orientalis* UQM 2921 and *V.tubiashii* UQM 2923, all hydrolyzed xanthine. Other differential characters uniformly positive in both phena included, weak indole, hydrolysis casein, gelatin, starch, SDS and horse serum growth 6 % sodium chloride, dichlorophene and d-ribose. Characters negative in both phena were: Indole production, urea hydrolysis, aesculin hydrolysis, arylsulphatase, growth 10 % sodium chloride, tetracycline, brilliant green, EDDA, lysine, l-citrulline, γ-amino-butyrate, δ-amino-valerate, norvaline, sarcosine, d-glucosamine, xylose, l-arabinose,

Table 3.14.1: **Attribute Percentages for *Vibrionaceae* Assayed in Batch [6.] by ALJ.**

Phenon:	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)	(I)
Genus:	*V.*	*V.*	*V.*	*V.*	*V.*	*V.*	*V.*	*V.*	*V.*
Species:	*vuln.*	*aest.*	*angu.*	*chol.*	*orie.*	*tubi.*	*algi.*	*sp.*	*carc.*
Number of OTU's:	n=5	n=2	n=2	n=1	n=2	n=4	n=1	N=2	N=8
Attribute.									
Colony diameter > 3 mm.	40/ 5	50/ 2	0/ 2	0/ 1	0/ 2	50/ 4	0/ 1	0/ 2	75/ 8
Colony mucoid.	0/ 5	0/ 2	0/ 2	0/ 1	50/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Colony opaque.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Colony entire.	80/ 5	100/ 2	100/ 2	100/ 1	100/ 2	100/ 4	100/ 1	100/ 2	100/ 8
Colony convex.	100/ 5	100/ 2	100/ 2	100/ 1	100/ 2	100/ 4	100/ 1	100/ 2	100/ 8
Swarming growth.	0/ 5	0/ 2	0/ 2	0/ 1	100/ 2	75/ 4	100/ 1	0/ 2	62/ 8
Luminous growth.	20/ 5	0/ 2	0/ 2	0/ 1	50/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Brown pigment.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Black pigment.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Yellow/orange.	0/ 2	ND/ 0	ND/ 0	ND/ 0	ND/ 0	ND/ 0	ND/ 0	ND/ 0	0/ 2
Broth growth mod. to heavy.	40/ 5	100/ 2	100/ 2	0/ 1	100/ 2	100/ 4	100/ 1	100/ 2	75/ 8
Pellicle.	0/ 5	0/ 2	100/ 2	0/ 1	0/ 2	75/ 4	100/ 1	0/ 2	25/ 8
Indole.	60/ 5	50/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	50/ 2	87/ 8
Weak indole.	80/ 5	50/ 2	100/ 2	100/ 1	100/ 2	100/ 4	100/ 1	100/ 2	100/ 8
5 day pH > 7.05.	0/ 4	0/ 1	100/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	14/ 7
5 day pH > 5.15.	25/ 4	0/ 1	100/ 2	0/ 1	50/ 2	0/ 4	100/ 1	0/ 2	14/ 7
Acetoin production.	0/ 5	0/ 2	100/ 2	0/ 1	0/ 2	0/ 4	100/ 1	0/ 2	0/ 8
Nitrite reduction.	100/ 5	0/ 2	50/ 2	0/ 1	100/ 2	100/ 4	100/ 1	100/ 2	100/ 8
Cholera-Red.	0/ 5	100/ 2	0/ 2	0/ 1	0/ 2	25/ 4	0/ 1	0/ 2	12/ 8
Gluconate oxidation.	20/ 5	0/ 2	100/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Arginine dihydrolase.	0/ 5	100/ 2	50/ 2	0/ 1	100/ 2	100/ 4	0/ 1	0/ 2	0/ 8
Lysine decarboxylase.	100/ 5	0/ 2	50/ 2	100/ 1	50/ 2	0/ 4	100/ 1	0/ 2	100/ 8
Ornithine decarboxylase.	100/ 5	0/ 2	50/ 2	100/ 1	0/ 2	0/ 4	0/ 1	0/ 2	100/ 8
Glucose fermentation.	100/ 5	100/ 2	100/ 2	100/ 1	100/ 2	100/ 4	100/ 1	100/ 2	100/ 8
Gas from glucose.	0/ 5	0/ 2	50/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
ONPG hydrolysis.	80/ 5	100/ 2	100/ 2	100/ 1	0/ 2	100/ 4	0/ 1	100/ 2	37/ 8
Urea hydrolysis.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	87/ 8
Aesculin hydrolysis.	40/ 5	0/ 2	50/ 2	0/ 1	0/ 2	0/ 4	0/ 1	100/ 2	62/ 8
Weak aesculin hydrolysis.	80/ 5	100/ 2	50/ 2	0/ 1	50/ 2	100/ 4	0/ 1	100/ 2	100/ 8
Agar hydrolysis.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Egg Albumen hydrolysis.	60/ 5	100/ 2	0/ 2	100/ 1	0/ 2	75/ 4	100/ 1	100/ 2	75/ 8
Arbutin hydrolysis.	80/ 5	0/ 2	0/ 2	0/ 1	0/ 2	75/ 4	0/ 1	100/ 2	75/ 8
Aryl-sulphatase.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	100/ 2	25/ 8
Casein hydrolysis.	100/ 5	100/ 2	100/ 2	100/ 1	100/ 2	100/ 4	100/ 1	0/ 2	100/ 8
Chitin hydrolysis.	100/ 5	100/ 2	100/ 2	100/ 1	100/ 2	75/ 4	100/ 1	0/ 2	100/ 8
DNA hydrolysis.	40/ 5	0/ 2	100/ 2	100/ 1	0/ 2	75/ 4	100/ 1	0/ 2	87/ 8
Gelatin hydrolysis.	100/ 5	100/ 2	100/ 2	100/ 1	100/ 2	100/ 4	100/ 1	100/ 2	100/ 8
Lecithinase.	100/ 5	100/ 1	50/ 2	100/ 1	50/ 2	100/ 2	100/ 1	100/ 2	100/ 3
Esterase (SDS).	100/ 5	50/ 2	50/ 2	100/ 1	100/ 2	100/ 4	100/ 1	100/ 2	100/ 8
Starch hydrolysis.	100/ 5	100/ 2	100/ 2	100/ 1	100/ 2	100/ 4	100/ 1	100/ 2	100/ 8
Sulphide production.	0/ 5	0/ 2	0/ 2	0/ 1	50/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Serum hydrolysis.	100/ 5	100/ 2	100/ 2	100/ 1	100/ 2	100/ 4	100/ 1	100/ 2	100/ 8
Tyrosine hydrolysis.	0/ 5	0/ 2	0/ 2	0/ 1	100/ 2	50/ 4	100/ 1	0/ 2	25/ 8
Tyrosine pigment.	0/ 5	0/ 2	0/ 2	0/ 1	100/ 2	25/ 4	100/ 1	0/ 2	12/ 8
Xanthine hydrolysis.	0/ 5	0/ 2	0/ 2	0/ 1	100/ 2	100/ 4	0/ 1	0/ 2	0/ 8
Many Sudanophilic Inclusions.	25/ 4	50/ 2	0/ 2	0/ 1	0/ 2	75/ 4	0/ 1	0/ 2	66/ 6
Growth 4°C.	0/ 5	100/ 2	50/ 2	0/ 1	50/ 2	0/ 4	0/ 1	100/ 2	0/ 8
Growth 37°C.	100/ 5	50/ 2	100/ 2	100/ 1	50/ 2	100/ 4	100/ 1	100/ 2	100/ 8
Growth 42°C.	ND/ 0	0/ 2	0/ 1	ND/ 0	0/ 2	0/ 3	ND/ 0	ND/ 0	0/ 5
Growth 0 % NaCl.	40/ 5	0/ 2	100/ 2	100/ 1	0/ 2	0/ 4	0/ 1	100/ 2	12/ 8
Growth 6.0 % NaCl.	100/ 5	100/ 2	100/ 2	100/ 1	100/ 2	100/ 4	100/ 1	100/ 2	100/ 8
Growth 10 % NaCl.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	12/ 8
Growth pH 4.5.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Growth Novobiocin.	0/ 5	0/ 2	50/ 2	0/ 1	0/ 2	25/ 4	100/ 1	100/ 2	0/ 8
Growth Polymyxin.	100/ 5	50/ 2	50/ 2	0/ 1	50/ 2	0/ 4	100/ 1	0/ 2	100/ 8

Table 3.14.1; (Cont.): **Attribute Percentages for *Vibrionaceae* Assayed in Batch [6.] by ALJ.**

Phenon:	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)	(I)
Genus:	*V.*	*V.*	*V.*	*V.*	*V.*	*V.*	*V.*	*V.*	*V.*
Species:	*vuln.*	*aest.*	*angu.*	*chol.*	*orie.*	*tubi.*	*algi.*	*sp.*	*carc.*
Number of OTU's:	n=5	n=2	n=2	n=1	n=2	n=4	n=1	n=2	n=8
Attribute.									
Growth Sulphamethoxazole.	100/ 5	100/ 2	100/ 2	100/ 1	100/ 2	100/ 4	100/ 1	100/ 2	100/ 8
Growth Tetracycline.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Growth Trimethoprim.	80/ 5	50/ 2	50/ 2	0/ 1	0/ 2	50/ 4	100/ 1	0/ 2	100/ 8
No growth 10 µg/ml 0/129.	80/ 5	100/ 2	100/ 2	100/ 1	100/ 2	75/ 4	0/ 1	0/ 2	37/ 8
No growth 150µg/ml 0/129.	100/ 5	100/ 2	100/ 2	100/ 1	50/ 2	75/ 4	0/ 1	100/ 2	87/ 8
Growth Brilliantgreen.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Growth Dichlorophene.	80/ 5	100/ 2	50/ 2	100/ 1	100/ 2	100/ 4	100/ 1	100/ 2	100/ 8
Growth EDDA.	0/ 5	0/ 2	50/ 2	100/ 1	0/ 2	0/ 4	0/ 1	0/ 2	25/ 8
Growth Haloquinol.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Growth 8-Hydroxyquinoline	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Growth Methylviolet.	0/ 5	100/ 2	100/ 2	100/ 1	0/ 2	50/ 4	100/ 1	50/ 2	100/ 8
Growth Pyronin-Y.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	25/ 4	100/ 1	0/ 2	100/ 8
Growth 0.2% SDS.	100/ 5	100/ 2	100/ 2	100/ 1	100/ 2	100/ 4	100/ 1	100/ 2	100/ 8
Growth Thionine.	20/ 5	50/ 2	0/ 2	100/ 1	0/ 2	25/ 4	100/ 1	0/ 2	75/ 8
Yellowon Thionine.	0/ 1	0/ 1	ND/ 0	0/ 1	ND/ 0	50/ 2	100/ 1	ND/ 0	85/ 7
Growth T.T.C.	100/ 5	100/ 2	100/ 2	100/ 1	100/ 2	100/ 4	100/ 1	100/ 2	100/ 8
Growth Glycine.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 1	25/ 4	0/ 1	0/ 2	50/ 8
Growth l-α-Alanine.	80/ 5	50/ 2	0/ 2	0/ 1	0/ 2	100/ 4	100/ 1	50/ 2	100/ 8
Growth d-α-Alanine.	0/ 4	0/ 2	0/ 2	ND/ 0	50/ 2	100/ 4	100/ 1	ND/ 0	100/ 8
Growth Serine.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	100/ 4	100/ 1	0/ 2	75/ 8
Growth l-Leucine.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	100/ 1	0/ 2	25/ 8
Growth Valine.	20/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Growth l+Glutamic acid.	80/ 5	50/ 2	50/ 2	100/ 1	100/ 1	100/ 4	100/ 1	100/ 2	100/ 8
Growth l-Lysine.	20/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	12/ 8
Growth l-Arginine.	20/ 5	50/ 2	0/ 2	100/ 1	0/ 2	25/ 4	0/ 1	100/ 2	37/ 8
Growth l-Ornithine.	0/ 5	0/ 2	0/ 2	0/ 1	100/ 1	75/ 4	0/ 1	100/ 2	12/ 8
Growth l-Citrulline.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	37/ 8
Growth γ-Amino-butyrate.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Growth δ-Amino-valerate.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	12/ 8
Growth Norvaline.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	12/ 8
Growth l-Proline.	60/ 5	50/ 2	0/ 2	0/ 1	0/ 2	100/ 4	100/ 1	100/ 2	100/ 8
Growth Putrescine.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	75/ 4	0/ 1	100/ 2	0/ 8
Growth Sarcosine.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Growth l-Glutamine.	60/ 5	50/ 2	50/ 2	0/ 1	0/ 1	75/ 4	0/ 1	0/ 2	100/ 8
Growth d-Glucosamine.	20/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	100/ 2	25/ 8
Growth n-Acetylglucosamine.	60/ 5	0/ 2	0/ 2	0/ 1	0/ 2	75/ 4	0/ 1	100/ 2	100/ 8
Growth d-Ribose.	100/ 5	100/ 2	50/ 2	100/ 1	100/ 2	100/ 4	100/ 1	50/ 2	100/ 8
Growth Xylose.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 1	0/ 4	0/ 1	100/ 2	0/ 8
Growth l-Arabinose.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	100/ 1	25/ 8
Growth l-Rhamnose.	0/ 4	0/ 2	0/ 1	0/ 1	0/ 2	0/ 3	0/ 1	0/ 1	12/ 8
Growth Glucose.	20/ 5	50/ 2	0/ 2	0/ 1	0/ 2	25/ 4	100/ 1	ND/ 0	62/ 8
Growth d-Mannose.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 1	25/ 4	0/ 1	0/ 2	0/ 8
Growth d-Galactose.	0/ 4	0/ 1	0/ 2	0/ 1	0/ 1	33/ 3	0/ 1	0/ 2	75/ 8
Growth Sucrose.	0/ 5	50/ 2	100/ 2	100/ 1	0/ 2	75/ 4	100/ 1	ND/ 0	37/ 8
Growth Trehalose.	40/ 5	0/ 2	100/ 2	0/ 1	0/ 1	75/ 4	0/ 1	50/ 2	37/ 8
Growth Maltose.	100/ 5	50/ 2	100/ 2	100/ 1	50/ 2	100/ 4	100/ 1	100/ 2	100/ 8
Growth Cellobiose.	80/ 5	0/ 2	50/ 2	0/ 1	0/ 1	100/ 4	100/ 1	100/ 2	100/ 8
Growth Lactose.	20/ 5	50/ 2	0/ 2	ND/ 0	0/ 2	25/ 4	0/ 1	50/ 2	0/ 8
Growth Gluconate.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 1	25/ 4	0/ 1	100/ 2	100/ 8
Growth Glucuronic acid.	80/ 5	50/ 2	0/ 2	0/ 1	0/ 1	75/ 4	0/ 1	100/ 2	100/ 8
Growth Salicin.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	75/ 4	0/ 1	100/ 2	25/ 8
Growth Starch.	66/ 3	100/ 1	0/ 1	ND/ 0	ND/ 0	33/ 3	ND/ 0	0/ 2	100/ 6
Growth Pullulan.	100/ 5	100/ 2	50/ 2	100/ 1	50/ 2	50/ 4	100/ 1	0/ 2	100/ 8
Growth Formate.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Growth Acetate.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	12/ 8

Table 3.14.1; (Cont.): **Attribute Percentages for *Vibrionaceae* Assayed in Batch [6.] by ALJ.**

Phenon:	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)	(I)
Genus:	*V.*	*V.*	*V.*	*V.*	*V.*	*V.*	*V.*	*V.*	*V.*
Species:	*vuln.*	*aest.*	*angu.*	*chol.*	*orie.*	*tubi.*	*algi.*	*sp.*	*carc.*
Number of OTU's:	n=5	n=2	n=2	n=1	n=2	n=4	n=1	n=2	n=8
Attribute.									
Growth Propionate.	40/ 5	0/ 2	50/ 2	0/ 1	0/ 2	25/ 4	100/ 1	100/ 2	50/ 8
Growth Hexanoate.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Growth Heptanoate.	20/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	100/ 1	0/ 2	37/ 8
Growth Caprylic acid.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Growth Nonanionic acid.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Growth Caprate.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Growth Succinate.	80/ 5	100/ 2	0/ 2	0/ 1	50/ 2	0/ 4	0/ 1	0/ 2	100/ 8
Growth Adipic acid.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	12/ 8
Growth Malate.	80/ 5	50/ 2	50/ 2	100/ 1	0/ 2	75/ 4	100/ 1	100/ 2	100/ 8
Growth Tartarate.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Growth dl-3-OH-butyrate.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
Growth 6-Hydroxy Caproate.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	ND/ 0	12/ 8
Growth Lactate.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	ND/ 0	62/ 8
Growth dl-Glyceric acid.	40/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	100/ 2	37/ 8
Growth Poly-β-OH-butyrate.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	12/ 8
GrowthCitrate.	100/ 5	100/ 2	100/ 2	100/ 1	0/ 2	75/ 4	100/ 1	0/ 2	100/ 8
Growthα-Ketoglutarate.	20/ 5	50/ 2	0/ 2	0/ 1	0/ 1	50/ 4	0/ 1	ND/ 0	75/ 8
GrowthPyruvate.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	100/ 8
GrowthErythritol.	0/ 3	0/ 1	0/ 1	0/ 1	0/ 1	0/ 2	0/ 1	0/ 2	100/ 1
GrowthSorbitol.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	ND/ 0	25/ 8
GrowthInositol.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8
GrowthEthanol.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 1	0/ 4	0/ 1	0/ 2	25/ 8
GrowthBenzoate.	20/ 5	0/ 2	0/ 2	0/ 1	50/ 2	0/ 4	100/ 1	0/ 2	0/ 8
GrowthPhenylaceticacid.	0/ 5	0/ 2	0/ 2	0/ 1	0/ 2	0/ 4	0/ 1	0/ 2	0/ 8

hexanoate, caprylate, nonanionate, caprate, adipic acid, tartarate, dl-3-hydroxybutyrate, 6-hydroxycaproate, lactate, dl-glyceric acid, PHB, pyruvate, sorbitol, inositol, ethanol and phenylacetic acid.

Apart from luminescence for which only the type culture of *V.orientalis* was positive, and production of LDC by one OTU, strains in phenon F were less versatile than those from *V.tubiashii*. Characters positive for all OTU's in phenon F, but negative in phenon E were for ONPG hydrolysis, growth l-α-alanine, l-serine, l-proline.

The group *Unassociated 1,* Phenon K, was gathered with an overall similarity, ($S_{j.}$), of 85 %, and comprised 15 OTU's, all provincial strains. The phenon most closely resembled *V.fluvialis* as amended by Brenner *et al.,* (1983). The replicated and representative OTU from this phenon, Stn 2440 was deposited as UQM 3240.

Table 3.14.2: Attribute Percentages for *Vibrionaceae* Assayed in Batch [6.] by ALJ.

Phenon:	(J)	(K)	(L)	(M)	(N)	(O)	(P)	(Q)
Genus	*V.*	*V.*	*A.*	*V.*	*V.*	*V.*	*Shew*	*V.*
Species:	*para.*	*fluv.*	*hydr.*	*natr.*	*sp.*	*sp.*	*sp.*	*nereis*
Number of OTU's.	n=33	n=15	n=2	n=2	n=4	n=3	n=4	n=1
Attribute.								
Colony diameter > 3 mm.	87/33	6/15	0/ 2	0/ 2	0/ 4	33/ 3	50/ 4	100/ 1
Colony mucoid.	6/33	0/15	0/ 2	0/ 2	0/ 4	0/ 3	0/ 4	0/ 1
Colony opaque.	3/33	0/15	0/ 2	0/ 2	0/ 4	0/ 3	0/ 4	0/ 1
Colony entire.	66/33	100/15	100/ 2	50/ 2	100/ 4	66/ 3	75/ 4	100/ 1
Colony convex.	60/33	100/15	100/ 2	50/ 2	100/ 4	100/ 3	100/ 4	100/ 1
Swarming growth.	84/33	73/15	0/ 2	100/ 2	0/ 4	0/ 3	25/ 4	100/ 1
Luminous growth.	0/33	0/15	0/ 2	0/ 2	0/ 4	0/ 3	0/ 4	0/ 1
Brown pigment.	0/33	0/15	0/ 2	0/ 2	0/ 4	0/ 3	0/ 4	0/ 1
Black pigment.	0/33	0/15	0/ 2	0/ 2	0/ 4	0/ 3	0/ 4	0/ 1
Yellow/orange.	0/ 6	0/ 1	ND/ 0	ND/ 0	0/ 3	0/ 2	ND/ 0	ND/ 0
Broth growth mod. to heavy.	93/33	100/15	100/ 2	100/ 2	100/ 4	66/ 3	100/ 4	100/ 1
Pellicle.	72/29	0/15	0/ 2	0/ 2	0/ 4	0/ 3	100/ 4	0/ 1
Indole.	12/33	13/15	0/ 2	0/ 2	0/ 4	100/ 3	0/ 4	0/ 1
Weak indole.	100/33	93/15	100/ 2	0/ 2	25/ 4	100/ 3	0/ 4	100/ 1
5 day pH > 7.05.	24/29	73/15	0/ 2	0/ 2	75/ 4	0/ 3	75/ 4	0/ 1
5 day pH > 5.15.	24/29	80/15	0/ 2	50/ 2	75/ 4	0/ 3	100/ 4	100/ 1
Acetoin production.	20/29	6/15	0/ 2	0/ 2	100/ 4	0/ 3	0/ 4	0/ 1
Nitrite reduction.	100/33	100/15	100/ 2	100/ 2	100/ 4	100/ 3	100/ 4	0/ 1
Cholera-Red.	3/33	6/15	0/ 2	50/ 2	25/ 4	0/ 3	0/ 4	0/ 1
Gluconate oxidation.	27/33	0/15	0/ 2	0/ 2	100/ 4	33/ 3	0/ 4	0/ 1
Arginine dihydrolase.	0/33	100/15	100/ 2	0/ 2	25/ 4	66/ 3	0/ 4	100/ 1
Lysine decarboxylase.	100/33	0/15	0/ 2	0/ 2	100/ 4	33/ 3	100/ 2	0/ 1
Ornithine decarboxylase.	84/33	13/15	0/ 2	0/ 2	100/ 4	33/ 3	100/ 2	0/ 1
Glucose fermentation.	100/33	100/15	100/ 2	100/ 2	100/ 4	100/ 3	25/ 4	100/ 1
Gas from glucose.	0/33	0/15	0/ 2	0/ 2	25/ 4	66/ 3	0/ 4	0/ 1
ONPG hydrolysis.	66/33	100/15	100/ 2	0/ 2	100/ 4	100/ 3	0/ 4	0/ 1
Urea hydrolysis.	0/33	0/15	0/ 2	0/ 2	0/ 4	0/ 3	0/ 4	0/ 1
Aesculin hydrolysis.	9/33	100/15	100/ 2	100/ 2	100/ 4	33/ 3	25/ 4	100/ 1
Weak aesculin hydrolysis.	48/33	100/15	100/ 2	100/ 2	100/ 4	33/ 3	25/ 4	100/ 1
Agar hydrolysis.	0/33	0/15	0/ 2	0/ 2	0/ 4	0/ 3	0/ 4	0/ 1
Egg Albumen hydrolysis.	78/33	60/15	0/ 2	100/ 2	100/ 4	100/ 3	75/ 4	100/ 1
Arbutin hydrolysis.	0/33	100/15	100/ 2	100/ 2	100/ 4	66/ 3	0/ 4	0/ 1
Aryl-sulphatase.	12/33	0/15	0/ 2	0/ 2	0/ 4	0/ 3	50/ 4	0/ 1
Casein hydrolysis.	100/33	100/15	100/ 2	100/ 2	100/ 4	66/ 3	100/ 4	0/ 1
Chitin hydrolysis.	100/33	100/15	100/ 2	50/ 2	100/ 4	66/ 3	75/ 4	100/ 1
DNA hydrolysis.	96/33	100/15	100/ 2	100/ 2	100/ 4	66/ 3	50/ 4	0/ 1
Gelatin hydrolysis.	100/33	86/15	100/ 2	100/ 2	100/ 4	100/ 3	100/ 4	100/ 1
Lecithinase.	100/28	100/15	100/ 2	50/ 2	100/ 4	100/ 3	100/ 3	100/ 1
Esterase (SDS).	84/33	100/15	50/ 2	100/ 2	50/ 4	66/ 3	66/ 3	ND/ 0
Starch hydrolysis.	96/33	100/15	100/ 2	0/ 2	50/ 4	33/ 3	0/ 4	0/ 1
Sulphide production.	3/33	0/15	0/ 2	0/ 2	0/ 4	0/ 3	50/ 4	100/ 1
Serum hydrolysis.	96/33	100/15	100/ 2	100/ 2	50/ 4	100/ 3	100/ 4	100/ 1
Tyrosine hydrolysis.	75/33	100/15	0/ 2	100/ 2	100/ 4	66/ 3	50/ 4	100/ 1
Tyrosine pigment.	36/33	0/15	0/ 2	100/ 2	50/ 4	0/ 3	25/ 4	100/ 1
Xanthine hydrolysis.	0/33	0/15	0/ 2	0/ 2	0/ 4	0/ 3	0/ 4	0/ 1
Many Sudanophilic Inclusions.	30/33	50/14	50/ 2	0/ 2	0/ 4	0/ 3	0/ 4	100/ 1
Growth 4°C.	24/33	0/15	0/ 2	50/ 2	100/ 4	100/ 3	75/ 4	0/ 1
Growth 37°C.	100/33	100/15	100/ 2	100/ 2	100/ 4	100/ 3	100/ 4	100/ 1
Growth 42°C.	ND/ 0	ND/ 0	ND/ 0	ND/ 0	ND/ 0	ND/ 0	0/ 1	ND/ 0
Growth 0 % NaCl.	6/33	93/15	50/ 2	0/ 2	100/ 4	100/ 3	100/ 4	0/ 1
Growth 6.0 % NaCl.	100/33	100/15	100/ 2	100/ 2	75/ 4	66/ 3	100/ 4	100/ 1
Growth 10 % NaCl.	81/33	53/15	0/ 2	100/ 2	0/ 4	33/ 3	25/ 4	100/ 1
Growth pH 4.5.	3/33	0/15	100/ 2	0/ 2	75/ 4	33/ 3	25/ 4	0/ 1
Growth Novobiocin.	6/33	100/15	50/ 2	100/ 2	75/ 4	100/ 3	100/ 4	0/ 1
Growth Polymyxin.	78/33	40/15	100/ 2	0/ 2	100/ 4	0/ 3	50/ 4	0/ 1

Table 3.14.2; (Cont.): Attribute Percentages for *Vibrionaceae* Assayed in Batch [6.] by ALJ.

Phenon:	(J)	(K)	(L)	(M)	(N)	(O)	(P)	(Q)
Genus	*V.*	*V.*	*A.*	*V.*	*V.*	*V.*	*Shew*	*V.*
Species:	*para.*	*fluv.*	*hydr.*	*natr.*	*sp.*	*sp.*	*sp.*	*nereis*
Number of OTU's.	n=33	n=15	n=2	n=2	n=4	n=3	n=4	n=1
Attribute.								
Growth Tetracycline.	0/33	0/15	100/ 2	0/ 2	25/ 4	0/ 3	25/ 4	0/ 1
Growth Trimethoprim.	84/33	93/15	100/ 2	100/ 2	100/ 4	100/ 3	100/ 4	100/ 1
No growth 10 µg/ml	0/129.	6/33	20/15	0/ 2	0/ 2	0/ 4	0/ 3	0/ 4	100/ 1
No growth 150µg/ml	0/129.	100/33	100/15	0/ 2	100/ 2	25/ 4	66/ 3	75/ 4	100/ 1
Growth Brilliantgreen.		21/33	86/15	100/ 1	0/ 2	100/ 4	33/ 3	0/ 4	0/ 1
Growth Dichlorophene.		100/33	100/15	100/ 2	100/ 2	100/ 4	100/ 3	100/ 4	100/ 1
Growth EDDA.		0/33	100/15	100/ 2	0/ 2	100/ 4	100/ 3	50/ 4	0/ 1
Growth Haloquinol.		0/33	0/15	100/ 2	0/ 2	100/ 4	0/ 3	0/ 4	0/ 1
Growth 8-Hydroxyquinoline		0/33	0/15	100/ 2	0/ 2	100/ 4	33/ 3	0/ 4	0/ 1
Growth Methylviolet.		100/33	100/15	100/ 2	100/ 2	100/ 4	100/ 3	100/ 4	0/ 1
Growth Pyronin-Y.		100/33	26/15	100/ 2	0/ 2	100/ 4	100/ 3	75/ 4	100/ 1
Growth 0.2% SDS.		100/33	100/15	100/ 2	100/ 2	100/ 4	100/ 3	75/ 4	0/ 1
Growth Thionine.		100/33	100/15	100/ 2	50/ 2	100/ 4	100/ 3	75/ 4	0/ 1
Yellowon Thionine.		100/33	100/15	100/ 2	0/ 1	0/ 4	33/ 3	0/ 3	ND/ 0
Growth T.T.C.		100/33	100/15	100/ 2	100/ 2	100/ 4	100/ 3	100/ 4	100/ 1
Growth Glycine.		96/33	100/15	100/ 2	100/ 2	75/ 4	0/ 3	0/ 4	100/ 1
Growth l-α-Alanine.		100/33	100/15	100/ 2	100/ 2	100/ 4	100/ 3	75/ 4	100/ 1
Growth d-α-Alanine.		100/33	100/15	100/ 2	100/ 2	100/ 4	100/ 3	0/ 2	100/ 1
Growth Serine.		100/33	73/15	100/ 2	100/ 2	100/ 4	100/ 3	50/ 4	100/ 1
Growth l-Leucine.		96/33	0/15	0/ 2	100/ 2	25/ 4	0/ 3	100/ 4	100/ 1
Growth Valine.		18/33	6/15	0/ 2	50/ 2	0/ 4	0/ 3	0/ 4	0/ 1
Growth l+Glutamic acid.		100/33	100/15	100/ 2	100/ 2	100/ 4	100/ 3	0/ 2	100/ 1
Growth l-Lysine.		15/33	0/15	50/ 2	100/ 2	25/ 4	0/ 3	0/ 4	100/ 1
Growth l-Arginine.		100/33	100/15	100/ 2	100/ 2	25/ 4	33/ 3	0/ 2	100/ 1
Growth l-Ornithine.		72/33	100/15	100/ 2	100/ 2	75/ 4	0/ 3	0/ 2	100/ 1
Growth l-Citrulline.		81/32	66/15	50/ 2	100/ 2	50/ 4	0/ 3	0/ 2	0/ 1
Growth γ-Amino-butyrate.		21/33	0/15	0/ 2	50/ 2	0/ 4	0/ 3	0/ 4	100/ 1
Growth δ-Amino-valerate.		33/33	73/15	0/ 2	0/ 2	50/ 4	0/ 3	25/ 4	0/ 1
Growth Norvaline.		6/33	0/15	0/ 2	50/ 2	0/ 4	0/ 3	0/ 4	0/ 1
Growth l-Proline.		96/33	100/15	100/ 2	100/ 2	100/ 4	100/ 3	0/ 3	100/ 1
Growth Putrescine.		90/33	100/15	100/ 2	100/ 2	100/ 4	0/ 3	100/ 4	100/ 1
Growth Sarcosine.		18/33	0/15	0/ 2	0/ 2	0/ 4	0/ 3	0/ 4	0/ 1
Growth l-Glutamine.		39/33	80/15	50/ 2	0/ 2	25/ 4	66/ 3	33/ 3	0/ 1
Growth d-Glucosamine.		100/33	80/15	100/ 2	100/ 2	100/ 4	66/ 3	0/ 3	0/ 1
Growth n-Acetylglucosamine.		100/33	100/15	100/ 2	100/ 2	100/ 4	100/ 3	75/ 4	0/ 1
Growth d-Ribose.		93/30	100/15	50/ 2	50/ 2	100/ 4	100/ 3	100/ 2	100/ 1
Growth Xylose.		12/33	13/15	0/ 2	100/ 2	0/ 4	50/ 2	0/ 4	0/ 1
Growth l-Arabinose.		57/33	13/15	100/ 2	100/ 2	50/ 4	33/ 3	50/ 4	0/ 1
Growth l-Rhamnose.		33/27	0/ 8	0/ 2	100/ 1	33/ 3	33/ 3	0/ 4	0/ 1
Growth Glucose.		93/33	100/13	100/ 2	100/ 2	100/ 4	100/ 3	100/ 4	0/ 1
Growth d-Mannose.		71/32	100/15	100/ 2	0/ 2	75/ 4	66/ 3	0/ 3	0/ 1
Growth d-Galactose.		87/32	100/15	100/ 2	0/ 2	100/ 4	100/ 3	0/ 2	0/ 1
Growth Sucrose.		46/30	100/15	100/ 2	100/ 2	75/ 4	66/ 3	66/ 3	100/ 1
Growth Trehalose.		93/33	93/15	100/ 2	50/ 2	75/ 4	66/ 3	75/ 4	100/ 1
Growth Maltose.		100/33	100/15	100/ 2	100/ 2	100/ 4	100/ 3	75/ 4	100/ 1
Growth Cellobiose.		54/33	93/15	0/ 2	100/ 2	0/ 4	100/ 3	0/ 4	0/ 1
Growth Lactose.		26/30	6/15	0/ 2	100/ 2	0/ 4	66/ 3	0/ 2	100/ 1
Growth Gluconate.		96/33	100/15	100/ 2	100/ 2	100/ 4	100/ 3	0/ 2	100/ 1
Growth Glucuronic acid.		36/33	93/15	100/ 2	50/ 2	75/ 4	33/ 3	50/ 4	0/ 1
Growth Salicin.		24/33	100/15	100/ 2	100/ 2	75/ 4	0/ 3	0/ 4	0/ 1
Growth Starch.		100/33	100/15	100/ 2	0/ 1	0/ 4	66/ 3	0/ 4	0/ 1
Growth Pullulan.		100/33	100/15	100/ 2	100/ 2	100/ 4	66/ 3	0/ 4	100/ 1
Growth Formate.		37/32	100/15	100/ 2	0/ 1	0/ 4	0/ 3	0/ 3	0/ 1
Growth Acetate.		96/33	100/15	100/ 2	100/ 2	0/ 4	33/ 3	25/ 4	0/ 1
Growth Propionate.		100/33	100/15	100/ 2	100/ 2	100/ 4	0/ 3	100/ 4	100/ 1

Table 3.14.2; (Cont.): **Attribute Percentages of *Vibrionaceae* Assayed in Batch [6.] by ALJ.**

Genus	(J) *V.*	(K) *V.*	(L) *A.*	(M) *V.*	(N) *V.*	(O) *V.*	(P) *Shew*	(Q) *V.*
Species:	*para.*	*fluv.*	*hydr.*	*natr.*	*sp.*	*sp.*	*sp.*	*nereis*
Number of OTU's.	N=33	N=15	N=2	N=2	N=4	N=3	N=4	N=1
Attribute.								
Growth Hexanoate.	0/33	6/15	0/ 2	0/ 2	0/ 4	0/ 3	0/ 3	0/ 1
Growth Heptanoate.	48/33	60/15	100/ 2	100/ 2	75/ 4	33/ 3	75/ 4	100/ 1
Growth Caprylic acid.	0/33	0/15	100/ 2	0/ 2	75/ 4	33/ 3	0/ 4	0/ 1
Growth Nonanionic acid.	0/33	0/15	100/ 2	0/ 2	50/ 4	0/ 3	0/ 4	0/ 1
Growth Caprate.	0/33	0/15	100/ 2	50/ 2	100/ 4	0/ 3	0/ 4	0/ 1
Growth Succinate.	100/33	86/15	50/ 2	100/ 2	100/ 4	100/ 3	75/ 4	100/ 1
Growth Adipic acid.	3/33	0/15	100/ 2	0/ 2	0/ 4	0/ 3	0/ 4	0/ 1
Growth Malate.	96/33	100/15	100/ 2	100/ 2	75/ 4	100/ 3	100/ 4	0/ 1
Growth Tartarate.	30/33	0/15	0/ 2	0/ 2	0/ 4	0/ 3	0/ 4	0/ 1
Growth dl-3-OH-butyrate.	0/33	0/15	0/ 2	50/ 2	0/ 4	0/ 3	0/ 4	0/ 1
Growth 6-Hydroxy Caproate.	12/33	0/15	0/ 2	0/ 2	0/ 4	0/ 3	0/ 3	0/ 1
Growth Lactate.	60/33	93/15	100/ 2	50/ 2	75/ 4	50/ 2	100/ 2	0/ 1
Growth dl-Glyceric acid.	93/33	100/15	100/ 2	100/ 2	75/ 4	66/ 3	100/ 4	0/ 1
Growth Poly-β-OH-butyrate.	33/33	40/15	50/ 2	100/ 2	75/ 4	33/ 3	0/ 4	0/ 1
Growth Citrate.	100/33	100/15	100/ 2	100/ 2	100/ 4	33/ 3	0/ 4	100/ 1
Growth α-Ketoglutarate.	93/33	100/15	100/ 2	50/ 2	75/ 4	0/ 3	100/ 4	0/ 1
Growth Pyruvate.	100/33	93/15	100/ 2	50/ 2	100/ 4	100/ 3	100/ 4	0/ 1
Growth Erythritol.	5/20	0/ 3	ND/ 0	50/ 2	0/ 3	0/ 1	0/ 2	0/ 1
Growth Sorbitol.	27/33	6/15	100/ 2	50/ 2	100/ 4	0/ 3	0/ 4	0/ 1
Growth Inositol.	12/33	0/15	0/ 2	0/ 2	50/ 4	0/ 3	0/ 4	100/ 1
Growth Ethanol.	48/33	6/15	0/ 2	0/ 2	25/ 4	0/ 3	0/ 4	0/ 1
Growth Benzoate.	3/33	0/15	0/ 2	0/ 2	0/ 4	0/ 3	0/ 4	0/ 1
Growth Phenylacetic acid.	3/33	0/15	0/ 2	0/ 2	75/ 4	0/ 3	0/ 4	0/ 1

This phenon could be differentiated by characters found to be greater than 90 % positive for constituent OTU's; weak indole, nitrate reduction, ONPG, aesculin, arbutin, casein, chitin, DNA, horse serum and tyrosine hydrolysis, susceptibility to 150 μg/ml 0/129 phosphate, growth on 0 and 6 % sodium chloride, at pH 10, in the presence of novobiocin and EDDA and utilization of glycine, d and l-α-alanine, l-glutamic acid, l-arginine, l-ornithine, l-proline, n-acetylglucosamine, d-ribose, glucose, mannose, d-galactose, sucrose, trehalose, maltose, cellobiose, gluconate, glucuronate, salicin, starch, pullulan, formate, acetate, propionate, malate, lactate, dl-glyceric acid, α-ketoglutarate and pyruvate. No OTU's in this phenon were measured for their utilization of dl-3-hydroxybutyrate, an attribute most commonly expressed by OTU's described by Lee *et al.*, 1981.

Phenon L, *(Unassociated 2)* comprised only the 90 % similar ($S_{j.}$) OTU's Stn 1960 and Stn 1990. Despite both OTU's being anaerogenic, they were

otherwise most like *A.hydrophila* as described by Popoff and Veron, (1976). These could be resolved from *V.fluvialis* (K) by: Growth at pH 4.5, in the presence 150 µg/ml 0/129, haloquinol, 8-hydroxyquinoline, separate utilization of leucine, and organic acid derivatives heptanoate, nonanionate, caprylate, caprate and adipate.

Phenon Q contained only the type culture for *V.nereis* UQM 2783, and was quite distinct from provincial OTU's. This OTU was not exceptional with respect to previous analyses (3.8.3) and so is not discussed further.

In phenon O, only the OTU's Stn 1280 and 2170 were ADH positive. These OTU's had their greatest affinity with the *V.fluvialis* group but could be separated from it according to reactions for; growth at 4°C., and in the presence of pyronin-Y, and the inability to separately utilize glycine, δ-aminovalerate, putrescine, salicin, acetate, propionate. One strain in this phenon additionally was able to grow in the presence of 150 µg/ml 0/129. Because these may represent new species the culture Stn 2170, was deposited as UQM 3371 and remaining OTU's remain stored under oil. Because only three OTU's are contained in phenon O tentative classification is as *V.fluvialis*-like until more, similar strains become available.

Phenon C containing only the type strain of *V.anguillarum,* UQM 2771 and the fish isolate Stn 2100 had similarities measured at 87 and 68 % for euclidean and Jaccard similarity coefficients respectively. The strain Stn 2100 showed variation in characters normally stable in *V.anguillarum* i.e. by gas production, absence of an ADH, and presence of ODC and LDC and consequently this atypical culture, remains stored under oil on SEHIA as an unidentified *Vibrio* sp.

3.8.4.2.3.2 Lysine and Ornithine Decarboxylating Phena.

Phenon A contained both type and reference cultures for *V.vulnificus* and three provincial OTU's, Stn 1380, 3010, and 7120. Differential characters positive for all OTU's were nitrite reduction, LDC, ODC, growth 50 i.u. polymyxin, no growth 150 µg/ml 0/129 phosphate, growth 0.2 % SDS, growth

ribose, maltose, pullulan and citrate. All OTU's in this phenon were negative for: 5 d pH > 7.05, production of pellicles, acetoin, and cholera-red, hydrolysis of urea, arylsulphate, tyrosine and xanthine, growth at 4°C., growth in 10 % sodium chloride, growth at pH 4.5, growth in the presence of novobiocin, tetracycline, basic fuchsin, EDDA, and separate utilization of d-α-alanine, serine, leucine, l-ornithine.

Phenon D contained only the type culture of *V.cholerae* UQM 2773, this OTU could be separated from other lysine and ornithine decarboxylating OTU's by its growth in the absence of sodium chloride and negative posture for production of acetoin and cholera-red; gluconate oxidation, growth in the presence of polymyxin, pyronin-Y, trimethoprim; separate utilization of glycine, leucine, serine, lysine, ornithine, citrulline, n-acetylglucosamine, gluconate, salicin and benzoate.

Phenon I contained the type culture for *V.carchariae* UQM 2919, and replicates of Stn 6670 and 7 other OTU's and combined with an overall $S_{j.}$ of *ca* 75 %. Apart from the production of urease by most OTU's in this phenon, other uniformly positive characters from this phenon which distinguish it from the phena A, D and J were: Production of weak indole, growth in the presence of of polymyxin, trimethoprim, pyronin-Y, and separate utilization of l-glutamic acid, l-proline, l-glutamine, n-acetylglucosamine, cellobiose, gluconate, glucuronic acid and pyruvate.

Only two attributes expressed by OTU's from this phenon conflicted with *V.carchariae* as described by Grimes *et al.*, (1984); all OTU's assayed here were denitrifying and none separately attacked ɣ-aminobutyrate. Other attributes were expressed at different frequencies to those found by Grimes *et al.* but otherwise consistent.

OTU's for type and reference cultures of *V.parahaemolyticus* were gathered into phenon J with an final $S_{j.}$ of 73 %. This phenon was distinct from the other ODC and LDC positive phena by susceptibility to 150μg/ml 0/129, methyl violet, pyronin-Y, glycine, d and l α-alanine, serine, leucine, l-arginine,

l-proline, d-glucosamine, n-acetylglucosamine, glucose, gluconate, starch, acetate, propionate, dl-glyceric acid, citrate, α-ketoglutarate and pyruvate.

The group, *Unassociated 5,* Phenon P, despite a similar decarboxylase pattern to the phena described above, was exceptional by presence of weakly fermentative OTU's, as measured in initial presumptive screening. These were grouped with the OTU Stn 8640, putatively designated as a *Shewanella* sp. isolated from a diseased goldfish. Apart from 50 % of this phenon producing sulphide, the phenon could further be differentiated by uniform positive reactions for the the following characters; pellicle production, nitrite reduction, growth in the absence of sodium chloride, growth at pH 10, in the presence of novobiocin, methyl violet, l-leucine, putrescine, d-ribose, glucose, propionate, malate, α-ketoglutarate and pyruvate.

Phenon N, *Unassociated 3,* was exceptional by its great nutritional versatility, consequently, its most close phenetic resemblance was to Phenon M, *V.natriegens.* All strains were positive in phenon N but negative in *V.natriegens* for the characters: Acetoin production, gluconate oxidation, lysine and ornithine decarboxylation, ONPG hydrolysis, growth 0 % saline, polymyxin, brilliant green, EDDA, haloquinol, 8-hydroxyquinoline, pyronin-Y and separate utilization of galactose. This group is classified as *V.natriegens*-like and the OTU's Stn 1020 and 1090 remain stored under paraffin.

3.8.4.2.3.3 Lysine Decarboxylating Phenon.

Phenon G, contained only the type culture for *V.alginolyticus* UQM 2770. Apart from its different ADH and LDC reactions this phenon was separable from the phenon holding *V.tubiashii* UQM 2923, by its growth in the presence of polymyxin, separate utilization of l-leucine and heptanoate, and by an inability to hydrolyze aesculin, arbutin or xanthine.

3.8.4.2.3.4 Decarboxylase Negative Phena.

The decarboxylase negative, unassociated *Vibrio* sp., phena H and M, *V.natriegens,* were both entirely positive for the characters nitrite reduction, hydrolysis of aesculin, egg albumen, arbutin, gelatin, SDS, horse serum, growth in the presence 6 % sodium chloride, susceptibility to 10 and 150 μg/ml 0/129, separate utilization of l-glutamic acid, l-arginine, l-ornithine, proline, putrescine, d-glucosamine, n-acetylglucosamine, xylose, arabinose, maltose, cellobiose, gluconate, salicin, propionate, malate and dl-glyceric acid. Diagnostic characters positive in phenon H but negative in phenon M were for ONPG hydrolysis, production aryl-sulphatase, and growth in the absence of sodium chloride. Characters positive in phenon M but negative in phenon H were for presence of swarming growth, hydrolysis of casein, DNA, tyrosine, growth in 10 % sodium chloride, growth in the presence of trimethoprim, separate utilization of glycine, serine, leucine, lysine, citrulline, pullulan, acetate and citrate.

Phenon H was associated with *V.alginolyticus* and *V.tubiashii* by ALJ but was distinct from this and previously described species (Baumann *et. al.,* 1984 and 2.7.2). The greatest phenetic resemblance is with *V.diazotrophicus.* One strain from this *V.diazotrophicus*-like phenon Stn 1870 was deposited as UQM 3367.

3.8.4.2.4 Summary of Results.

Placements in dendrograms originated from the $S_{j.}$ coefficient were generally more consistent with recognized taxa than those produced from $S_{e.}$. Clusters formed by the trimmed single link and median linkage protocols were loosely bound and did not resolve all recognized taxa. The complete linkage analyses were more rigorous, particularly CLE, and split replicated OTU's of Stn 6421 and/or 6670 into discrete sub-phena. The ALJ analysis resolved replicates into single phena but did not aggregate OTU's from the xanthine hydrolytic phena for *V.orientalis* and *V.tubiashii.*

Most of the seventeen resolved phena resembled previously described species. Three phena *V.parahaemolyticus, V.fluvialis* and *V.carchariae* contained more than 60 % of provincial OTU's. No local OTU's were found which corresponded to reference cultures of *V.alginolyticus, V.anguillarum, V.cholerae* or *V.nereis*. The phena (C,O,H,N) contained provincial OTU's which did not correspond to any previously described *Vibrionaceae* species and representatives of these and strains resembling *V.carchariae* from above were held in storage under oil or deposited in the departmental culture collection.

3.8.4.3 Discussion.

The quality of all analyses as measured by the placement of duplicate and reference cultures was significantly improved by sorting from the S_j coefficient consistent with recommendation for its use by Sneath (1957).

The best resolution of phena was consistent with the empirical findings of Williams *et al.,* (1971) and Everitt (1979) which favoured average linkage analysis. This was despite the additional experimental error imposed by an impure distilled water supply. These analyses would not have been feasible if it were not for the use of standardizing procedures such as the inclusion of duplicate OTU's, co-specific reference cultures, and from the use of both negative- and positive- controls in carbon assimilation assays. Use of 5 % "trimming" was not sufficient to prevent chaining under the single linkage protocol. Perhaps a higher trimming percentage might lead to better analyses, but at the expense of losing additional OTU's from analysis.

Because resultant taxa were generally consistent with previously described species it can be inferred that sufficient differential characters were present for an internally consistent analysis which was stable to the skewing effects of impure distilled water, and which assimilated data with missing values without too much disruption. However the non-adjacent placement of rare and otherwise similar xanthinolytic OTU's, e.g. *V.tubiashii* and as shown here *V.orientalis*, despite high phenetic similarity may be an artifact of skewed testing or sorting procedure.

The frequent occurrence of *V.parahaemolyticus* rather than the other common species of *V.alginolyticus* and *V.harveyi* may have reflected the pathologic rather than environmental origin of most OTU's from Batch [6].

3.8.4.4 Conclusion.

The average linkage protocol sorting a Jaccard matrix, assembled from Batch [6.] data with missing values and using specially written accessory computer programs, produced results most consistent with previously classified and replicate OTU's.

These results constitute the first Australian records for the species *V.aestuarianus, V.orientalis, V.tubiashii,* and *V.carchariae.* Strains from these and atypical phena were deposited in the departmental culture collection or stored under oil for verification or further analysis.

3.8.5.0 Evaluation of Replicate Consistency Within- and Across- Batches.

3.8.5.1 Experimental Design.

Phenotypic characterization of OTU's in chronologically, (and/or geographically), discrete batches introduces problems of cross-batch reproducibility. These problems resemble those encountered when rapid identification of bacterial isolates is attempted. Because the primary purpose of numerical analysis in bacteriology in the past has been to formulate objective classificatory schemes (2.9.3.1), by the incorporation of batches into the classificatory regimen, conditions are created for the evolution of diagnostic schemes whose component tests are intrinsically more robust to the problems of discrete identifications (Appendix 8.).

Within batches the factors which contribute to noise (2.9.3.6.1) are equalized, but not controlled, by internal standardization. Hidden internal noise which may become apparent with cross-batch replication, is composed of intrinsic variations induced by weak or otherwise variable (e.g. plasmid bearing) or mixed OTU's, and scoring errors, as defined by Sneath and Johnson (1972) composed of reading errors and test variability. These invisible test errors in batches may also appear in characters which are uniformly positive or negative amongst all internal replicates; and may also illustrate areas of skewed readings such as where a replicate is weak for an attribute in one analysis and positive for the same attribute in a different analysis.

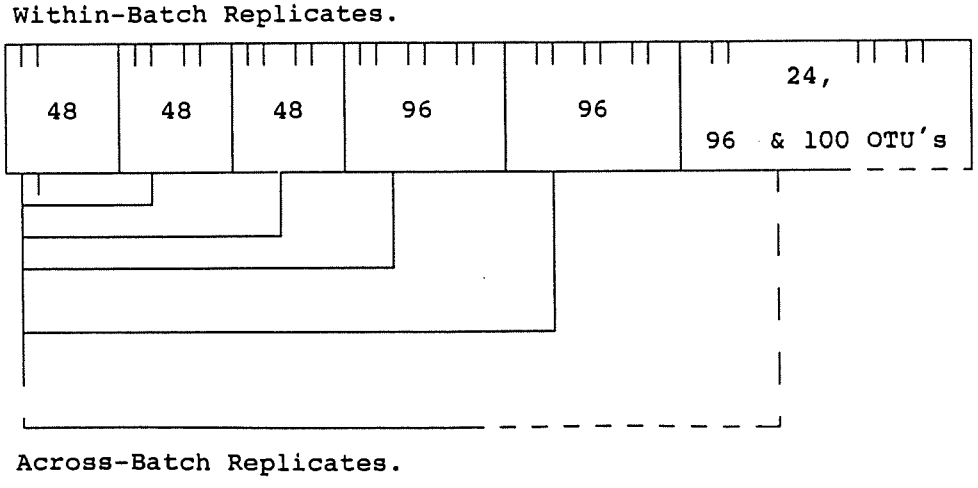
This problem was shown by controlled changes to test incubation temperatures; in work by Davis, Wilson, Fomin and Newton (1969) and iterated by Sneath and Johnson (1972), causing shifts into new taxonomic space. This skewing may be treated by recoding characters in entire batches to ND where replicates are inconsistent. However such recoding while improving across-batch miscibility is at the expense of resolution i.e. by removing information from locally valid data.

The effects of such codings can be minimized when it is considered that each character locus can function at least as a place marker, as well as to hold only positive or negative information or both positive- and negative-

information. The corollary from this is that noise damping transformations can be made which remove all information or only part of the positive- and negative- information found at each character locus i.e. if for a character discrepant-replicate-OTU's occurs across different batches, damping across those batches can be undertaken in a manner which confines the amount of information which is removed to that which is inconsistent. Obviously these procedures are most objectively implemented by a program similar to that used by Bryant *et al.,* (1986a) with standardized variance calculations adjusted to accommodate attribute frequencies. However no program to date has facility to standardize variances for duplicates within- and across-batches separately, nor is there one capable of examining multiple replications of OTU's.

The second method, adopted here, relied upon the manual examination for deviant attributes amongst replicates, and recoding these to ND where possible. Complete and partial replicates, (to gauge the effects of missing value treatments), for both type and unknown provincial OTU's were included both within- and across-batches as specified in 3.8.2.1 and schematically depicted in Figure 3.6 so that at some later stage these could be used as markers to identify and perform damping transformations upon "noisy" attributes within- and across-batches. This methodology is detailed in

Figure 3.6: Schematic Origins of Replicate Data from Within and Across Batches.



3.8.2.3. The analysis was then tested for its resolution of discrete species.

To empirically establish across-batch miscibility, species which were more or less stable to such pooled analysis, as measured by partial and complete replication of OTU's across-batches 277 OTU's were imported from 12 native batches into a hybrid dataset, using the *SOS* **(DEC)** line editor, for analysis using average linkage sorts of the Jaccard matrix as comparatively indicated in 3.8.4. Replicates in Batches [5a.] and [6a.]; and [7a.] and [8a.], respectively included carbon assimilation tests, and all tray tests. Tests in Batch [5a.] were inoculated from a suspension which contained growth supplement (50 mg/ml pyridoxal hydrochloride) to previously assayed but weak OTU's. Results obtained from this analysis were analyzed by overlaying OTU's replicate information onto the original data. Table 3.15 lists OTU's examined, their native batch, and whether they were putatively mixed by streak-plate examination.

The replicate OTU's used to assess test variation were *V.anguillarum,* UQM 2771, UQM 2843; *V.parahaemolyticus* UQM 2776, UQM 2125, UQM 2126, UQM 2201. Information removed in cross-batch damping transformations coded in Batches [1.] and [6.] are listed in sections 3.8.3.2 and 3.8.4.1.

Non-exclusively, strings which follow list all characters or attributes for which all OTU's in specified batches were recoded to ND. In Batch [2.] all OTU's for the characters of colony opaque; cell length greater than twice width, and separate utilization of sarcosine were scored as ND. All OTU's for the attributes colony diameter of greater than 3 mm, for which sudanophilic inclusions were found, produced sulphide, and which separately utilized inulin, adipic acid, galactarate, malonate and tartarate were scored as ND. OTU's which were negative for growth on TTC, separate utilization of citrate, glucose and glutamine were scored as ND. Weakly positive OTU's for the attributes growth in the presence of brilliant green, separate utilization of pyruvate, and malate were scored as positive. Weakly positive OTU's for the separate utilization of ornithine were scored as negative.

Table 3.15: OTU's Examined, Their Native Batch and Whether Streak-Plates Indicated: Monomorphic, (pure) (+); or Dimorphic, (potentially mixed) Cultures (o).

OTU.	Batch.	Purity.	OTU.	Batch.	Purity.	OTU.	Batch.	Purity.
Stn 1470	6	+	Stn 6780	7	+	Stn 8190	4	+
Stn 1470	8a	+	Stn 6780	5	+	Stn 8190	7a	+
Stn 2300	7	+	Stn 7100	5	+	Stn 8200	4	+
Stn 5580	8	+	Stn 7100	6	+	Stn 8200	5	+
Stn 2440	6	+	Stn 7120	5	o	Stn 8260	2	+
Stn 2440	6	+	Stn 7120	6	+	Stn 8260	2	+
Stn 3330	7	o	Stn 7170	5	o	Stn 8270	2	+
Stn 3330	8a	+	Stn 7170	6	+	Stn 8270	2	+
Stn 3560	7	o	Stn 7260	4	+	Stn 8370	4	+
Stn 3560	8a	+	Stn 7260	7	o	Stn 8370	7a	+
Stn 3681	7	o	Stn 7260	8a	+	Stn 8380	4	+
Stn 3681	8a	+	Stn 7280	4	+	Stn 8380	7a	o
Stn 5860	8	+	Stn 7280	7a	+	Stn 8380	8a	+
Stn 4830	8	+	Stn 7300	4	+	Stn 8490	4	+
Stn 6421	8	+	Stn 7300	7a	+	Stn 8490	7a	+
Stn 6421	6	+	Stn 7310	4	+	Stn 8500	4	+
Stn 6430	6	+	Stn 7310	7a	+	Stn 3521	7	+
Stn 6430	6	+	Stn 7320	4	+	Stn 8510	5	o
Stn 6500	5	+	Stn 7320	4	+	Stn 8510	5a	+
Stn 6500	8	+	Stn 7320	5	+	Stn 8550	7a	+
Stn 6510	5	+	Stn 7360	2	+	Stn 8550	8a	+
Stn 6510	8	+	Stn 7360	2	+	Stn 8570	4	+
Stn 6550	1	+	Stn 7360	5	+	Stn 8570	7a	+
Stn 6550	4	+	Stn 7450	5	o	Stn 8600	4	+
Stn 6550	5	+	Stn 7450	6	+	Stn 8600	7a	+
Stn 6550	5a	+	Stn 7450	7	o	Stn 8640	5	+
Stn 6560	5	+	Stn 7600	4	+	Stn 8640	6	o
Stn 6560	8	+	Stn 7600	5	o	Stn 8680	4	+
Stn 6561	5	o	Stn 7600	7	o	Stn 8680	7	o
Stn 6561	7	o	Stn 7600	8a	+	Stn 8680	8	+
Stn 6561	8	+	Stn 7630	4	+	Stn 8810	4	+
Stn 6580	5	+	Stn 7630	7	+	Stn 8810	7	+
Stn 6580	8	+	Stn 7700	5	o	Stn 8830	4	+
Stn 6590	5	+	Stn 7700	7	+	Stn 8830	7a	+
Stn 6590	8	+	Stn 7710	2	+	Stn 8950	8	+
Stn 6600	8	+	Stn 7710	5	+	Stn 8950	8	+
Stn 6600	8	+	Stn 7760	4	+	Stn 9200	7	+
Stn 6610	2	+	Stn 7760	7a	+	Stn 9200	8a	o
Stn 6610	8	+	Stn 7920	2	+	B 1	4	+
Stn 6611	5	+	Stn 7930	2	+	B 1	7a	o
Stn 6611	8	+	Stn 7940	5	+	B 51	4	+
Stn 6670	6	+	Stn 7940	8a	+	B 51	7a	+
Stn 6670	6	+	Stn 7960	2	+	Be 07	5	+
Stn 6710	5	+	Stn 7960	2	+	Be 07	5a	+
Stn 6710	8	+	Stn 8010	5	+	Be 08	5	+
Stn 6720	5	o	Stn 8010	5	+	Be 08	5a	+
Stn 6720	6	+	Stn 8030	2	+	Be 09	5	+
Stn 6720	7	+	Stn 8030	2	+	Be 09	5a	+
Stn 6730	8	+	Stn 8120	4	+	Be 10	5	+
Stn 6730	5	+	Stn 8120	7a	+	Be 10	5a	+
			Stn 8090	4	o			

Table 3.15; (cont.): OTU's Examined, Their Native Batch and Whether Streak-Plates Indicated: Monomorphic, (pure) (+); or Dimorphic, (potentially mixed) Cultures (o).

OTU.	Batch.	Purity.	OTU.	Batch.	Purity.	OTU.	Batch.	Purity.
Be 11	5	+	UQM 2744	1	+	UQM 2784	1	+
Be 11	5a	+	UQM 2744	6	+	UQM 2784	6a	+
Be 12	5	+	UQM 2745	1	+	UQM 2784	7	o
Be 12	5a	+	UQM 2745	6	+	UQM 2785	8a	+
Be 13	5	+	UQM 2769	1	+	UQM 2785	1	+
Be 13	5a	+	UQM 2769	6a	+	UQM 2785	7	o
Be 14	5	+	UQM 2770	1	+	UQM 2785	8	+
Be 14	5a	+	UQM 2770	6	+	UQM 2786	1	+
Be 16	5	+	UQM 2770	6a	+	UQM 2838	2	+
Be 16	5a	+	UQM 2770	7	+	UQM 2838	2	+
Be 17	5	+	UQM 2771	1	+	Stn 180	1	+
Be 17	5a	+	UQM 2771	1	+	Stn 180	1	+
Be 18	5	+	UQM 2771	2	+	Stn 170	3	+
Be 18	5a	+	UQM 2771	3	+	Stn 170	3	+
Be 19	5	+	UQM 2771	4	+	UQM 2843	7a	+
Be 19	5a	+	UQM 2771	5	+	UQM 2843	1	+
Be 20	5	+	UQM 2771	5a	+	UQM 2843	2	+
Be 20	5a	+	UQM 2771	7	+	UQM 2843	6a	+
DA 11	7	o	UQM 2771	6a	+	UQM 2849	7a	+
DA 11	8a	+	UQM 2771	6a	+	UQM 2849	2	+
UQM 2125	4	+	UQM 2771	7	+	UQM 2852	2	+
UQM 2125	6	+	UQM 2771	8	o	UQM 2852	4	+
UQM 2126	4	+	UQM 2773	6	+	UQM 2853	7	+
UQM 2126	6	+	UQM 2773	6a	+	UQM 2853	2	+
UQM 2201	4	+	UQM 2774	1	+	UQM 2888	5	o
UQM 2201	6	+	UQM 2774	6a	+	UQM 2888	7	+
UQM 2628	5	+	UQM 2775	1	+	UQM 2888	8a	+
UQM 2628	5	+	UQM 2775	6a	+	UQM 2889	4	+
UQM 2730	1	+	UQM 2776	1	+	UQM 2889	7	+
UQM 2730	6	+	UQM 2776	6	+	UQM 2890	4	+
UQM 2731	4	+	UQM 2776	6a	+	UQM 2890	4	+
UQM 2731	7a	+	UQM 2779	1	+	UQM 2890	7	+
UQM 2732	4	+	UQM 2779	6a	+	UQM 2890	8a	+
UQM 2732	7a	+	UQM 2780	2	+	UQM 2906	5	+
UQM 2740	1	+	UQM 2780	7	+	UQM 2906	7	+
UQM 2740	6	+	UQM 2781	1	+	UQM 2921	6	+
UQM 2741	1	+	UQM 2781	6a	+	UQM 2921	7	+
UQM 2741	6	+	UQM 2782	1	+	UQM 2923	6	+
UQM 2742	1	+	UQM 2782	6	+	UQM 2923	7	+
UQM 2742	6	+	UQM 2782	6a	+	UQM 2954	7	o
UQM 2743	1	+	UQM 2783	1	+	UQM 2954	8a	+
Stn 3700	2	+	UQM 2783	6	+	Stn 1620	1	+
						Stn 1620	8	+

In Batch [3.] all OTU's for the characters of nitrate and nitrite reduction and separate utilization of acetate were scored as ND. Strains which grew only weakly in the presence of streptomycin were scored as negative. Strains initially scored as positive for sulphide production were recoded to ND. OTU's which failed to hydrolyze albumen or grew in the presence of TTC were

scored as ND. OTU's which were weakly positive for separate utilization of citrate and gluconate were respectively coded as positive and negative.

In Batch [4.] all OTU's were coded as ND for the characters, length greater than twice width, both sudanophilic inclusion assays, and growth at 4°C. OTU's which were positive for; colonies larger than 3mm, mucoid broth sediment, non-uniform turbidity in broth, growth at 42°C., and which grew in the presence of brilliant green were scored as ND for those attributes. OTU's which did not separately utilize glutamine, glucose, maltose, starch, dextrin, succinate, lactate, dl-glyceric acid, pyruvate, mannitol, sorbitol were coded as ND for those attributes. In Batch [5.] all OTU's were coded to ND for cell length greater than twice width, fast yellow, separate utilization of galactarate, dextrin, and ethanol. All OTU's positive for the attributes of mucoid broth growth, sulphide production, separate growth on valine, raffinose and adipic acid and OTU's negative for the attributes, separate utilization of glucosamine, glucose, starch, formate, nonanionate, caprate, citrate, pyruvate and succinate; were also coded to ND. In Batch [5a.] all OTU's for separate utilization of serine were coded as ND, as were OTU's which failed to grow on l-alanine, dl-glyceric acid, n-acetyl glucosamine, mannose, galactose, inulin, caprate, nonanionic acid, succinate, lactate and citrate. In Batches [7.] and [7a.] all OTU's were coded as ND for: Colony diameter greater than 3mm, cell length more than twice width, growth in 0.5 % NaCl, serum hydrolysis, production of many sudanophilic inclusions and growth in the presence of sulphamethoxazole, and dichlorophene and separate utilization of dextrin as were OTU's which produced mucoid broth growth, grew at 42°C. or which failed to produce lipase, grow at pH 10, or separately utilize cellobiose and starch were coded as ND. All OTU's in Batch [8.] were recoded to ND for characters: Colonies yellow, length greater than twice width, broth sediment mucoid, and production of many sudanophilic inclusions.

Data from 277 replicates from batches coded as specified in Table 3.15 was produced into a top and bottom Jaccard distance matrix and this sorted by

the UPGMA method with "k-" linkage list length set to 8, using *SAS*. 5.08. The dendrogram Figure 3.7 is plotted on a scale indicating % similarity and OTU's were assigned to phena according to the distribution of type cultures. Subsequent to analysis it was discovered that the OTU's Stn 5580, 5860, 7930, 8090, 3521, 3700 and UQM 2786 were read into the dataset instead of replicates for respectively, Stn 2300, 4830, 7920, 8120, 8500, UQM 2743, UQM 2766 no data was entered for replicates of the OTU's Stn 760 and 770 and apart from different replicates of *V.anguillarum* ATCC 19264, no replicated type cultures of the same species with different UQM numbers e.g. *V.campbellii* and *V.harveyi* were included. OTU identifiers to unmatched OTU's either from these clerical errors or corresponding to potentially unmatchable dimorphic or mixed colonies shown in Table 3.15 and the dendrogram Figure 3.7 with a "o" have been prefaced on the dendrogram with a "▼". Because of the time involved in processing the dendrograms; finding discrepancies and attributing them as keying errors, and because PCC IBM 3083 operated on a monthly fixed rental basis, it was not possible to edit and reanalyse the data in the time available.

Attribute variances were not calculated because it was reasoned that cross- and within-batch species resolution, if successful, would justify itself, and average group homogeneity would be shown upon aggregation of phena. Frequency tables for phena were not prepared because these could be shown for more diverse ranges of OTU's in later analyses.

3.8.5.2.1 **Results.**

All 277 OTU's were fused into the dendrogram by a similarity of *ca* 20 % and are located by 33 phena, A - AG. Most of these phena were formed at similarities of 60 % or greater, reflecting the use of the Jaccard rather than the euclidean distance coefficient. Table 3.16 summarizes information from the dendrogram by listing reference species and indicating correspondence between replicated OTU's and locating other phena which contain common OTU's. The fifteen latterly fused OTU's from phenon AG, with two exceptions, were comprised of only partially characterized strains,

(carbon assimilation tests, in media prepared from impure distilled water), from Batch [6a.]. Within this orphaned aggregate; different OTU's from *V.anguillarum* were variably placed. Of the remaining phena, seventeen, A,B,H,I,K,M,N,O,Q,R V,Y,Z,AB,AC,AD,AE and AF contained type or reference OTU's from only one species. All reference cultures for the species *V.gazogenes, V.costicola, V.natriegens, V.nereis, P.fischeri,* and *V.ordalii* were robust to the crossed-batch treatment and represented in single phena. The remaining phena A, *V.vulnificus;* K, *V.campbellii;* O, *A.hydrophila;* V, *V.pelagius;* Y, *V.alginolyticus;* Z, *V.damsela;* AC, *V.nigripulchritudo* and AD *V.hollisae* contained reference cultures which appeared in at least one other phenon.

Four phena, D,P,T and W contained type or reference cultures from two species, and four C,E,X, and AA type or reference OTU's from 3 or more species. No type or reference OTU's were associated with the six remaining phena F,G,J,L,S and U. Of these, the phena F and U contained mainly dimorphic or mixed OTU's from Batch [7.], and phenon G contained an unreplicated OTU, and others only replicated in Batch [6.] so that all could be effectively discounted from analysis. The remaining phena J,L and S all contained OTU's in common with phenon H.

The phena F,G,J,U, and X contained OTU's from only one batch and A,B,K,Q,Y and AB from two; C,L,M,N,O,S T,V,W,AC,AD,AF and AG from three and D,H,I,P,R,Z and AE from four to seven. The phena E and AA respectively contained OTU's from nine and eleven batches.

Dimorphic OTU's were placed in the phena B-F, H-J, M-N, S-T, W, Z, AA, AC, AE-AF. But replicates only from the identified species *V.fluvialis/-furnissii, V.mimicus, V.ordalii* from the phena E, AA and AE; and the unidentified *Vibrio* spp. Stn 9200, 6561 and 8380 respectively from the phena S, W and Z were placed together i.e. were shown unaffected by contaminants.

Table 3.16: Summary of Phenon Size, Species and Batch Composition, and Number of OTU's Replicated Within and Outside these Phena, and Listing of other Phena with Overlapping OTU's.

Phenon	Species Present	Batch Numbers.	Fusion Similarity %.	Number Pure OTU's.	Number. Grouped/Ungrouped	Other Phena Shared OTU's (u-Unassociated).
A	*V.gazogenes*	1,3	65	4	4/0	-
B	*V.vulnificus*	1,5,5a,8	60	9	4/5	X,AG
C	*V.orientalis, V.tubiashii*	2,4,7	60	6	0/6	T,X,U,Z
D	*V.parahaemolyticus* *V.alginolyticus*	1,2,4,5,6, 7a,8a	70	28	27/1	U,X,Y
E	*V.fluvialis, V.furnissii* *A.hydrophila*	1,4,5,6a,7a,8a	70	17	16/1	O
F	All mixed	7	70	3	NA	NA
G	*Vibrio* sp.	1,8	70	2	0/2	I,H
H	*V.harveyi*	1,2,5,5a	75	14	5/9	I
I	*V.harveyi,V.carchariae*	1,4,5,6,7,8,8a	75	17	12/5	H
J	*V.harveyi,V.carchariae*	5a,7	70	3	0/3	H,L
K	*V.campbellii,V.damsela* *V.hollisae*	1,7	65	3	0/3	Z,AD,u
L	*Vibrio* sp.	2,6	60	4	2/2	J,AC
M	*V.diazotrophicus*	2,7	80	2	2/0	-
N	*V.natriegens*	1,6,6a	70	3	3/0	-
-	*V.parahaemolyticus*	6a	NA	1	0	D
O	*A.hydrophila*	7a	65	3	0/3	E,Y
P	*A.hydrophila,Vibrio* sp.	1,6,6a	65	3	2/1	AG
Q	*V.nereis*	1,6a	65	2	2/0	-
R	*P.fischeri*	4,5,7	60	4	4/0	-
S	*Vibrio* sp.	6	55	3	2/1	H
T	*V.anguillarum, V.cholerae*	7a,8,8a	65	10	1/8	T
U	Unassociated	7a,7a	65	2	0/2	C,Z
V	*V.pelagius*	1,4,8,8a	60	6	6/0	AG
W	*V.nigripulchritudo*	1,4,5	60	3	0/3	AC,J,u
X	*V.vulnificus,V.alginolyticus* *V.tubiashii,V.orientalis*	6	60	4	0/4	A,C,D,Y,AG
Y	*V.alginolyticus*	4,7	60	3	0/3	D,O,X,AG
Z	*V.damsela*	2,4,7a,8a	60	10	7/3	A,C,K
AA	*V.anguillarum,V.cholerae* *V.mimicus*	1,2,3,4,5, 5a,6,7,8a	60	20	12/8	T,A,G
AB	*V.anguillarum*	5,5a	60	22	22/0	-
AC	*V.nigripulchritudo*	2,7	60	2	2/0	L,W
AD	*V.hollisae*	4,5	50	3	1/1	K
AE	*V.ordalii*	4,5,7	50	8	8/0	-
AF	*V.costicola*	5,6a	45	2	2/0	AG
-	*V.campbellii*	6a	NA	1	NA	K
AG	*V.vulnificus,Vibrio* sp. *V.cholerae,V.pelagius,* *V.anguillarum,V.vulnificus* *V.alginolyticus,V.costicola.*	5,6a,7	30	13	2/11	B,D,P,T,V, X,Y,AF

Grouped: Replicates placed into the same phenon.
Ungrouped: Replicates not placed into the same phenon.

Repeated spherical clusters i.e. indicating provincial correspondence, but across-batch immiscibility, occurred for replicates from several species. In the phena T and AA replicates of *V.anguillarum,* and *V.cholerae* were placed repeatedly as discrete groups. Phenon T represented a skewed phenon because most OTU's were partial replicates from Batches [7a.] and [8a.]. In the latter phenon *V.mimicus* was associated with *V.cholerae* before juncture with *V.anguillarum* replicates. Replicates of the other type culture of *V.anguillarum* UQM 2628, derived less directly from the originating culture collection than UQM 2771, was associated with phenon AA only after forming with the unidentified but cohesive phenon AB which otherwise contained only Benalla isolates.

Similar repeated resolution of the same reference OTU's from different batches occurred in the Phena C, V, W, X and AC. Additionally provincial OTU's common between these phena were also present in phena J and L. Of these phena, C J W and AC additionally carried OTU's which were putatively mixed, as interpreted from di-morphic colony appearances on streak-plates of SENA medium. Type or reference cultures in these phena were *V.orientalis, V.tubiashii, V.pelagius,* the Tasmanian oyster OTU's B1, B2, and B51, and *V.splendidus* with *V.nigripulchritudo* and *V.alginolyticus.* The compositions (Batch, OTU) of these phena are listed in Table 3.17

OTU's from Batch [6.] were found only in the phena X and L, neither with matching replicate OTU's. A consistent spherical association existed between replicate OTU's for *V.tubiashii* UQM 2923 and *V.orientalis* UQM 2921 from Batches [6.] and [7.] (Phena X and C). Cross-batch replicates were present in Batches [1.] and [8a.] for *V.pelagius* UQM 2785 in phenon V, and Batches [5.] and [8.] for Stn 6561 in phenon W. These results indicate either that differential attributes used to assemble similarity coefficients between these species might be highly variable in across-batch analyses of these species, or that the formation of phena for these species was distorted by the introduction of one or a combination of mixed and variable cultures into these phena; or induced through skewing as developing phena gathered

replicates from Batch [6.]. Other phena showed associations between OTU's from most batches i.e. [2. & 7.]; [7 .& 5a.]; [8.,4., 8a. & 1.]; [5.,8.,4. & 1.]; [2.,7. & 7a.].

Table 3.17: Compositions of other Phena with OTU's in Common with the Phena C and X.

Phenon C	Phenon J	Phenon L	Phenon V	Phenon W	Phenon X	Phenon AC
7, UQM 2921	o7, Stn 6561	6, Stn 6720	8, Stn 6600	o5, Stn 6561	6, Stn 7450	2, Stn 8260
7, UQM 2923	7, Stn 6780	2, Stn 8260	8, Stn 6600	o5, Stn 6720	6, UQM 2740	o7, DA 11
o7, UQM 2785	7, Stn 6720	2, Stn 8270	4, B 1	5, Be 07	6, UQM 2770	o7, Stn 8680
2, Stn 6610	5a,Be 07	2, Stn 8270	8a,Stn 8680	8, Stn 6561	6, UQM 2923	7a,B 1
4, Stn 7300			4, Stn 8680	o5, Stn 7450	6, UQM 2921	7a,B 51
4, Stn 7310			8a,UQM 2785	4, B 51		7, UQM 2784
			1, UQM 2785	1, UQM 2786		
				1, UQM 2784		

V.alginolyticus UQM 2770 from three assays was placed into three phena, D i.e. with *V.parahaemolyticus,* and X and Y. These later placements indicated some consistency for this species cross-Batches [6.] and [7.] but with insufficient resolution to separate other species.

The Batch [1.] replicate of *V.alginolyticus* interrupted the type and reference cultures of *V.parahaemolyticus,* in phenon, D. The only other inconsistent OTU from this phenon was the partial replicate of *V.parahaemolyticus* UQM 2776 from Batch [6a.]. This was consistent with the spurious placements of other OTU's from this batch. All of nine replicated provincial OTU's, from all except Batches [3.] and [7.] i.e. Stn 1470, 6421, 6430, 6560, 6510, 7360, 7940, 7960, and 8030, were gathered only into this phenon.

Phenon E was gathered by an average similarity of 75 % and contained provincial OTU's and predominantly type and reference OTU's from *V.fluvialis* and *V.furnissii, (V.fluvialis Biogroup II)* and a single OTU for *A.hydrophila* UQM 2838 from Batch [2.]. All eight replicated provincial OTU's Stn 2440, 3330, 3560, 7000, 7710, 7760, and 8950 from Batches [4.], [5.], [6.], [7.], [8.], [7a.] and [8a.] were placed only in Phenon E. The

remaining replicated cultures of *A.hydrophila* from Batches [1.], [7a.] and [6a.] were placed in Phenon P with the *Vibrio* sp. UQM 2744 from Batch [1.]. No provincial OTU's were placed in this phenon.

The phena H and I respectively contained reference and type cultures from *V.carchariae* UQM 2849 and *V.harveyi* UQM 2781, however some provincially isolated replicate OTU's were dispersed across both these phena. Of 16 replicated OTU's located in these adjacent phena eleven had at least one corresponding replicate. The non-corresponding (dispersed) replicate OTU's from these phena were: Stn 6580, and 6730, and (Phenon G); Stn 6780 (Phenon J).

The presence of the vitamin B_6 growth supplement in carbon assimilation media affected the placement of only one of the replicate pairs, Be 7, across-Batches [5.] and [5a.]. However most of these replicates were gathered into the unclassified group phenon U associated with *V.cholerae* and *V.anguillarum.*

Partially replicated, but unoverlaid results for OTU's, from different species in some cases were stable to the effects of the various noises present in this analysis e.g. the Batch [6a.] (carbon sources only) replicates for *V.fluvialis,* UQM 2774 and *V.harveyi* UQM 2781 were faithful to their respective phena but *V.parahaemolyticus* UQM 2776 was not. Of 26 "unmixed" complete tray test replicates from batch 7a and 8a, 15; *V.vulnificus* UQM 3032, *V.parahaemolyticus* Stn 1470, 7940, 6560; *V.fluvialis* Stn 3560, 3330, 7760; *V.harveyi/carchariae* Stn 7260, 7600; *Vibrio* sp. Stn 9200; *V.anguillarum* Stn 8550, *Vibrio* sp. Stn 8680, *Vibrio* sp. Stn 8370, 8380, and *V.mimicus* UQM 2954 were placed into the same phenon; and 11 UQM 2838, *A.hydrophila* Stn 8120, 8190, 3681, *V.anguillarum* UQM 2843, Stn 8570, 8600, *V.cholerae* UQM 2731-2, Stn 7280 and 7310 were isolated into local optima. Of these, OTU's from *V.cholerae* and *V.anguillarum* were aggregated into different divisible hierarchies of the same species.

3.8.5.2.2 **Summary.**

The analysis depicted here indicated that only some of the species represented from variously replicated and dimorphic or putatively mixed OTU's from 12 batches were consistently resolved as discrete taxa. Apart from random noise across-batches, phena from this hybrid analysis were additionally distorted by the presence of "atypical" OTU's formed from mixed cultures, partial replication, or from non-standard culture conditions i.e. from Batch [6].

After discounting results from a separate phenon of primarily Batch [6a.] OTU's this latter effect was not generally distributed amongst all species, all OTU's of the species *V.gazogenes, V.costicola, V.natriegens, V.nereis, P.fischeri,* and *V.ordalii* were partitioned discretely. Others including *V.parahaemolyticus* and *V.alginolyticus; V.harveyi* and *V.carchariae; V.fluvialis* and *V.furnissii;* were non-divisively merged into single phena. The species *V.cholerae, V.anguillarum* and on one occasion also *V.mimicus* were repeatedly resolved as separable, but associated phena.

Single OTU's from type cultures of *V.tubiashii* and *V.orientalis* from the same batches were fused first, but replicates were then placed into separate apparently unordered phena with other OTU's, most notably the Tasmanian oyster OTU's, and *V.pelagius, V.nigripulchritudo* and *V.splendidus* which were repeatedly resolved as unassociated small phena rather than being merged with phena formed by larger phena unrelated species. The exception to this was phenon X which was immiscible to other OTU's except for those assayed in Batch [6.]. Replicates of *V.alginolyticus* UQM 2770 used in Batches [6.] and [7.] by their aggregation into neighbouring phena but discretely from phenon D are consistent with the atypical presentation of this species in 3.8.4. A separate phenon (F) of unpaired dimorphic OTU's from Batch [7.] was resolved indicating that in this case all OTU's were mixed.

3.8.5.3 **Discussion.**

Some of the concepts underlying this work were retrospectively acted upon by Bryant *et al.,* (1986a,b), whose highly sophisticated analysis across 5 batches was derived from an average linkage rendering of a euclidean distance matrix. This was formed from 90 strains represented in 194 OTU's each truncated to comprise up to 111 characters. These "stable" characters were deduced from all replicates in two batches and selected replicates from the remaining three which had attribute variance, (s_i^2), of 0.1 or less as calculated using the formula $s_i^2 = p_i(1 - p_i)$ where i is a binary character state and p is is the probability of an error averaged over n tests (Sneath and Johnson, 1972). The well distributed but incomplete dendrogram from their analysis, i.e. representing 71 replicate OTU's from 31 strains, prevented evaluation of the distribution, of the remaining 59 strains and 123 replicates with respect to batch origins and identities and placement of replicate type cultures e.g. from *A.hydrophila, V.campbellii* and *V.metschnikovii,* each of which were included in at least 2 of the 3 generally published studies imported into this analysis i.e. Lee *et al.,* (1981), West *et al.,* (1983) and West *et al.,* (1986).

On the data presented, by Bryant *et al.,* the species of *Photobacterium* i.e. *P.fischeri, P.leiognathi,* or *P.phosphoreum* were presented in heterogeneous rather than discrete phena. Type cultures for *V.splendidus* Biogroup I from ATCC and NCMB were placed respectively with the *Photobacterium* spp. and with *V.harveyi. V.campbellii* also interrupted replicates in the phenon formed by *V.harveyi.*

Because the study of Bryant *et al.,* was conducted retrospectively insufficient replicates were included across-batches to make a valid measurement of across-batch variance i.e. no replicates were present between their Batches [1.] & [4.], [3.] & [5.] and [4.] & [5.], and ten or less replicates were included across-Batches [1.] & [3.], [3.] & [4.] and [2.] & [5.]. Character deletions across all batches based on high variances calculated from so few replicates would carry such a high statistical error

that the validity of at least some deletions may not have been valid. Other data retained from attributes uniformly negative or rarely positive across batches i.e. which otherwise carry less information than attributes with a higher frequency might appear have a low or 0 or high variance because sample size is so small. Evidence supporting this conclusion can be seen when based on these results a reduction of 20 % of characters (from 142 to 111) only improved the co-phenetic correlation coefficient, (across-batch miscibility,) of Bryant *et al.,* from 0.82 to 0.85 i.e. by 3%. Subject to problems of small sample size Sneath *et al.,* (1972) proposed that high or low attribute frequencies, could be adjusted to equivalent frequencies i.e. 0.5 by dividing s_i^2 by twice the proportion of positive or negative attributes, whichever is less i.e. to create a standardized variance useful in exploratory datasets. The conceptual framework upon which this analysis was based was different from that of Bryant *et al.,* (1986) i.e. by the use of multiwell trays and with respect to the number of batches, by the inclusion of partially replicated, potentially mixed OTU's, and 2 batches of inordinately skewed data and by the sorting similarity coefficient.

The similarities shown by OTU's here was generally lower than would have been obtained using the euclidean distance coefficient, but the diagnostic range of this exploratory analysis was across 80 % of the potential range cf. 35 % of Bryant *et al.,* (1986). Most species were discernible either as discrete, or as phena or sub-phena and so indicated that the data is fragmented, but also highly structured. Sufficient non-overlapping taxonomic space existed for local pockets of OTU's, each with intrinsic local hierarchies, to be resolved discretely.

Overlaid replicates from Batch [5a.] were most reliable and those from Batch [6a.] variably reliable between species. The precept of stability (2.9.3.5) relied here upon to permit association by replicates was probably affected by the adoption of the Jaccard coefficient. For replicates determined from only *ca* 70 carbon assimilation tests nutritionally diverse species; such as *V.natriegens, V.fluvialis, V.furnissii, V.parahaemolyticus,* and *V.harveyi,*

would have been less subject to the effects of partial replication than less diverse species such as *V.cholerae* and *V.vulnificus;* which when the Jaccard coefficient was calculated, would have similarities assembled from far fewer than 70 positive matches.

The removal of cross-feeding and potential access to shared enzymes, i.e. extracellular permeases, which may have been lost by some of the replicated OTU's, has probably resulted in the analysis being less affected than if the euclidean similarity were used i.e. by removal of spurious matching negative scores a bias has been imposed in similarity coefficients towards pattern over vigour (See 2.9.3.3).

Partially replicated OTU's from less vigorous species and/or OTU's from putatively dimorphic or truly mixed cultures could in these circumstances act as spurious, or distorting cluster nuclei which might affect the aggregation of at least some across-batch clusters such as was reasoned from the immiscibility of OTU's from Batches [6.] and [6a.]. The permeation of some across-batch replicate OTU's into phena holding other completely replicated OTU's from the same or related species could indicate that in the absence of partial replicates and OTU's from Batch [6.] the remaining OTU's might be presented in a different classification which conforms more closely to hierarchies produced in unit-batch analyses.

The species which were resolved into inseparable aggregates of species i.e. *V.alginolyticus* and *V.parahaemolyticus;* and *V.fluvialis* and *V.furnissii* have been shown by other methods; i.e. immunological distance (Baumann *et al.,* 1980), and DNA hybridization (Brenner *et al.,* 1983) to have been closely related and consequently the placement by this analysis is quite natural with respect to these species.

In the analysis of Bryant *et al.,* (1986, Figure 1.) replicates of *V.pelagius* were fused with a euclidean similarity of 80 % . With the Jaccard coefficient here an across-batch fusion for *V.pelagius* was achieved at a similarity of 65 %. Consequently it is evident both from this and also

implied from external studies that generalizations about the extent of acceptable phenetic variation within replicated datasets depend upon the number of differential characters and underlying similarities to other species rather than by conformity with any arbitrarily established similarity values and test regimens.

The high phenetic variation of *V.pelagius* across-studies and between laboratories indicates a functional sensitivity easily perturbed even in apparently standard conditions. Whether this condition is a result of an unstable plasmid or genome, and whether these are local-species- or general *Vibrionaceae*- qualities requires further investigation; and (3.8.6.2.3.1).

3.8.5.4 Conclusion.

It seems from this analysis that geographically and temporally separate batches of data can be meaningfully analyzed simultaneously; however, in the absence of programmed assistance to objectively choke or standardize datasets there is a tendency for related species not always to be separably resolved. Across-batch-resolution of OTU's with replication only of carbon assimilation tests under this regimen seems dependent upon the enzymic diversity of species and number of characters replicated.

Such analysis, perhaps in combination with Wishart's (1986) method for assigning missing values (modified as suggested in 3.8.3.3) might more objectively improve data quality without removing too much information.

Because of the tentative and speculative nature of findings from this analysis and the necessity to use analyses ultimately to identify provincial OTU's the remaining undiagnosed OTU's were analyzed in two equal sized blocks, in the absence of partial replicates and OTU's from Batch [6.], but including all other replicated OTU's, both to act as cluster nuclei and markers to fragmented clusters.

3.8.6.0 Species Identification Among Provincial OTU's.

3.8.6.1 Experimental Design.

Considering the computational impracticality of an entire cross-batch analysis the data were divided into two subsets each of *ca* 240 OTU's and analyzed under average linkage sorts of Jaccard matrices with *Rosetta* OTU's from Batch [1.]. Incomplete replicates and OTU's from Batch [6.], found in (3.8.5) to be only partially miscible with OTU's from other batches were excluded from these analyses. The first analysis upon OTU's from batches 1,2,4 & 5 was performed using **SAS**$^{r.}$ on an IBM 3083 mainframe computer and the second using OTU's from Batches [1., 3., 7. & 8.] using **Microcluster** $^{r.}$ on a personal computer (See 3.8.2.4). Interpretation of these numerical analyses was prefaced by an evaluation of within- and across-batch replicate consistency which for consistency with other numerical taxonomic literature was by the euclidean distance coefficient. Additional evaluation was made on the basis of within- and across-batch reference-OTU placements.

3.8.6.2.0 Diagnosis of Species and Phena from Batch 1, 2, 4 & 5 OTU's.

3.8.6.2.1 Experimental Design.

Two hundred and seven unidentified OTU's from Batches [1., 2., 4. & 5.], and 71 reference cultures representing 28 known species, were assembled into a top and bottom Jaccard distance matrix (Appendix A11.4) and sorted by UPGMA **SAS**$^{r.}$ with the "k-"linkage list length set to 8. The resultant dendrogram (Figure 3.8) was plotted against a similarity scale. Dubious OTU's deduced as mixed cultures are marked with an "o". Attribute frequencies from all except single or paired provincial OTU's were calculated as previously for up to 186 characters and phena were evaluated and differentiated according to the most discriminatory attributes, shown in frequency Tables 3.19.1-3. Results for three tests in which the author had little confidence; collagen hydrolysis, and colonies red or white on congo red medium, were respectivelly deleted from all but the last 48 unidentified OTU's in Batch [4.], and all OTU's in Batch [2.]; so it could be established if these characters had any differential taxonomic significance.

The data contained 22 replicated OTU's, including 10 cross-batch replicates. euclidean similarities and batch origins for these are shown in Table 3.18.1. Separate OTU's with different UQM numbers but common ATCC accession codes were used for *V.anguillarum* ATCC 19264 (UQM 2628 and UQM 2771); *V.harveyi* ATCC 1426 (UQM 2766 and UQM 2781); and *V.natriegens* ATCC 14048 (UQM 879 and UQM 2782). A preliminary abbreviated testing system for more rapid identification of *Vibrionaceae* phena was developed from the data.

3.8.6.2.2 Results.

3.8.6.2.2.1 Review of Replicates.

In this analysis reference cultures from within- and across- batches fell into concurrent phena except the already discussed (3.8.3) type culture of *V.vulnificus* [Batch 1.], Baumann's blister isolate of *V.alginolyticus* UQM 2675 [Batch 4.], *V.anguillarum* UQM 2628, [Batch 4.]; *V.natriegens* UQM 879, [Batch 4.] and *P.phosphoreum* UQM 2482. Within-batch similarities in (Table 3.18.2) were highest in Batch [1.] and lowest in Batch [5] where only 2 cross-batch replicates were available. $S_{e.}$ values of cross-batch replicates including ATCC 19264 as UQM 2628 but not UQM 2482 ? (See below) ranged between 76 and 89 %.

The CDC blister isolate of *V.alginolyticus* UQM 2675 from Batch [4.] nested with two UQM preparations of *V.harveyi* ATCC 14126; UQM 2766 [Batch 4.] and UQM 2781 [Batch 1.], but was separated from phenon K into which the type culture of *V.alginolyticus* UQM 2770 [Batch 1.] fell. This infers initial mis-classification of this culture by Baumann *et al.,* (1971).

V.natriegens UQM 879 (ATCC 14048) clustered with *(P.phosphoreum)* UQM 2482 (ATCC 11040) rather than with its more recent homologue from Batch [1.], UQM 2782. The putative type culture of *P.phosphoreum* UQM 2482 conformed more closely with *V.natriegens* as described in Baumann *et al.,* (1984) than with *P.phosphoreum* as summarized by Yang *et al.,* (1983) and *P.phosphoreum* UQM 140 from phenon F, which strongly implies that it was not *P.phosphoreum* UQM 2482 but possibly *V.natriegens* UQM 2782. The low similarity between UQM

Table 3.18.1: Comparative Euclidean Similarities for OTU's from Batches 1,2,4 & 5 Replicated Within- and Across- Batches.

OTU.	Accession Number.	Batch.	Euclidean Similarity %. Batch 1.	Batch 2.	Batch 4.	Batch. 5.
V.anguillarum	UQM 2771	1	94	94	94	79
ATCC 19264	UQM 2771	1	*	94	94	78
	UQM 2771	2	*	*	94	76
	UQM 2771	4	*	*	*	84
	UQM 2628	5	*	*	*	*
V.anguillarum	UQM 2843	1	NA	82	NA	NA
	UQM 2843	2	NA	*	NA	NA
V.cholerae	UQM 2772	2	NA	NA	88	NA
	UQM 2772	4	NA	*	NA	NA
A.hydrophila	UQM 2838	1	NA	90	NA	NA
	UQM 2838	2	NA	*	NA	NA
V.gazogenes	UQM 2840	1	97	NA	NA	NA
	UQM 2840	1	*	NA	NA	NA
V.natriegens	UQM 2782	1	NA	NA	*	NA
ATCC 14048	UQM 879	4	82	NA	75	NA
	UQM 2482 ?	4	65	NA	*	NA
V.ordalii	UQM 2890	4	NA	NA	90	NA
	UQM 2890	4	NA	NA	*	NA
V.harveyi	UQM 2781	1	NA	NA	86	NA
ATCC 14126	UQM 2766	4	*	NA	NA	NA
Vibrio sp.	UQM 2849	2	NA	99	NA	NA
	UQM 2849	2	NA	*	NA	NA
	B 1	4	NA	NA	80	NA
	B 1	4	NA	NA	*	NA
	B 2	4	NA	NA	96	NA
	B 2	4	NA	NA	*	NA
	Stn 6550	1	NA	NA	88	88
	Stn 6550	4	NA	NA	NA	89
	Stn 6550	5	*	NA	*	NA
	Stn 7320	4	NA	NA	94	89
	Stn 7320	4	NA	NA	*	92
	Stn 7320	5	NA	NA	*	*
	Stn 7360	2	NA	96	NA	NA
	Stn 7360	2	NA	*	NA	NA
	Stn 7600	4	NA	NA	NA	*
	Stn 7600	5	NA	NA	84	NA
	Stn 7920	2	NA	87	NA	NA
	Stn 7920	2	NA	*	NA	NA
	Stn 7960	2	NA	93	NA	NA
	Stn 7960	2	NA	*	NA	NA
	Stn 8010	5	NA	NA	NA	86
	Stn 8010	5	NA	NA	NA	*
	Stn 8030	2	NA	95	NA	NA
	Stn 8030	2	NA	*	NA	NA
	Stn 8200	5	NA	NA	NA	92
	Stn 8200	5	NA	NA	NA	*
	Stn 8260	2	NA	90	NA	NA
	Stn 8260	2	NA	*	NA	NA
	Stn 8270	2	NA	93	NA	NA
	Stn 8270	2	NA	NA	NA	NA

Table 3.18.2: Average Similarity ($S_{e.}$) for OTU's Replicated Within- and Across-Batches [1,2,4 & 5].

Batch.	Average Euclidean Similarity[α]. 1.	2.	4.	5.
1.	(2) 96 ± 1	(3) 89 ± 5	(3) 89 ± 3	(2) 82 ± 4
2.	*	(7) 93 ± 4	(1) 88	(1) 76 ?
4.	*	*	(4) 90 ± 6	(4) 89 ± 3
5.	*	*	*	(2) 89 ± 3

(n) Number of Determinations.
α. Including all ATCC homologues except UQM 2482?.

preparations of *V.natriegens* ATCC 14048 assayed in Batch [4.], cf. other internal Batch [4.] replicates indicates that the two cultures were phenetically divergent i.e. through genomic instability. Phenon U therefore represents *V.natriegens* rather than *P.phosphoreum* which was otherwise represented by the more typical, i.e. less nutritionally diverse, reference culture UQM 140 from Phenon F.

V.anguillarum UQM 2628 (ATCC 19264) was metabolically less versatile, i.e. than the other UQM preparation of the same culture. This may have been an artifact of its circuitous deposition (See Table 3.4). Because it was not directly associated with any OTU's, it is excluded from further discussion.

3.8.6.2.2.2 Diagnosis and Description of Phena.

Twenty-six phena and seven unassociated reference cultures were discretely resolved and all OTU's were fused at a similarity of 40 %. No provincially isolated OTU's were placed with *V.cholerae, V.metschnikovii, V.damsela, V.nereis, P.leiognathi, P.angustum, P.fischeri* or *P.shigelloides.* All OTU's grew at 25°C., and excluding the *Micrococcus* sp. Stn 8660, all were Gram-negative, and produced catalase; and all but one weak OTU fermented glucose. All grew in 1 and 3 % saline, but none produced brown pigment, could grow in the presence of haloquinol or could separately assimilate n-butyric acid, hexanoate or benzoate.

The Phena A and Z which held reference OTU's for *V.anguillarum* and *V.ordalii, (V.anguillarum Biovar II),* contained OTU's isolated only from Fish 32 and 33. These phena were quite distinct from each other but were respectively most like OTU's from phenon B, *V.cholerae,* and Phenon C, *Vibrio* sp. and OTU's from *V.splendidus* and Phenon E.

All OTU's from phena A,B and C were exceptional by their proportionally high production of acetoin and cholera-red; and absence of aesculin, arbutin and urea hydrolysis. Differential characters with attribute frequencies of 80 % or greater in phenon [A,B] included pellicle production, pH greater than 7.05 in MRVP medium, DNA hydrolysis, growth at pH 10, and separate utilization of l-glutamine, succinate, pyruvate and mannitol.

OTU's from phenon A produced ADH, oxidized gluconate, grew in the presence of 6 % saline and at 4°C. and separately attacked glutamic acid, glutamine and sorbitol. Those from phena B and C grew in the presence of polymyxin and at 42°C., and produced lipase. OTU's from phenon B differed from those in A and C by their positive cholera-red reaction, growth in the presence of dichlorophene, and upon 0.2 % SDS. OTU's from phenon C in general were less versatile than those in the A & B phena. With exception of production of arylsulphatase and growth on tetracycline by OTU's from phenon C, where comparisons were possible the positive attributes expressed in this phenon represented only a subset of those expressed in Phena A and B. The optimal growth temperature of OTU's from phenon C appeared nearer 37°C. than the usual assay temperature of 25°C. This implies that some test results may have suffered by culturing at less than 37°C.

Phenon C was not typical of any previously described species of *Vibrionaceae.* Apart from their higher optimal growth temperatures no OTU's from this phenon produced ADH, and only some decarboxylated ornithine and lysine at 25°C. All OTU's were susceptible to 0/129 phosphate at 10 µg/ml, and to novobiocin and trimethoprim, but all were resistant to streptomycin and penicillin. This phenon is like *V.cholerae* but atypical by its variable decarboxylation patterns. The recently described species *A.veronii*

(Hickmann-Brenner *et al.,* 1987) assayed at 36°C. was also characterized as *V.cholerae* like, but Phenon C differed from this species by absence of gas production, susceptibility to 0/129, and by not hydrolyzing aesculin. To validly compare OTU's from phenon C with *V.cholerae, A.veronii* and other *Vibrionaceae,* further work is required by molecular methods, but first through electron microscopy (i.e. for sheathed or unsheathed flagellum), and phenetically at optimum growth temperature. Because this phenon did not contain any provincial OTU's such effort was not justified here.

Phenon D, represented by type cultures from *V.nigripulchritudo* and *V.splendidus,* and phenon E, comprised provincial OTU's and the reference culture assigned UQM 3376 as *V.aestuarianus* Stn 7450 (3.8.4), were formed respectively with S_j coefficients of 65 and 60 %, and fused at *ca* 60 %. Eighty percent or more OTU's from these phena were positive for cytochrome oxidase ADH, hydrolysis of chitin, DNA and gelatin, growth in the presence of 0.2 % SDS and separate utilization of starch and succinate. Twenty percent or less OTU's from phena D and E were positive for luminescence, swarming, ODC, xanthine hydrolysis, sudanophilic inclusions, growth in 10 % saline, growth on tetracycline, haloquinol, separate utilization of remaining aliphatic amino acids except serine, all amines, all carbohydrates and sugar derivatives except the pentose arabinose, the hexoses glucose, mannose, galactose, and the disaccharides sucrose and trehalose, all aliphatic organic acids, dicarboxylic acids, and hydroxy acids, and remaining Kreb's cycle substrates, poly-alcohols, and aromatic acids and their derivatives. OTU's from phenon E were slightly more nutritionally diverse than those from Phenon D, but characters possessed by 80 % or more OTU's from phenon E, and by a lesser proportion of OTU's from Phenon D included growth at 37°C. and in 0.5 % saline, growth in the presence of brilliant green, SDS and separate utilization of maltose. Attributes expressed by 80 % or more OTU's from Phenon D, and by a lesser proportion of OTU's from phenon E included weak aesculin hydrolysis, egg yolk proteolysis, starch and serum hydrolysis growth in the presence of dichlorophene, and separate utilization of pyruvate.

OTU's from the phena D *[V.splendidus,/V.nigripulchritudo]* and E respectively attacked up to 15 and 24 of 59 comparable carbon sources also reported in Baumann *et al.,* (1984). These respectively did not correspond to the reported 32 and 23 of these comparable C sources reported by Baumann *et al.,* for Biovars I and II of *V.splendidus.* Most OTU's in phenon E further differed from Biovar II by the presence of an ADH reaction, and so would appear to belong to another non-luminescent species.

The pattern of attributes; ADH positive, resistance to polymyxin and sensitivity to 10 or less μg/ml 0/129 found for phenon E, was earlier also expressed by the unidentified *phenon 14* of West *et al.* (1986). Although differing by their acetoin production and their lesser nutritional facility, OTU's from phenon E seem more similar to those of West *et al.'s* phenon 14, than to other published descriptions; West *et al.* however did not comparatively examine type material from *V.aestuarianus,* so the possibility occurs that their *phenon 14* and phenon E here, may represent non-lactose utilizing OTU's of *V.aestuarianus* which supports the view that the provincial ADH positive and polymyxin resistant OTU, Stn 7450 (UQM 3376 from (3.8.4) is *V.aestuarianus.* Consequently Phenon E is assigned as lactose negative *V.aestuarianus.* A further OTU from this phenon, Stn 8370, was deposited as UQM 3359, *V.aestuarianus.*

Phenon F formed with a $S_{j.}$ of 60 %, and contained the reference culture *P.phosphoreum* UQM 140 and differed from OTU's in the phena D and E by its ONPG hydrolysis, narrower sodium tolerance (> 0 to < 6 % saline), by susceptibility to trimethoprim, and 0/129 at 10 or less μg/ml, by an absence of growth in the presence of basic fuchsin, brilliant green, dichlorophene and pyronin-Y, and by the failure of all OTU's to separately utilize any supplied carbon source.

This phenon differed from the growth factor-supplemented *P.phosphoreum* as described by Yang *et al.,* (1983) by attributes including ADH, hydrolysis of starch and gelatin, and by growth at 42°C. Typical and *exceptional,* nutritional facilities of *P.phosphoreum sensu stricto* expressed in this

Table 3.19.1: Batch 1,2,4 & 5, Attribute Frequencies for Phena A - I and Discrete Type or Reference OTU's.

Phenon.	A	B	C			D	E	F	G	H	I
Species.	*V.angu*	*V.chol*		*V.mets*	*V.dams*	*V.sple*	*V.aest*	*P.phos*	*A.hydr*	*V.natr*	*V.flu/fur*
Number of OTU's (n).	n = 12	n = 9	n = 12	n = 1	n = 1	n = 5	n = 13	n = 3	n = 19	n = 2	n = 14
Attribute.											
Colony diameter > 3 mm.	0	0	41	0	0	0	30	33	22/18	0	14
Colony mucoid.	8	0	0	0	0	0	23	0	0	0	0
Colony matt.	0	0	0	0	0	0	0	0	0	0	0
Colony opaque.	0	0	8	0	0	0	30	0	26	100	0/13
Colony entire.	100	100	91	100	100	80	100	100	100	100	100
Colony convex.	100	100	91	100	100	100	84	100	100	100	100
Swarming growth.	0	0	0	0	0	0	0	0	21	50	21
Luminous growth.	0	0	0	0	0	0	0	0	0	0	0
Red pigment.	0	0	0	0	0	0	0	0	0	0	0
Brown pigment.	0	0	0	0	0	0	0	0	0	0	0
Black pigment.	0	0	0	0	0	20	0	0	0	0	0
Yellow/orange.	0	0	0	0	0	0	0	0	0	0	0
Gram-positive.	0	0	0	0	0	0	0	0	0	0	0
Length > 2 times width.	100/ 3	100/ 1	ND	100	ND	100/ 2	ND	ND	60/ 5	0	66/ 6
Catalase.	100	100	100	100	100	100	100	100	100	100	100
Strong catalase.	0	11	0	0	0	0	0	0	10	0	7
Oxidase.	100	100	100	0	0	100	92	100	100	100	100
Oxidase (Toluene).	100	100	100	0	100	100	100	100	100	100	100
Broth uniform turbidity.	100	100	100	100	100	100	100	100	94	100	71
Broth with sediment.	91	55	100	0	100	100	100	66	100	100	92
Broth sediment mucoid.	0	22	0/ 2	0	100	20	0/ 8	0/ 1	0/18	0	16/12
Broth growth mod. to heavy.	66	87/ 8	0	0	0	100	92	100	100	100	92
Pellicle.	100	88	0	0	0	0	0	0	31	0	0
Indole.	91	44	83	100	0	0	30	33	10	0	7
Weak indole.	100	100	100	100	0	40	38	66	73	50	42
Motile.	100	100	100	100	100	100	100	100	94	100	92
Polar Flagellation.	100	100	100	100	100	100	100	100	100	100	100/13
5 day pH > 7.05.	91	100	0	0	0	0	0	0	42	0	50
5 day pH > 5.15.	100	100	66	100	100	20	7	0	52	50	92
Acetoin production.	75	88	91	100	100	0	38	0	21	0	28
Nitrate reduction.	100	100	100	0	100	100	100	100	94	100	100
Nitrite reduction.	66	33	0	0	0	60	61	66	89	100	92
Cholera-Red.	41	88	66	100	0	0	0	33	5	0	0
Gluconate oxidation.	83	66	16	0	0	0	0	0	36	0	42
Arginine dihydrolase.	91	0	0	100	100	100	84	33	100	0	100
Lysine decarboxylase.	8	100	66	100	100	0	23	100	57	50	0
Ornithine decarboxylase.	25	100	58	0	0	0	0	66	26	50	7
Glucose fermentation.	100	100	100	100	100	100	100	100	100	100	100
Gas from glucose.	0	0	0	0	0	0	0	0	47	0	0
ONPG hydrolysis.	100	100	83	100	100	100	23	100	84	50	92
Urea hydrolysis.	0	0	0	0	0	0	30	0	0	0	7
Aesculin hydrolysis.	0	0	0	0	0	40	7	66	68	50	92
Weak aesculin hydrolysis.	8	0	0	ND	0	100	76	66	68	50	92
Agar hydrolysis.	0	0	0	0	0	0	0	0	0	0	0
Egg Albumen hydrolysis.	100	66	75	0	0	40	23	33	15	0	78
Arbutin hydrolysis.	0	0	0	0	0	40	15	66	68	100	85
Aryl-sulphatase.	0	0	8	0	0	0	0	33	0	0	0
Casein hydrolysis.	100	88	100	100	100	100	36/11	50/ 3	93/15	100	83/12
Chitin hydrolysis.	100	100	100	100	0	100	84	100	100	100	100
Collagen hydrolysis.	80/ 5	ND	ND	ND	ND	0/ 2	0/ 6	ND	0/ 1	ND	0/ 3
DNA hydrolysis.	91	100	58	100	0	100	84	66	84	100	100
Gelatin hydrolysis.	100	100	100	100	0	100	100	100	100	50	100
Lecithinase.	100	100	80/10	100	0	20	69	100	84	0/ 1	100
Lipase.	58	100	91	100	100	100	100	100	78	100	100
Esterase (SDS).	0/10	100	50/ 2	0	100	40	76	100	42	100	71

Table 3.19.1; (cont.): Batch 1,2,4 & 5, Attribute Frequencies for Phena A - I and Discrete Type or Reference OTU's.

Phenon.	A	B	C			D	E	F	G	H	I
Species.	*V.angu*	*V.chol*		*V.mets*	*V.dams*	*V.sple*	*V.aest*	*P.phos*	*A.hydr*	*V.natr*	*V.flu/fur*
Number of OTU's (n).	n = 12	n = 9	n = 12	n = 1	n = 1	n = 5	n = 13	n = 3	n = 19	n = 2	n = 14
Attribute.											
Egg proteolysis.	100	88	100/11	100	100	100	70/10	33	100	100	100
Phosphatase.	100/11	100	88/ 9	100	100	100	100	100/ 2	100/18	100/ 1	100/12
Starch hydrolysis.	100	77	81/11	100	100	100	38	33	78	100	92
Sulphide production.	0	0	0/ 7	0	0	0	0/11	0/ 1	0/ 5	0	0/ 3
Serum hydrolysis.	81/11	100	ND	ND	0	100	33/ 6	0/ 1	63/11	ND	100/10
Tyrosine hydrolysis.	0	11	8	0	0	60	53	0	89	100	100
Tyrosine pigment.	8	0	0	100	0	60	23	33	21	100	71
Xanthine hydrolysis.	0	0	0	0	0	0	7	0	0	0	0
Congo Red Colonies Red.	0/ 2	100/ 3	ND	ND	100	ND	ND	ND	75/ 8	ND	50/ 2
Congo Red Colonies White.	50/ 2	0/ 3	ND	ND	0	ND	ND	ND	25/ 8	ND	50/ 2
Sudanophilic inclusions.	0	11	0	0	0	0	0	33	0/17	0	0
Growth 4°C.	100/ 5	0/ 4	8	0	0	0/ 2	0/ 7	0/ 2	44/18	0	0/11
Growth15°C.	100	100	72/11	100	100	100	92	33	100	100	100
Growth30°C.	100	100	91	100	100	100	100	100	100	100	100
Growth37°C.	100	100	100	100	100	40	92	66	100	100	100
Growth42°C.	0/ 9	100/ 4	91	100	100	0/ 3	0/ 7	33	83/18	100	90/11
Growth 0 % NaCl.	91	100	75	0	0	60	7	0	100	0	100
Growth 0.5 % NaCl.	100	100	91	100	100	40	100	66	100	100	100
Growth 1.0 % NaCl.	100	100	100	100	100	100	100	100	100	100	100
Growth 3.0 % NaCl.	100	100	83	100	100	100	100	100	100	100	100
Growth 6.0 % NaCl.	91	55	8	100	100	40	53	0	26	100	100
Growth10 % NaCl.	0	0	0	0	0	0	0	0	0	0	14
GrowthpH 10.	91	100	8	100	100	40	30	0	68	50	85
GrowthpH 4.5.	0	0	16	0	0	0	0	0	0	0	0
GrowthNovobiocin.	0	0	0	0	0	40	0	0	57	0	50
GrowthPenicillin.	91	77	100	0	100	80	100	100	100	100	100
GrowthPolymyxin.	8	100	100	0	100	0	76	33	26	50	7
GrowthStreptomycin.	100	100	100	100	100	100	100	100	100	100	100
GrowthSulphamethoxazole.	100	100	75	100	100	100	100	100	100	100	92
GrowthTetracycline.	0	0	50	0	0	0	7	0	0	0	21
GrowthTrimethoprim.	8	22	0	0	0	40	46	0	94	100	50
No growth10 µg/ml	0/129.	100	88	100	100	100	20	100	100	0	0	35
No growth150µg/ml	0/129.	100	100	100	100	100	60	100	100	5	100	85
GrowthBasic fuchsin.	58	100	41	0	0	100	84	0	100	50	78
GrowthBrilliantgreen.	60/ 5	0/ 6	0	0	0	0/ 4	85/ 7	0	61/18	100	100/11
GrowthDichlorophene.	16	88	8	0	100	100	61	0	100	100	92
GrowthEDDA.	41	33	8	0	0	40	15	33	21	100	57
Growthon Fast yellow.	100	88	ND	0	100	100	100/ 6	100/ 1	92/14	100	100/11
GrowthHaloquinol.	0	0	0	0	0	0	0	0	0	0	0
Growth8-Hydroxyquinoline	0	11	0	0	0	0	0	0	52	0	7
GrowthMethylviolet.	100/10	100/ 7	66	0	ND	100	100	33	94/17	100	100/13
GrowthPyronin-Y.	0	0	0	0	0	40	0	0	84	0	7
Growth0.2% SDS.	66	100	16	0	100	60	92	100	100	100	100
GrowthThionine.	50	55	0	0	0	40	46	0	57	50	100
Yellowon Thionine.	0/ 6	0/ 5	ND	ND	ND	0/ 2	0/11	0/ 1	33/ 6	ND	50/ 6
GrowthT.T.C.	100	100	100/11	0	100	100	100	100/ 2	100	100	100
Acid from Xylose.	0/ 3	0/ 1	ND	0	ND	0/ 2	ND	ND	0/ 3	0/ 1	ND
Acid from Arabinose.	100/ 3	0/ 1	ND	0	ND	0/ 2	ND	ND	33/ 3	100/ 1	ND
Acid from Mannose.	100/ 3	0/ 1	ND	100	ND	0/ 2	ND	ND	100/ 3	0/ 1	ND
Acid from Sucrose.	100/ 3	100/ 1	ND	100	ND	0/ 2	ND	ND	100/ 3	100/ 1	ND
Acid from Maltose.	100/ 3	100/ 1	ND	100	ND	100/ 2	ND	ND	100/ 3	100/ 1	ND
Acid from Cellobiose.	100/ 3	0/ 1	ND	0	ND	100/ 2	ND	ND	33/ 3	100/ 1	ND
Acid from Salicin.	0/ 3	0/ 1	ND	0	ND	0/ 2	ND	ND	100/ 3	100/ 1	ND
Acid from Mannitol.	100/ 3	100/ 1	ND	100	ND	100/ 2	ND	ND	100/ 3	0/ 1	ND
Acid from Sorbitol.	0/ 3	0/ 1	ND	0	ND	0/ 2	ND	ND	66/ 3	0/ 1	ND

Table 3.19.1; (cont.): Batch 1,2,4 & 5, Attribute Frequencies for Phena A - I and Discrete Type or Reference OTU's.

Phenon.	A	B	C			D	E	F	G	H	I
Species.	*V.angu*	*V.chol*		*V.mets*	*V.dams*	*V.sple*	*V.aest*	*P.phos*	*A.hydr*	*V.natr*	*V.flu/fur*
Number of OTU's (n).	n = 12	n = 9	n = 12	n = 1	n = 1	n = 5	n = 13	n = 3	n = 19	n = 2	n = 14
Attribute.											
Acid from Inositol.	0/ 3	0/ 1	ND	100	ND	0/ 2	ND	ND	0/ 3	0/ 1	0/ 2
Acid from Dulcitol.	0/ 3	0/ 1	ND	0	ND	0/ 2	ND	ND	0/ 3	0/ 1	ND
Growth Glycine.	0	11	0	0	0	20	0	0/ 2	57	100	100
Growth l-α-Alanine.	16	44	8	ND	0	0	7	0	78	100	100
Growthd-α-Alanine.	8	33	0	0	0	20	0	0	78	50	100
Growthdl-β-Alanine.	0	0	0	0	0	0	0	0	0/16	0/ 1	0/ 7
GrowthSerine.	8	0/ 8	8	ND	0	60	0	0	89	50	84/13
Growthl-Leucine.	0	0	0	0	0	0	0	0	5	0	57
GrowthValine.	0	0	0	0	0	0	0	0	5	100	28
Growthl+Glutamicacid.	100	77	41	100	0	60	30	0	94	100	100
Growthl-Lysine.	0	0	0	100	0	0	7	0	10	0	66/12
Growthl-Arginine.	66	11	8	0	0	20	0	0	63	100	100
Growthl-Ornithine.	0	0/ 8	8	100	0	0	0	0	5	50	100
Growthl-Citrulline.	25	22	8	0	0	0	0	0	42	50	92
Growth γ-Amino-butyrate.	0	0	0	0	0	0	0	0	44/18	0	92
Growthδ-Amino-valerate.	0	0/ 8	0	ND	0	0	0	0	0	100	28
Growthl-Proline.	58	55	0	0	100	0	7	0	100	100	100
GrowthPutrescine.	0	0	0	0	0	0	0	0	26	100	85
GrowthSarcosine.	0/10	0/ 7	0	0	ND	0	0	0	0/11	100	50/12
Growthl-Glutamine.	100/ 9	100/ 5	25	100	ND	0/ 2	14/ 7	0/ 2	100	100	100
Growthd-Glucosamine.	8	33	ND	0	100	0	14/ 7	0/ 1	88/17	0	83/12
Growthn-Acetylglucosamine	41	12/ 8	50	ND	100	0	30	0	78	100	84/13
Growthd-Ribose.	8	37/ 8	8	0	0	0	16/12	0	84	100	92
GrowthXylose.	0	0	0	0	0	0	0	0	0	0	21
Growthl-Arabinose.	41	0	0	0	0	0	0	33	57	100	100
Growthl-Rhamnose.	0	0	0	100	0	0	0	0	5/18	100	28
GrowthGlucose.	60/ 5	33/ 3	ND	ND	100	0/ 2	ND	ND	100/18	100	100
Growthd-Mannose.	33	12/ 8	8	100	100	40	30	0	68	50	92
Growthd-Galactose.	41	44	8	0	100	0	38	33	100	100	100
GrowthSucrose.	83	37/ 8	16	100	0	0	33/12	0/ 3	100	50	100
GrowthTrehalose.	25	44	8	ND	100	0	54/11	33	100	100	100
GrowthMaltose.	83/ 6	60/ 5	16	100	100	66/ 3	81/11	100/ 2	100	100	100
GrowthCellobiose.	58	0	0/ 7	ND	0	0	0/10	0/ 2	61/18	50	53/13
GrowthLactose.	0	0	0	0	0	0	0	0	5	0	7
GrowthRaffinose.	0	0	0	0	0	0	0	0	0	0	28
GrowthGalactarate.	0	0	ND	0	0	0	16/ 6	0/ 1	0/14	0	30/10
GrowthGluconate.	75	55	8	100	0	0	7	0	94	50	100
GrowthGlucuronicacid.	0/11	11	0	0	0	0	7	0	0	50	92
GrowthSalicin.	0	0	8	0	0	20	0	0	68	100	100
GrowthStarch.	60/ 5	25/ 4	100/ 2	100	0	100/ 4	100/ 1	ND	84	100	92/13
GrowthDextrin.	88/ 9	75/ 4	ND	100	100	0/ 2	ND	ND	100/14	100	100/11
GrowthInulin.	0	0/ 8	0	ND	0	0	0	0	0/18	0	0/13
GrowthPullulan.	8	0	0	100	100	20	30	0	89	100	92
GrowthFormate.	0	22	ND	100	0	0	0/ 6	0/ 1	14/14	0	58/12
GrowthAcetate.	0	0	0	100	0	0	7	0	52	100	100
GrowthPropionate.	0	0	0	0	0	0	0	0	5	0	35
Growthn-Butyricacid.	0	0	0	0	0	0	0	0	0	0	0
GrowthHexanoate.	0	0	0	0	0	0	0	0	0	0	0
GrowthHeptanoate.	0	0	0	0	0	0	0	0	0	0	0
GrowthCaprylicacid.	0	0	0	0	0	0	0	0	0	0	7
GrowthNonanionicacid.	0	11	ND	100	0	0	0/ 6	0/ 1	0/14	0	8/12
GrowthCaprate.	0	11	0	100	0	20	0/ 8	0/ 2	66/18	0	15/13
GrowthMalonate.	0	0	0	0	0	0	0	0	0	50	33/12
GrowthSuccinate.	100/ 7	100/ 5	0	100	100	100/ 1	88/ 9	0/ 1	100/14	ND	100/ 8
GrowthMaleate.	30/10	0/ 7	0	0	ND	40	7	0	66/ 9	0/ 1	85/ 7

Table 3.19.1; (cont.): Batch 1,2,4 & 5, Attribute Frequencies for Phena A - I and Discrete Type or Reference OTU's.

Phenon.	A	B	C			D	E	F	G	H	I
Species.	*V.angu*	*V.chol*		*V.mets*	*V.dams*	*V.sple*	*V.aest*	*P.phos*	*A.hydr*	*V.natr*	*V.flu/fur*
Number of OTU's (n).	n = 12	n = 9	n = 12	n = 1	n = 1	n = 5	n = 13	n = 3	n = 19	n = 2	n = 14
Attribute.											
Growth Adipic acid.	0	0	0	0	0	0	0	0	0	50	7
Growth Malate.	33	25/ 8	0	ND	0	0	0	0	89	100	92
Growth Tartarate.	0	0	0	0	0	0	0	0	0	0	11/ 9
Growth dl-3-OH-butyrate.	0	0	0	0	0	0	7	0	21	50	50
Growth 6-Hydroxycaproate.	0	0	0	0	0	0	0	0	0	0	0
Growth Lactate.	83/ 6	60/ 5	16	ND	100	0/ 2	71/ 7	50/ 2	88/18	100	100
Growth dl-Glyceric acid.	80/10	75/ 4	0	ND	0	50/ 2	50/ 8	0/ 2	89	50	100
Growth Poly-β-OH-butyrate.	0	0	0	0	0	0	0	0	0	0	14
GrowthCitrate.	10/10	14/ 7	100/ 1	ND	ND	0	33/ 9	0/ 1	71/14	100	100/13
Growthα-Ketoglutarate.	8	11	0	0	0	0	0	0	5	100	84/13
GrowthPyruvate.	100/ 3	100/ 4	0	ND	0	100/ 1	71/ 7	50/ 2	100/18	100	100
GrowthErythritol.	0/10	0/ 7	0	0	ND	0	0	0	0/ 8	ND	50/ 6
GrowthDulcitol.	0	0	0	0	0	0	0	0	0/16	0/ 1	12/ 8
GrowthMannitol.	83/ 6	80/ 5	8	100	0	0/ 2	28/ 7	0/ 2	100	100	100/13
GrowthSorbitol.	100/ 6	0/ 4	0	0	0	0/ 2	0/ 7	0/ 2	22/18	0	66/12
GrowthInositol.	0	0	0	0	0	0	0	0	0/16	ND	10/10
GrowthEthanol.	0/ 9	0/ 8	ND	0	0	0/ 3	0/ 6	0/ 1	22/ 9	ND	100/ 6
GrowthBenzoate.	0	0	0	0	0	0	0	0	0	0	0
GrowthHydroxybenzoate.	0	0	0	0	0	0	0	0	10	100	71
GrowthPhenylacetic acid.	0	0	0	0	0	0	0	0	5	0	0

phenon were, separate utilization of *arabinose,* galactose, *trehalose,* maltose, lactate, and pyruvate. Because Yang *et al.,* reported results from nearly 100 diversely isolated OTU's, phenon F most likely represents a poly-specific group, i.e. of *P.phosphoreum* and other *P.phosphoreum*-like OTU's.

Phenon G comprised 19 OTU's gathered at a $S_{j,}$ of 65 % and represented in similar proportions of both aero- and anaerogenic OTU's of *A.hydrophila*. Eighty percent or more OTU's from this phenon were positive for cytochrome oxidase, nitrate reduction to gas, ADH, ONPG, casein, chitin, DNA, gelatin, lecithin, starch and tyrosine hydrolysis, egg proteolysis, growth at 42°C., in the absence of sodium, in the presence of the folic acid emulators trimethoprim, and 0/129, in the presence of basic fuchsin, dichlorophene, methyl violet, pyronin-Y, SDS, and by separate utilization of the amino-acids of serine, l-glutamic acid, proline, and the amines l-glutamine, d-glucosamine, the pentose d-ribose, and hexoses glucose, galactose, the disaccharides sucrose, trehalose, maltose, and the sugar derivatives gluconate, starch, dextrin, pullulan, and the dicarboxylic acids succinate,

malate, lactate, dl-glyceric acid, pyruvate and mannitol. Twenty percent or less of OTU's were positive for cholera-red production, egg albumen hydrolysis, sulphide production, sudanophilic inclusions, growth in the presence of haloquinol, separate utilization of the aliphatic amino acids dl-β-alanine, leucine, valine, lysine, ornithine, δ-aminovalerate, sarcosine, glutamine; the sugars xylose, l-rhamnose, and lactose, and the sugar derivatives glucuronic acid, inulin, all aliphatic organic acids except caprate, malonate, tartarate, 6-hydroxycaprate, poly-β-hydroxy-butyrate, α-ketoglutarate, remaining polyalcohols except sorbitol, and all aromatic organic acids and their derivatives.

Phenon H comprised only the Batch [1.] OTU's *V.natriegens* UQM 2782 and *V.harveyi* Stn 6390, and was aggregated at a $S_{j.}$ of 75 %. In previous analyses (3.8.3) this doublet was only discerned by the ESS protocol.

Phenon U, ($S_{j.}$ *ca* 70 %), contained two provincially isolated OTU's and remaining reference OTU's for *V.natriegens* ATCC 14048, all assayed in Batch [4.] It differed from phenon H *[V.natriegens]* principally by attributes variably expressed in that phenon. Only starch hydrolysis and growth at 42°C. were expressed by all OTU's in that in Phenon H and 25 % or less in Phenon U. Characters positive in 75 % or more OTU's from Phenon U; but not expressed in Phenon H were separate utilization of dl-β-alanine, τ-aminobutyrate, d-glucosamine, inulin, pullulan, formate, tartarate and poly-β-hydroxybutyrate.

Phenon I, ($S_{j.}$ *ca* 75 %); contained 11 provincial isolates and type cultures for the ADH positive species *V.fluvialis* and *V.furnissii.* The dispersal of replicated OTU's of Stn 7710 from Batches [2.] and [5.] within this phenon prevented resolution of these species.

Phenon I was associated closely with the predominantly LDC positive phenon, J, containing 23 OTU's bound at $S_{j.}$ of *ca* 70 %, and represented by *V.alginolyticus* UQM 2770 and four correspondent replicates. Apart from their different decarboxylation patterns phena I and J had similar

Table 3.19.2: Batch 1,2,4 & 5, Attribute Frequencies for Phena J - S and Discrete Type or Reference OTU's.

Phenon.	J	K	L	M	N	O	P	Q	R		S
Species.	*V.algi*	*V.para*	*V.harv*				*V.comb*	*V.vuln*	*V.diaz*	*V.nere*	*V.pele*
Number of OTU's (n).	n = 23	n = 6	n = 43	n = 32	n = 6	n = 13	n = 6	n = 13	n = 5	n = 1	n = 2
Attribute.											
Colony diameter > 3 mm.	50/16	20/ 5	3/26	40	0/ 5	8/12	0	7	0	0	0
Colony mucoid.	0	0	2/42	0	0	0	0	0	0	0	0
Colony matt.	0	0	0/42	0	0	0	0	0	0	0	0
Colony opaque.	0/21	50	4/41	3/30	0/ 3	23	20/ 5	15	0	100	100
Colony entire.	60	100	80/42	84	100	100	100	100	100	100	100
Colony convex.	65	100	66/42	100	100	100	100	100	100	100	100
Swarming growth.	91	16	38/42	25	33	0	0	7	20	100	50
Luminous growth.	0	0	4/42	3	0	0	0	7	0	0	0
Red pigment.	0	0	0/42	0	0	0	0	0	0	0	0
Brown pigment.	0	0	0/42	0	0	0	0	0	0	0	0
Black pigment.	0	0	0/42	0	0	0	0	0	0	0	0
Yellow/orange.	0	0	0/42	0	0	0	0	0	20	0	0
Gram-positive.	0	0	0	0	0	0	0	0	0	0	0
Length > 2 times width.	100/ 1	100/ 1	37/ 8	ND	ND	100/ 1	100/ 4	50/ 8	ND	0	100
Catalase.	100	100	100	100	100	100	100	100	100	100	100
Strong catalase.	0	0	4	0	80/ 5	7	16	0	0	0	0
Oxidase.	100	100	100	100	100	100	100	100	100	100	100
Oxidase (Toluene).	100	100	100	100	100	100	100	100	100	100	100
Broth uniform turbidity.	100	100	90	96	100	100	100	92	100	100	100
Broth with sediment.	95	100	95	96	100	92	100	84	100	100	100
Broth sediment mucoid.	5/17	16	27/40	0/21	0	16/12	16	0/11	0	0	0
Broth growth mod. to heavy.	100	100	95	100	33	100	50	69	60	100	100
Pellicle.	65	66	25	9	0	0	0	7	0	0	0
Indole.	17	16	46	81	0	15	0	69	20	0	0
Weak indole.	95	100	100	100	83	69	50	100	80	100	0
Motile.	100	100	100	100	100	100/12	83	100	100	100	100
Polar Flagellation.	100/22	100	100	100	100/ 5	100/12	100/ 4	100/12	100	100	100
5 day pH > 7.05.	39	0	13	0	16	0	0	0	20	0	0
5 day pH > 5.15.	43	50	30/42	15	16	15	83	76	20	100	50
Acetoin production.	47	0	11	0	0	0	0	0	20	0	0
Nitrate reduction.	100	100	97	96	83	100	100	100	100	100	100
Nitrite reduction.	95	100	93	93	100	100	0	23	100	0	50
Cholera-Red.	0	0	27	0	0	0	0	0	40	0	0
Gluconate oxidation.	43	0	11	0	83	7	0	7	20	0	0
Arginine dihydrolase.	4	0	6	3	100	100	50	0	60	100	50
Lysine decarboxylase.	86	100	100	87	0	0	33	92	0	0	0
Ornithine decarboxylase.	69	100	100/42	78	0	0	0	100	20	0	0
Glucose fermentation.	100	100	100	100	100	100	100	100	100	100	100
Gas from glucose.	0	0	0	0	0	0	0	0	20	0	0
ONPG hydrolysis.	43	0	60	18	0	92	16	84	100	0	100
Urea hydrolysis.	0	16	51	68	0	7	0	0	0	0	0
Aesculin hydrolysis.	0	0	34	96	0	30	16	46	80	ND	50
Weak aesculin hydrolysis.	86	0	97	100	0	92	16	61	100	ND	100
Agar hydrolysis.	0	0	0	0	0	0	0	0	0	0	50
Egg Albumen hydrolysis.	47/21	66	60	0	100	69	16	38	0	0	0
Arbutin hydrolysis.	26	16	34	53	0	30	16	61	100	0	50
Aryl-sulphatase.	30	0	51	53	100	46	0	23	60	0	50
Casein hydrolysis.	94/18	100/ 5	93	96/29	100	91/12	100	90/10	25/ 4	100	0
Chitin hydrolysis.	100	100/ 5	95	90	100	76	100	100	20	100	100
Collagen hydrolysis.	ND	ND	43/16	ND	100/ 1	33/ 3	ND	ND	0/ 2	ND	ND
DNA hydrolysis.	86	83	97	81	16	92	100	84	40	100	100
Gelatin hydrolysis.	100	100	97/42	96/31	83	100	100	100	60	100	0
Lecithinase.	77/ 9	100/ 5	100/20	87/24	100	100/12	100	100/12	0	0	100
Lipase.	100	100	100	100	100	100	100	100	40	100	100
Esterase (SDS).	86	83	95	100	33	100	50	91/12	75/ 4	0	100

Table 3.19.2; (Cont.): Batch 1,2,4 & 5, Attribute Frequencies for Phena J - S and Discrete Type or Reference OTU's.

Phenon.	J	K	L	M	N	O	P	Q	R		S
Species.	*V.algi*	*V.para*	*V.harv*				*V.camb*	*V.vuln*	*V.diaz*	*V.nere*	*V.pele*
Number of OTU's (n).	n = 23	n = 6	n = 43	n = 32	n = 6	n = 13	n = 6	n = 13	n = 5	n = 1	n = 2
Attribute.											
Egg proteolysis.	100	50	53/30	53	100	100	100	92	100	0	100
Phosphatase.	100	100	100	96/30	100	100	100	100/12	80	ND	100/ 1
Starch hydrolysis.	95	83	100	100	66	100	100	76	60	0	50
Sulphide production.	0/11	0/ 5	0/37	0/ 3	0/ 4	0/11	0/ 5	0/ 8	0/ 3	0	0
Serum hydrolysis.	66/12	80/ 5	70/40	25/ 4	100	90/10	0/ 2	100/ 8	0/ 4	100	ND
Tyrosine hydrolysis.	95	100	97	96	83	69	83	30	0	100	100
Tyrosine pigment.	95	50	34	100	100	61	50	61	20	100	100
Xanthine hydrolysis.	17	0	0	0	66	76	0	0	20	0	0
Congo Red Colonies Red.	50/12	ND	100/ 4	50/ 4	60/ 5	ND	100/ 1	100/ 2	100/ 2	ND	ND
Congo Red Colonies White.	41/12	ND	0/ 4	0/ 4	40/ 5	ND	0/ 1	0/ 2	0/ 2	ND	ND
Sudanophilic inclusions.	0/21	0	2/42	0/30	0/ 5	0	0	15	0	0	0
Growth 4°C.	8	0/ 2	6/16	0	100/ 5	0/ 4	20/ 5	0	100/ 3	0	0
Growth15°C.	100	83	97	96	100	100	100	100	100	100	100
Growth30°C.	100	100	100	100	100	100	100	100	100	100	100
Growth37°C.	100	100	100	96	0	38	66	100	100	100	100
Growth42°C.	95	50/ 2	29/17	0	0	9/11	16	100	66/ 3	0	0
Growth 0 % NaCl.	4	50	2	0	0	7	0	92	100	0	50
Growth 0.5 % NaCl.	100	100	100	100	83	76	100	100	100	0	100
Growth 1.0 % NaCl.	100	100	100	100	100	100	100	100	100	100	100
Growth 3.0 % NaCl.	100	100	100	100	100	100	100	100	100	100	100
Growth 6.0 % NaCl.	100	100	93	93	100	69	83	15	100	100	50
Growth10 % NaCl.	60	0	9	0	83	0	16	0	20	0	0
GrowthpH 10.	82	66	93	25	100	46	16	92	40	100	100
GrowthpH 4.5.	0	0	0	6	0	7	0	7	0	0	0
GrowthNovobiocin.	0	0	0	3	0	0	0	7	20	0	0
GrowthPenicillin.	100	100	100	100	100	69	66	92	100	0	50
GrowthPolymyxin.	95	83	97	100	0	7	100	100	0	0	0
GrowthStreptomycin.	100	100	100	100	100	100	100	100	100	100	100
GrowthSulphamethoxazole.	100	100	97	100	100	100	100	100	100	100	100
GrowthTetracycline.	8	0	9/42	3	0	0	16	7	0	0	0
GrowthTrimethoprim.	65	100	100	96	66	23	66	53	0	100	100
No growth10 µg/ml 0/129.	60	0	4	78	33	84	50	84	100	100	50
No growth150µg/ml0/129.	95	100	90	100	33	100	100	100	100	100	100
GrowthBasic fuchsin.	65	100	93	81	33	38	0	15	0	0	0
GrowthBrilliantgreen.	95	50/ 2	61/13	53	80/ 5	8/12	0	0	100/ 3	100	0
GrowthDichlorophene.	69	100	95	50	83	100	33	0	40	0	100
GrowthEDDA.	39	16	59/42	9	0	15	50	38	20	0	50
Growthon Fast yellow.	100/13	100/ 5	100/42	100/ 4	83	100/10	100	88/ 9	100/ 4	100	50
GrowthHaloquinol.	0	0	0/42	0	0	0	0	0	0	0	0
Growth8-Hydroxyquinoline.	4	0	0/42	0	0	0	0	0	0	0	0
GrowthMethylviolet.	100/13	100	97/39	100/28	100/ 1	92	0/ 5	18/11	100/ 3	0	50
GrowthPyronin-Y.	8	16	21/42	0	0	0	0	0	0	0	0
Growth0.2% SDS.	100	100	100	100	100	100	100	92	60	0	100
GrowthThionine.	43	83	76	40	16	30	16	0	60	0	0
Yellowon Thionine.	77/ 9	100/ 5	80/31	45/22	0/ 1	60/ 5	100/ 1	0/ 3	0/ 3	ND	ND
GrowthT.T.C.	100/22	100	100	100/31	100	100	100	100	100/ 4	100	100
Acid from Xylose.	100/ 1	ND	0/ 4	ND	ND	0/ 1	0/ 4	0/ 7	ND	0	50
Acid from Arabinose.	100/ 1	ND	0/ 7	ND	ND	0/ 1	0/ 4	0/ 7	ND	0	0
Acid from Mannose.	100/ 1	ND	100/ 4	ND	ND	100/ 1	75/ 4	100/ 7	ND	100	100
Acid from Sucrose.	100/ 1	ND	50/ 4	ND	ND	0/ 1	25/ 4	14/ 7	ND	100	100
Acid from Maltose.	100/ 1	ND	100/ 4	ND	ND	100/ 1	100/ 4	100/ 7	ND	100	100
Acid from Cellobiose.	100/ 1	ND	100/ 4	ND	ND	100/ 1	75/ 4	71/ 7	ND	ND	100
Acid from Salicin.	0/ 1	ND	100/ 4	ND	ND	0/ 1	25/ 4	28/ 7	ND	100	100
Acid from Mannitol.	100/ 1	ND	100/ 4	ND	ND	100/ 1	75/ 4	100/ 7	ND	100	100
Acid from Sorbitol.	100/ 1	ND	100/ 4	ND	ND	0/ 1	0/ 4	14/ 7	ND	0	100

Table 3.19.2; (Cont.): Batch 1,2,4 & 5, Attribute Frequencies for Phena J - S and Discrete Type or Reference OTU's.

Phenon.	J	K	L	M	N	O	P	Q	R	S	
Species.	*V.algi*	*V.para*	*V.harv*				*V.camb*	*V.vuln*	*V.diaz*	*V.nere*	*V.pele*
Number of OTU's (n).	n = 23	n = 6	n = 43	n = 32	n = 6	n = 13	n = 6	n = 13	n = 5	n = 1	n = 2
Attribute.											
Acid from Inositol.	0/ 1	ND	0/ 4	ND	ND	0/ 1	0/ 4	0/ 7	ND	0	50
Acid from Dulcitol.	0/ 1	ND	0/ 4	ND	ND	0/ 1	0/ 4	14/ 7	ND	0	0
Growth Glycine.	100	83	95	100	100	69	66	0	0	100	50
Growth l-α-Alanine.	100	80/ 5	97	71	100	91/12	100	69	80	100	50
Growthd-α-Alanine.	100	83	97	96	100	92	83	53	60	100	50
Growthdl-β-Alanine.	31/16	16	0/36	0	0	0	0	0/11	0	0	0
GrowthSerine.	95	80/ 5	100	6	100	83/12	83	7	40	100	100
Growthl-Leucine.	100	33	23	0	0	0	0	0	0	100	0
GrowthValine.	0/18	0	9	0	0	0	0	7	0	0	50
Growthl+Glutamicacid.	100	100	100	96	100	100	100	100	100	100	100
Growthl-Lysine.	66/15	50	25/40	43	0	7	33	7	0/ 3	100	100
Growthl-Arginine.	100	83	92/42	53	100	61	83	23	80	100	100
Growthl-Ornithine.	78	83	48	0	0	30	0	15	80	100	100
Growthl-Citrulline.	95	66	65	3	66	27/11	66	46	60	100	100
Growthγ-Amino-butyrate.	47	50	6	0	16	0	0	0	20	0	50
Growthδ-Amino-valerate.	47	0	0	0	0	0	0	0	0	100	0
Growthl-Proline.	100	100	97	93	100	100	100	84	100	100	50
GrowthPutrescine.	82	83	4	0	16	7	0	0	40	100	100
GrowthSarcosine.	0/11	16	7/39	0/28	50/ 2	0/12	0/ 5	0/11	0/ 3	0	0
Growthl-Glutamine.	100	100	100	51/31	100	92	100	84	100	100	50
Growthd-Glucosamine.	95/21	100	45/42	100/ 4	83	0/11	33	90/10	80	100	100
Growthn-Acetylglucosamine.	100	100	95	78	100	76	50	69	100	100	50
Growthd-Ribose.	95	100	97	34	100	46	100	53	100	100	0
GrowthXylose.	17	0	2	0	0	0	0	0	80	0	0
Growthl-Arabinose.	82	83	6	0	100	7	0	0	80	0	50
Growthl-Rhamnose.	5/19	33	4/42	0	0	0	0	0	0	0	50
GrowthGlucose.	100	100	100/42	100/24	100	100/ 6	100	100/11	100	100	0
Growthd-Mannose.	86	83	62	100	0	100	83	92	60	0	100
Growthd-Galactose.	86	83	83	96	83	92	50	69	100	0	100
GrowthSucrose.	95/21	100	88	55/27	100	83/12	66	7	100	100	50
GrowthTrehalose.	100	100	69	100/30	100	84	83	92	80	100	100
GrowthMaltose.	100/22	100	100/42	85/28	100	100/ 8	100/ 5	100	100/ 3	100	100
GrowthCellobiose.	94/19	33	97	50/22	0	100/12	100	41/12	100	0	50
GrowthLactose.	21	0	4	0	0	38	0	15	80	100	0
GrowthRaffinose.	0	16	2	0	0	0	0	0	0	0	50
GrowthGalactarate.	0/ 9	0	17/41	0/ 4	0	0/11	0	11/ 9	0/ 3	0	0
GrowthGluconate.	100	100	100	100	100	100	50	92	100	100	50
GrowthGlucuronicacid.	73	83	93	87/31	16	23	0	15	40	0	0/ 1
GrowthSalicin.	69	16	27	18	0	23	16	23	60	0	50
GrowthStarch.	100	100	100	100/29	0/ 5	100/ 7	100	77/ 9	75/ 4	100	100
GrowthDextrin.	100/13	100/ 5	100/42	100/ 4	80/ 5	100/ 6	100	100/ 9	100/ 4	100	100
GrowthInulin.	0/21	ND	2	0	0	0/12	0	0	0	0	0
GrowthPullulan.	91	0/ 5	95	87	50	15	83	61	0	100	50
GrowthFormate.	92/14	66	4/42	0/ 4	50	9/11	0	0/ 9	50/ 4	0	0
GrowthAcetate.	95	16	0	3	50	0	0	7	60	0	0
GrowthPropionate.	17	33	0	0	0	0	0	0	0	0	0
Growthn-Butyricacid.	0	0	0	0	0	0	0	0	0	0	0
GrowthHexanoate.	0	0	0	0	0	0	0	0	0	0	0
GrowthHeptanoate.	0	0	0	0	0	0	0	0	0	0	0
GrowthCaprylicacid.	0	0	0	0	0	0	0	0	0	0	0
GrowthNonanionicacid.	14/14	0	0/42	0/ 4	0	9/11	0	0/ 9	25/ 4	0	0
GrowthCaprate.	0/18	0/ 5	0/42	0/14	0	8/12	0	0/10	0/ 4	0	0
GrowthMalonate.	33/18	16	10/39	0	0	0	0	0	0	0	0
GrowthSuccinate.	100/22	33	100/35	100	100	100/11	100/ 2	100/ 4	100	ND	ND
GrowthMaleate.	94/19	100/ 5	51/37	53/28	0/ 1	30	0/ 5	0/11	66/ 3	0	0

Table 3.19.2; (Cont.): Batch 1,2,4 & 5, Attribute Frequencies for Phena J - S and Discrete Type or Reference OTU's.

Phenon.	J	K	L	M	N	O	P	Q	R		S
Species.	*V.algi*	*V.para*	*V.harv*				*V.camb*	*V.vuln*	*V.diaz*	*V.nere*	*V.pele*
Number of OTU's (n).	n = 23	n = 6	n = 43	n = 32	n = 6	n = 13	n = 6	n = 13	n = 5	n = 1	n = 2
Attribute.											
Growth Adipic acid.	0/14	0	6	0	0	0	0	0	0	0	50
Growth Malate.	100	0	100	78	100	83/12	66	46	100	0	100
Growth Tartarate.	5/20	100	0/38	0	0	0	0	0	0	0	0/ 1
Growth dl-3-OH-butyrate.	21	0/ 5	0	0	0	0	0	0	20	0	0
Growth 6-Hydroxycaproate.	21	0	2	0	0	0	0	0	0	0	0
Growth Lactate.	100	0	100/42	100	100	100	100	92	100/ 4	100	100
Growth dl-Glyceric acid.	91	100	90/33	40	100/ 5	66/ 6	60/ 5	69	33/ 3	0	50
Growth Poly-β-OH-butyrate.	21	100	13	0	0	0	0	0	0	0	0
GrowthCitrate.	100/19	33	97/40	100/13	100	38	100	71/ 7	100/ 4	100	0
Growthα-Ketoglutarate.	4	66	20	0	0	7	66	53	0	100	100
GrowthPyruvate.	91	16	100	68	100	92	100/ 5	88/ 9	66/ 3	100	100/ 1
GrowthErythritol.	90/11	100	2/35	0/28	0/ 1	0	0/ 5	0/11	0/ 3	0	0
GrowthDulcitol.	21	0	0/41	0	0	0	0	0/12	0	0	0
GrowthMannitol.	100	0	100/35	62	100	91/12	83	69	100/ 4	0	50
GrowthSorbitol.	69	100	77/22	40	0/ 5	0/ 4	0/ 5	0	0/ 3	0	50
GrowthInositol.	26	100/ 2	2/42	0	0	0	0	0	0	0	0
GrowthEthanol.	91/12	0	11/34	0/ 4	0	0/10	0/ 2	0/ 2	0/ 4	ND	ND
GrowthBenzoate.	0	40/ 5	0	0	0	0	0	0	0	0	0
GrowthHydroxybenzoate.	21	0	6	0	0	0	0	0	0	0	0
GrowthPhenylaceticacid.	0	0	0	0	0	0	0	0	0	0	0

nutritional facility, but could be distinguished by characters positive in 90 % or more OTU's from I, but less common amongst OTU's from phenon J including ONPG, aesculin, DNA and lecithin hydrolysis, growth 0 % saline, growth ɣ-aminobutyrate, mannose, galactose and glucuronic acid. Characters positive in 90 % or more OTU's from phenon J but less common in phenon I included weak indole, casein hydrolysis, growth polymyxin, no growth 150 μg/ml 0/129, separate utilization l-leucine, d-glucosamine, cellobiose, formate and maleate.

The phena K, L and M with 6, 43 and 33 OTU's respectively were each formed with internal similarities of *ca* 75 % before juncture at *ca* 70 % S_j. Eighty percent or more OTU's from these phena separately utilized glucuronate, an attribute slightly less common in Phenon J. Other characters positive in 80 % or more OTU's from Phenon J and less common or not expressed in the phena K, L & M included growth on brilliant green and separate utilization of citrulline, formate, acetate, and ethanol.

OTU's replicated within the phena K, L and M (UQM 2849, ATCC 14126, Stn 6550, 8010, 8030, 7920, 7600, 7960) were placed discretely in concurrent phena with the exception of two variably dimorphic OTU's, Stn 6550, and Stn 7600. The isolated replicates, Stn 6550 from Batch [5.] (Phenon M) and Stn 7600 from Batch [4.] (Phenon L) were tentatively considered as mixed and removed from consideration.

Phenon K, contained all type and reference cultures of *V.parahaemolyticus.* Eighty percent or more OTU's from phenon K were positive for characters absent or variable in phenon L and M including serum hydrolysis, susceptibility to 10 μg/ml 0/129, separate utilization of ornithine, putrescine, l-arabinose, maleate, tartarate, poly-β-hydroxybutyrate, and the polyalcohols erythritol, sorbitol and inositol. Three attributes, weak aesculin hydrolysis and separate utilization of lactate, succinate and citrate were present in 80 % or more OTU's from phena L and M but absent from phenon K.

Additional characters positive as above in phena K and L but less common in Phenon M included growth in the presence of dichlorophene, and separate utilization of serine, arginine, ribose and dl-glyceric acid. Only three attributes, (separate utilization of d-glucosamine, mannose, and trehalose), were positive in phena K and M but not in phenon L. Six attributes, growth at pH 10, separate utilization cellobiose, malate, lactate, pyruvate and mannitol, were positive as above in phenon L but less common in K and M. Only one attribute, aesculin hydrolysis, was present in phenon M and but not in the phena K and L.

The location of the OTU, UQM 2849 putatively *V.harveyi,* but with a G + C of 41.9 mole % $T_{m.}$ (See A8.2) and similar to *V.carchariae* UQM 2920, into a phenon adjoining *V.parahaemolyticus* and *V.harveyi* strongly infers the possibility that phenon M may represent *V.carchariae.* Comparison of phenetic attribute frequencies of phena L and M with phenon I, (Table 3.14.1), comprising eight OTU's of *V.carchariae* from a previous analysis were insufficient to conclusively differentiate phena.

Comparison of DNA base ratios of the OTU Stn 6550, present in phena L and M, from (A9.2) indicated a G + C of 41.9, mole % $T_{m.}$ This is equivalent to that obtained from *V.carchariae,* UQM 2920 and consequently indicating either that species overlap occurred between these phena or Stn 6550 in phenon L was mixed. The former possibility though seems more probable when the incidence of urease production in the phena L and M, (respectively 51 and 66 %) is considered. Urease production is absent in *V.harveyi, sensu stricto,* but occurs in *V.carchariae.*

More than 50 % of OTU's from phena N and O hydrolyzed xanthine. Further common traits shared by 80 % or more OTU's from these phena were: Decarboxylation pattern of ADH + LDC - & ODC -, serum hydrolysis, growth on SDS and separate utilization of the amino acids l-α-alanine, d-α-alanine, serine, l+glutamic acid, l-proline, the amine glutamine, the sugars and sugar derivatives glucose, sucrose, trehalose, maltose, gluconate, and amongst dicarboxylic acids and hydroxy acids succinate, malate, lactate, pyruvate and only mannitol amongst polyalcohols. Less than 20 % of OTU's were positive for indole, acetoin, cholera-red and urease production, gas from glucose, agar hydrolysis, sudanophilic inclusions, growth at 42°C., growth in the presence of novobiocin, polymyxin, tetracycline, EDDA, haloquinol, 8-hydroxyquinoline, and pyronin-Y, and were unable to separately utilize the aliphatic amino-acids dl-β-alanine, leucine, valine, lysine, δ-aminovalerate, γ-aminobutyrate, the pentose xylose, the hexoses rhamnose, the trisaccharide raffinose, and the sugar derivatives galactarate and inulin, any aliphatic organic acids, the dicarboxylic acids malonate, adipate and tartarate, and the hydroxy acids dl-3-hydroxybutyrate, 6-hydroxycaproate, poly-β-hydroxybutyrate, the Kreb's cycle intermediate α-ketoglutarate, the remaining polyalcohols, or any aromatic organic acid derivatives.

Divisive attributes expressed in 80 % or more OTU's from Phenon N and absent or *variable* amongst OTU's from Phenon O included gluconate oxidation, *aryl-sulphatase, chitin* and *tyrosine hydrolysis,* tyrosine pigment, growth at

4°C.; in *6* and 10 % saline, at *pH 10,* in the presence of *penicillin,* and brilliant green, and by the separate attack of l-arginine, d-glucosamine, *n-acetylglucosamine, d-ribose,* arabinose and citrate. Characters positive in 80 % or more OTU's from Phenon O and *variable* or absent amongst OTU's from Phenon N included *DNA, SDS,* and *starch hydrolysis, susceptibility to 10 µg/ml 0/129* and by separate utilization of d-mannose, cellobiose and starch. The OTU's B1, B2 and B55 and tentatively identified in Batch [6.] (3.8.4) as *V.orientalis* were contained exclusively by phenon O. Phenon N contained OTU's examined in Batches [2.] and [4.] while phenon O contained OTU's from Batches [1.] and [4.]. It would seem on the basis of this overlap between batches that the phena N and O probably represent separate non-luminescent biogroups of *V.orientalis.*

Phenon P representing *V.campbellii,* and containing 6 OTU's was fused with phenon [N,O] at a $S_{j.}$ of *ca* 65 %. Eighty percent or more OTU's from this non-xanthinolytic phenon were positive for three characters absent or *variable* in the phenon [N,O] i.e. MRVP-pH greater than 5.15, and separate utilization of *mannose* and *pullulan.* Seven characters absent or *variable* in phenon P but positive in phenon [N,O] were nitrite reduction, *ADH,* serum hydrolysis, growth on *dichlorophene,* and methyl violet, and separate utilization of *sucrose,* and *malate.*

The association of the type culture for *V.vulnificus* with type cultures of *V.campbellii* assayed in Batches [1.] and [4.], rather than with other OTU's from *V.vulnificus* is consistent with its placement in the Batch [1.] analyses and also supports the earlier assignment of UQM 2730 to *V.vulnificus* (See 3.8.3).

Phenon Q, ($S_{j.}$ *ca* 70 %), grouped *V.vulnificus* UQM 2730 and twelve other OTU's. Eighty percent or more OTU's from this phenon were positive for the differential characters LDC, ODC, and hydrolysis of casein, DNA, ONPG, gelatin, lecithin, SDS and serum; egg proteolysis, growth at 42°C., at pH 10, in the absence of saline, in the presence of penicillin, polymyxin, streptomycin, susceptibility to 10 µg/ml 0/129, and separate utilization of l+glutamic acid, l-proline, l-glutamine, d-glucosamine, glucose, mannose,

trehalose, maltose, dextrin, succinate, lactate, and pyruvate. Twenty percent or less OTU's were positive for the characters indole, MRVP-pH > 7.05, production of acetoin and cholera-red, gluconate oxidation, arginine dihydrolase, sulphide production, xanthine hydrolysis, sudanophilic inclusions, growth at 4°C., in 10 % saline, at pH 4.5, in the presence of novobiocin, tetracycline, basic fuchsin, brilliant green, dichlorophene, haloquinol, 8-hydroxyquinoline, pyronin-Y, and separate utilization of glycine, dl-β-alanine, serine, leucine, valine, lysine, γ-aminobutyrate, δ-aminovalerate, putrescine, sarcosine, all pentoses except ribose, and the remaining sugars and sugar derivatives rhamnose, sucrose, lactose, galactarate, glucuronic acid, inulin, formate, all aliphatic organic acids, all remaining hydroxy-acids, all alcohols and polyalcohols except mannitol, and all aromatic acid derivatives.

These results were consistent with those of Reichelt *et al.,* (1976) by showing that most strains from *V.vulnificus* were unable separately to utilize sucrose or lactose. It is apparent that some strains from *V.vulnificus* can also be luminescent and produce lateral flagellation. This indicates an inadequacy in differential criteria proposed by Yang *et al.,* (1983) to distinguish *V.harveyi* from *V.vulnificus.* Here it is indicated that these species can be distinguished by the failure of *V.vulnificus* strains to utilize glucuronic acid.

The type culture of *V.diazotrophicus,* UQM 2780, was aggregated with four provincially isolated OTU's into phenon R ($S_{j.}$, 65 %). Eighty percent or more OTU's from phenon R were positive for the characters ONPG, weak aesculin hydrolysis, arbutin, egg proteolysis, growth at 4°C., in the absence of saline, in the presence of penicillin, streptomycin, and sulphamethoxazole, and were susceptible to 10 μg/ml 0/129, and separately able to utilize l-α-alanine, l-glutamic acid, l-arginine, l-ornithine, l-proline, l-glutamine, d-glucosamine, n-acetyl glucosamine, d-ribose, xylose, and arabinose, glucose, galactose, sucrose, trehalose, maltose,

cellobiose, lactose, gluconate, dextrin, succinate, malate, lactate, citrate, and mannitol. Twenty percent or fewer OTU's were positive for the characters, indole, gluconate reduction, ODC, gas from glucose, hydrolysis of urea, chitin, lecithin, serum, tyrosine, xanthine, production of sudanophilic inclusions, growth in 10 % saline, at pH 4.5, in the presence of novobiocin, polymyxin, tetracycline, trimethoprim, EDDA, haloquinol, 8-hydroxyquinoline, pyronin-Y. Twenty percent or fewer OTU's were able to separately utilize glycine, dl-β-alanine, leucine, valine, ɣ-aminobutyrate, δ-aminovalerate, sarcosine, rhamnose, raffinose, galactarate, inulin, pullulan, all aliphatic organic acids with the exception of nonanionate, and no remaining hydroxy-acid with the exception of maleate, α-ketoglutarate, and no remaining alcohols or polyalcohols or aromatic acid derivatives.

Phenon S contained only two OTU's, UQM 2785 *V.pelagius* and the tentatively designated (3.8.3) *V.nereis* Stn 7650, which were fused at a $S_{j.}$ of *ca* 63 % and placed adjacent to the type culture for *V.nereis* UQM 2783. The OTU Stn 7650 had intermediate phenetic appearance between these two species but apart from its lateral flagellation and ADH system Stn 7650 here more closely resembled *V.pelagius*. Other attributes which differentiate *V.pelagius* from *V.nereis* include weak indole production, ONPG hydrolysis, an absence of casein and gelatin hydrolysis, production of lipase, growth in 0.5 % saline, growth in the presence of dichlorophene and 0.2 % SDS, and acid production from sorbitol. Neither OTU from phenon S was able to separately utilize serine, δ-aminovalerate, glucose lactose or citrate.

Phenon T, formed with a $S_{j.}$ of *ca* 70 %, did not contain any previously described reference cultures. This phenon was associated with the cluster formed by [G-R] rather than with any single group. Despite the separation shown in Figure (3.8) between phenon T and phenon E and cluster [N,O] OTU's from these ADH positive and 0/129 sensitive phena were phenetically similar. Like Phenon E the oxidase reaction of OTU's from phenon T was enhanced through the action of toluene. OTU's from phenon T were distinguishable by the absence of xanthine hydrolysis, production of urease, resistance to polymyxin and greater nutritional facility compared to phenon E. At least 80

% of OTU's from phenon T could separately utilize glycine, dl-β-alanine, l-leucine, valine, l-citrulline, γ-aminobutyrate, δ-aminovalerate, putrescine, sarcosine, xylose, l-arabinose, rhamnose, lactose, raffinose, galactarate, glucuronic acid, salicin, growth inulin, formate, no aliphatic organic acids, adipic acid, malate, tartarate, dl-3-hydroxybutyrate, 6-hydroxycaproate, poly-β-hydroxybutyrate, α-ketoglutarate, erythritol, dulcitol, mannitol, inositol, ethanol, or aromatic acid derivatives. Consequently this phenon is also designated as a lactose negative biogroup of *V.aestuarianus* orphaned from phenon E by its greater nutritional facility. The OTU's from *V.aestuarianus II* deposited at UQM were Stn 6610, 7060 and 7300, as UQM 3358, 3361, and 3360 respectively.

The type culture of *P.fischeri* UQM 2889 was substantially different from the description of Baumann *et al.,* (1984). Characters for this OTU exceptional to that description have been *italicized.* UQM 2889 was positive for nitrate reduction, *gas production, gelatin hydrolysis, growth in the absence saline,* separate utilization of *l-α-alanine,* proline, n-acetylglucosamine, ribose, *xylose, l-arabinose, l-rhamnose, sucrose, gluconate, glucuronic acid, acetate, malate, lactate,* dl-glyceric acid, *citrate, α-ketoglutarate, pyruvate,* mannitol, and *sorbitol.* This OTU was negative for characters including swarming growth, *luminous growth, oxidase reaction, lipase,* growth at 4°C. no growth upon glycine, dl-β-alanine, serine, l-leucine, l+glutamic acid, l-arginine, l-ornithine, l-citrulline, γ-aminobutyrate, δ-aminovalerate, putrescine, sarcosine, *d- galactose, cellobiose,* salicin, n-butyrate, hexanoate, heptanoate, caprylate, nonanionate, caprate, malonate, tartarate, dl-3-hydroxybutyrate, citrate, inositol, ethanol, benzoate and hydroxybenzoate. These differences comprised almost a third of verifiable traits and in view of the additional hydrolytic enzyme facility and resistance to antibiotics compared to results from the replicate analysis conducted later (3.8.6.3) suggest that this OTU was mixed for at least some tests. If mixing did occur effects upon final distribution were not sufficient to prevent primary association of these replicates in the dendrogram Figure (3.7).

Table 3.19.3: Batch 1,2,4 & 5, Attribute Frequencies for Phena T - Z and Discrete Type or Reference OTU's.

Phenon.	T	U	V		W	X			Y	Z	
Species.		*V.natr*		*P.fisc*	*V.gazo*	*V.holl*	*P.leio*	*P.angu*	*V.cost*	*V.orda*	*P.shig.*
Number of OTU's (n).	n = 5	n = 4	n = 9	n = 1	n = 2	n = 2	n = 1	n = 1	n = 5	n = 4	n = 1
Attribute.											
Colony diameter > 3 mm.	20	0/ 2	0	0	0	50	0	0	0	0	0
Colony mucoid.	0	0	0	0	100	0	0	0	0	0	0
Colony matt.	40	25	22	0	0	0	0	0	0	25	0
Colony opaque.	100/ 4	50	11	0	100	0	100	0	0	50	0
Colony entire.	100	100	89	100	100	100	100	100	100	100	100
Colony convex.	100	100	89	100	100	100	100	100	100	100	0
Swarming growth.	0	0	0	0	0	0	0	0	0	0	0
Luminous growth.	0	0	0	0	0	0	0	0	0	0	0
Red pigment.	0	0	11	0	100	0	0	0	0	0	0
Brown pigment.	0	0	0	0	0	0	0	0	0	0	0
Black pigment.	0	0	0	0	0	0	0	0	0	0	0
Yellow/orange.	40	50	11	0	0	0	0	0	0	0	0
Gram-positive.	0	0	0	0	0	0	0	0	0	0	0
Length > 2 times width.	ND	ND	50/ 2	ND	100	ND	ND	ND	ND	100/ 1	ND
Catalase.	100	100	100	100	100	100	100	100	100	100	100
Strong catalase.	0	25	44	100	0	0	0	0	0	100	0
Oxidase.	80	75	22	0	0	100	100	100	80	0	100
Oxidase (Toluene).	100	75	56	0	0	100	100	100	100	75	100
Broth uniform turbidity.	100	100	100	100	100	100	100	100	100	75	100
Broth with sediment.	60	100	100	100	0	50	0	100	100	75	100
Broth sediment mucoid.	33/ 3	25	0	0	100	0/ 1	0	0	0	0	ND
Broth growth mod. to heavy.	100	100	89	0	0	0	0	0	80	100	100
Pellicle.	0	0	33	0	0	0	0	0	0	0	0
Indole.	20	0	0	0	0	100	0	0	0	25	0
Weak indole.	100	0	12/ 8	100	0	100	0	0	60	75	0
Motile.	100	100	89	100	100	100	0	100	100	75	0
Polar Flagellation.	100	100	89	100	100	100	100	100	100	75	100
5 day pH > 7.05.	0	75	78	0	100	0	0	0	0	0	0
5 day pH > 5.15.	60	75	89	0	100	100	0	0	80	25	0
Acetoin production.	0	25	68	0	100	50	100	100	20	0	0
Nitrate reduction.	100	100	100	100	0	100	100	100	100	100	100
Nitrite reduction.	60	100	100	100	0	0	0	0	60	75	0
Cholera-Red.	0	25	0	0	0	50	0	0	80	0	0
Gluconate oxidation.	20	50	78	0	0	0	0	0	0	0	0
Arginine dihydrolase.	100	25	89	0	0	0	0	0	20	0	100
Lysine decarboxylase.	0	0	22	100	0	0	0	0	0	100	100
Ornithine decarboxylase.	0	0	56	0	0	0	0	0	0	50	100
Glucose fermentation.	100	100	100	100	100	100	100	100	100	100	100
Gas from glucose.	0	0	78	100	0	0	0	0	0	25	0
ONPG hydrolysis.	0	50	89	0	100	0	0	100	0	25	0
Urea hydrolysis.	80	0	0	0	0	0	0	0	0	0	0
Aesculin hydrolysis.	60	75	22	0	0	0	0	0	0	25	0
Weak aesculin hydrolysis.	100	75	56	0	100	0	0	100	40	25	0
Agar hydrolysis.	0	0	0	0	0	0	0	0	0	0	0
Egg Albumen hydrolysis.	40	50	33	0	100	0/ 1	0	0	0	0	0
Arbutin hydrolysis.	80	75	56	0	100	0	0	0	20	0	0
Aryl-sulphatase.	0	0	22	0	0	0/ 1	0	0	0	0	0
Casein hydrolysis.	100	50	57	0	100	0/ 1	0	0	0	0	0
Chitin hydrolysis.	40	0	50	0	ND	50	100	0	20	0	0
Collagen hydrolysis.	0/ 2	100/ 2	0/ 3	ND	ND	ND	ND	ND	0/ 1	ND	0
DNA hydrolysis.	100	100	78	100	100	50	100	100	100	50	0
Gelatin hydrolysis.	100	100	78	100	100	100	0	0	60	50	100
Lecithinase.	100	33/ 3	44	0	0	100	0	0	20	0	0
Lipase.	100	50	56	0	100	0/ 1	0	0	0	0	0
Esterase (SDS).	100	50	11	0	0	ND	100	0	100/ 4	0	100

Table 3.19.3; (Cont.): Batch 1,2,4 & 5, Attribute Frequencies for Phena T - Z and Discrete Type or Reference OTU's.

Phenon.	T	U	V	W	X			Y	Z	
Species.		*V.natr*		*P.fisc*	*V.gazo*	*V.holl*	*P.leio*	*P.angu*	*V.cost*	*V.orda*	*P.shig.*
Number of OTU's (n).	n = 5	n = 4	n = 9	n = 1	n = 2	n = 2	n = 1	n = 1	n = 5	n = 4	n = 1
Attribute.											
Egg proteolysis.	60	100	100/ 8	100	100	100/ 1	0	0	0/ 4	75	0
Phosphatase.	100/ 3	100	100/ 8	100	100/ 1	100/ 1	100	100	100	75	100
Starch hydrolysis.	100	0	11	0	100	0/ 1	0	0	0	0	0
Sulphide production.	0/ 3	0/ 3	0/ 4	0	0	0/ 1	0	0	0	0/ 3	0
Serum hydrolysis.	33/ 3	25	83/ 6	0	100/ 1	0/ 1	0	0	0	0/ 2	ND
Tyrosine hydrolysis.	100	100	56	100	0	0	0	100	75/ 4	25	0
Tyrosine pigment.	100	75	11	0	0	0	0	0	0/ 4	0	0
Xanthine hydrolysis.	0/ 4	0	0	0	0	0/ 1	0	0	0	25	0
Congo Red Colonies Red.	0/ 1	ND	0/ 1	ND	ND	ND	ND	ND	ND	ND	ND
Congo Red Colonies White.	100/ 1	ND	0/ 1	ND	ND	ND	ND	ND	ND	ND	ND
Sudanophilic inclusions.	0	0	0	0	0	0	100	100	0	0	0
Growth 4°C.	0/ 3	ND	60/ 5	0	0	0	ND	ND	ND	0	0
Growth15°C.	100	100	100	100	100	0	100	100	100	100	100
Growth30°C.	100	100	100	100	100	100	100	100	100	100	100
Growth37°C.	60	100	100	100	100	100	0	0	60	100	100
Growth42°C.	0	0/ 2	ND	ND	100	0/ 1	0	0	0/ 2	100	100
Growth 0 % NaCl.	0	75	100	100	0	0	0	0	0	100	100
Growth 0.5 % NaCl.	100	100	100	100	100	50	100	100	80	100	100
Growth 1.0 % NaCl.	100	100	100	100	100	50	100	100	100	100	100
Growth 3.0 % NaCl.	100	100	100	100	100	50	100	100	100	100	100
Growth 6.0 % NaCl.	80	100	100	100	100	0	0	0	60	100	0
Growth10 % NaCl.	0	0	22	0	100	0	0	0	20	0	0
GrowthpH 10.	60	25	44	0	0	0	0	0	60	0	0
GrowthpH 4.5.	0	50	22	0	0	0	0	0	0	0	0
GrowthNovobiocin.	0	75	100	100	0	0	0	0	0	75	0
GrowthPenicillin.	60	100	100	100	100	0	100	100	0	100	100
GrowthPolymyxin.	100	0	57	0	0	0	0	0	0	25	0
GrowthStreptomycin.	100	75	100	100	100	50	100	100	100	75	100
GrowthSulphamethoxazole.	100	100	100	100	100	100	100	100	100	100	100
GrowthTetracycline.	0	25	78	100	0	0	0	0	20	100	0
GrowthTrimethoprim.	20	100	67	100	0	50	0	0	80	50	0
No growth10 µg/ml 0/129.	80	0	0	0	0	100	100	100	0	0	100
No growth150µg/ml0/129.	100	50	44	0	100	100	100	100	100	25	100
GrowthBasic fuchsin.	0	50	100	100	0	50	100	100	60	100	0
GrowthBrilliantgreen.	0	ND	80/ 5	ND	0	0	ND	ND	0/ 2	100/ 2	0
GrowthDichlorophene.	0	100	100	100	100	0	100	100	80	100	0
GrowthEDDA.	0	50	100	100	100	0	0	0	0	50	0
Growthon Fast yellow.	66/ 3	100	100/ 4	100	0	100/ 1	100	100	100	100/ 2	ND
GrowthHaloquinol.	0	0	22	0	0	0	0	0	0	0	0
Growth8-Hydroxyquinoline.	0	0	78	0	0	0	0	0	0	50	0
GrowthMethylviolet.	0/ 4	100	100	100	50	0	0	0	80	100	100
GrowthPyronin-Y.	0	50	78	100	0	0	0	0	0	75	0
Growth0.2% SDS.	100	100	100	100	0	0	100	100	80	100	100
GrowthThionine.	20	100	100	100	0	0	100	100	100	75	0
Yellowon Thionine.	0/ 4	25	57/ 7	100	ND	ND	0	100	60	66/ 3	ND
GrowthT.T.C.	100/ 4	100	100	100	100	0/ 1	100	100	100	100	100
Acid from Xylose.	ND	ND	100/ 1	ND	100	ND	ND	ND	ND	ND	ND
Acid from Arabinose.	ND	ND	100/ 1	ND	100	ND	ND	ND	ND	ND	ND
Acid from Mannose.	ND	ND	100/ 1	ND	100	ND	ND	ND	ND	ND	ND
Acid from Sucrose.	ND	ND	100/ 1	ND	100	ND	ND	ND	ND	ND	ND
Acid from Maltose.	ND	ND	100/ 1	ND	100	ND	ND	ND	ND	ND	ND
Acid from Cellobiose.	ND	ND	100/ 1	ND	100	ND	ND	ND	ND	ND	ND
Acid from Salicin.	ND	ND	100/ 1	ND	100	ND	ND	ND	ND	ND	ND
Acid from Mannitol.	ND	ND	100/ 1	ND	100	ND	ND	ND	ND	ND	ND
Acid from Sorbitol.	ND	ND	100/ 1	ND	0	ND	ND	ND	ND	ND	ND

Table 3.19.3; (Cont.): Batch 1,2,4 & 5, Attribute Frequencies for Phena T-Z and Discrete Type or Reference OTU's.

Phenon.	T	U	V	W	X			Y	Z	
Species.		*V.natr*		*P.fisc*	*V.gazo*	*V.holl*	*P.leio*	*P.angu*	*V.cost*	*V.orda*	*P.shig.*
Number of OTU's (n).	n = 5	n = 4	n = 9	n = 1	n = 2	n = 2	n = 1	n = 1	n = 5	n = 4	n = 1
Attribute.											
Acid from Inositol.	ND	ND	0/ 1	ND	0	ND	ND	ND	ND	ND	ND
Acid from Dulcitol.	ND	ND	0/ 1	ND	0	ND	ND	ND	ND	ND	ND
Growth Glycine.	0	100	11	0	0	0	0	0	0	0	0
Growth l-α-Alanine.	80	100	100	100	100	50	0	0	40	0	0
Growthd-α-Alanine.	40	100	100	0	0	0	0	0	0	0	0
Growthdl-β-Alanine.	0	75	11	0	0	0	0	0	0	0	0
GrowthSerine.	60	100	78	0	0	50	0	0	0	0	0
Growthl-Leucine.	0	50	11	0	0	0	0	0	0	0	0
GrowthValine.	0	75	0	0	0	50	0	0	0	0	0
Growthl+Glutamicacid.	100	100	100	0	100	50	0	ND	100	0/ 3	0
Growthl-Lysine.	25/ 4	75	0	0	0	0	0	0	0	0	0
Growthl-Arginine.	40	100	11	0	0	0	0	0	0	0	0
Growthl-Ornithine.	40	100	0	0	0	0	0	0	0	0	0
Growthl-Citrulline.	0	100	0	0	0	0	0	0	0	0/ 3	0
Growthγ-Amino-butyrate.	0	100	67	0	50	0	0	0	0	0	0
Growthδ-Amino-valerate.	0	50	33	0	0	0	0	0	0	0	0
Growthl-Proline.	40	100	100	100	50	50	0	100	20	50	0
GrowthPutrescine.	0	75	67	0	0	0	0	0	0	0	0
GrowthSarcosine.	0/ 4	75	0/ 8	0	0	0	0	0	0	0	0
Growthl-Glutamine.	50/ 4	100	100	ND	100	50	ND	ND	100/ 2	0/ 2	0
Growthd-Glucosamine.	33/ 3	100	100	100	0	100/ 1	0	0	20	0/ 2	ND
Growthn-Acetylglucosamine.	40	100	100	100	100	50	0	0	40	0	0
Growthd-Ribose.	20	100	100	100	100	0	0	0	40	0	0
GrowthXylose.	0	50	78	100	0	0	0	0	0	0	0
Growthl-Arabinose.	0	100	67	100	100	50	0	0	0	0	0
Growthl-Rhamnose.	0	75	67	100	0	0	0	0	0	0	0
GrowthGlucose.	ND	100	100	100	50	ND	ND	ND	ND	ND	ND
Growthd-Mannose.	40	100	100	100	100	50	0	0	40	0	0
Growthd-Galactose.	80	100	100	0	100	50	0	0	0	0	0
GrowthSucrose.	80	100	100	100	100	50	0	0	80	0/ 3	0
GrowthTrehalose.	60	100	100	100	100	50	0	0	40	0	0
GrowthMaltose.	75/ 4	100/ 3	100	100	100	50	ND	ND	100/ 2	0/ 1	0
GrowthCellobiose.	60	100	100/ 6	0	100	0	0	0	20	0/ 3	0
GrowthLactose.	0	25	78	0	0	0	0	0	0	0	0
GrowthRaffinose.	0	75	0/ 8	0	0	0	0	0	0	0	0
GrowthGalactarate.	0/ 3	75	40/ 5	100	50	0/ 1	0	0	0	0/ 2	ND
GrowthGluconate.	80	100	100	100	0	50	0	100	40	0	0
GrowthGlucuronicacid.	0	100	100	100	100	0	0	0	60	0	0
GrowthSalicin.	0	75	89	0	100	0	0	0	0	0	0
GrowthStarch.	100	100/ 3	66/ 3	ND	100	ND	ND	ND	ND	ND	ND
GrowthDextrin.	100/ 3	100/ 3	100/ 5	ND	100	ND	ND	ND	ND	ND	ND
GrowthInulin.	0	100	0	0	0	0	0	0	0	0	0
GrowthPullulan.	40	75	11	0	0	0	0	0	0	0	0
GrowthFormate.	0/ 3	75	0/ 6	0	0	0/ 1	0	0	0	0/ 2	ND
GrowthAcetate.	0	25	78	100	100	0	0	0	0	0	0
GrowthPropionate.	0	25	44	0	0	0	0	0	0	0	0
Growthn-Butyricacid.	0	0	0	0	0	0	0	0	0	0	0
GrowthHexanoate.	0	25	0	0	0	0	0	0	0	0	0
GrowthHeptanoate.	0	25	22	0	0	0	0	0	0	0	0
GrowthCaprylicacid.	0	25	22	0	0	0	0	0	0	0	0
GrowthNonanionicacid.	0/ 3	50	57/ 7	0	0	0/ 1	0	0	0	0/ 2	ND
GrowthCaprate.	0/ 3	33/ 3	44	0	0	0	0	0	0	0	0
GrowthMalonate.	0	75	22	0	0	0	0	0	0/ 3	0	0
GrowthSuccinate.	100/ 4	100	100/ 8	ND	ND	50	ND	ND	ND	0/ 2	0
GrowthMaleate.	75/ 4	100	88/ 8	0	0	0	0	0	20	0	0

Table 3.19.3: (Cont.): Batch 1,2,4 & 5, Attribute Frequencies for Phena T - Z and Discrete Type or Reference OTU's.

Phenon.	T	U	V	W	X			Y	Z	
Species.		*V.natr*		*P.fisc*	*V.gazo*	*V.holl*	*P.leio*	*P.angu*	*V.cost*	*V.orda*	*P.shig.*
Number of OTU's (n).	n = 5	n = 4	n = 9	n = 1	n = 2	n = 2	n = 1	n = 1	n = 5	n = 4	n = 1
Attribute.											
Growth Adipic acid.	0	50	0	0	0	0	0	0	0	0	0
Growth Malate.	0	100	100	100	0	50	0	0	40	0	0
Growth Tartarate.	0	75	0	0	0	0	0	0	0	0	0
Growth dl-3-OH-butyrate.	20	75	44	0	0	0	0	0	0	0	0
Growth 6-Hydroxycaproate.	0	50	0	0	0	0	0	0	0	0	0
Growth Lactate.	33/ 3	100	100	100	100	50	ND	ND	100/ 2	0/ 2	0
Growth dl-Glyceric acid.	60	100	88/ 8	100	0	50	ND	ND	ND	0/ 2	0
Growth Poly-β-OH-butyrate.	0	100	0	0	0	0	0	0	0	25	0
GrowthCitrate.	33/ 3	100	100	0	100	100/ 1	0	0	0	0/ 2	ND
Growthα-Ketoglutarate.	0	25	56	100	100	0	0	0	0	0	0
GrowthPyruvate.	100	100	100	100	100	50	ND	ND	100/ 1	0/ 2	0
GrowthErythritol.	0/ 4	25	0/ 7	0	0	0	0	0	0	0	0
GrowthDulcitol.	0	66/ 3	25/ 8	0	0	0	0	0	0	0/ 3	0
GrowthMannitol.	0/ 3	100	100	100	100	0/ 1	ND	ND	100/ 3	0/ 2	0
GrowthSorbitol.	33/ 3	100	100	100	100	0/ 1	ND	ND	ND	0/ 2	0
GrowthInositol.	0	75	38/ 8	0	0	0	0	0	0	0	0
GrowthEthanol.	0/ 3	75	0/ 5	0	ND	0/ 1	0	0	0	0/ 2	ND
GrowthBenzoate.	0	0	0	0	0	0	0	0	0	0	0
GrowthHydroxybenzoate.	0	25	0	0	0	0	0	0	0	0	0
GrowthPhenylaceticacid.	0	0	22	0	0	0	0	0	0	0	0

Phenon V comprised nine OTU's fused at a similarity of *ca* 65 % and then joined with the potentially mixed culture of *P.fischeri* UQM 2889 at *ca* 63 % similarity. Attributes shown by some OTU's from phenon V were exceptional to *P.fischeri* and *Vibrionaceae sensu stricto* and so were examined separately.

Some OTU's from phenon V in primary screening (Table 3.6) and subsequent assay (Table 3.19.3) were oxidase-negative and appeared to have peritrichous flagellation, however electron-micrographic examination of the red pigmented OTU, Stn 2000 deposited as UQM 3256, indicated sheathed polar as well as unsheathed lateral flagellation (Plate 3.8), indicating that at least some of these OTU's were not from the *Enterobacteriaceae.*

Seventy percent or more OTU's from Phenon V were positive for 15 differential characters absent in *P.fischeri* including 5d pH greater than 7.05, gluconate oxidation, ADH, ONPG, serum hydrolysis, growth in the presence of 8-hydroxyquinoline, separate attack of d-α-alanine, serine, l+glutamic acid, d-galactose, cellobiose, lactose, salicin, maleate, and citrate. *P.fischeri* UQM 2889 was positive, and OTU's from phenon V negative

or *variable,* for characters including *LDC, tyrosine hydrolysis* and *growth in the presence of trimethoprim.*

The ephemeral presence of luminescence in Stn 1800, UQM 3242, at presumptive screening was exceptional to other OTU's in this phenon and may be a rare trait, or an indication that phenon V is polyspecific. Further taxonomic evaluation of remaining OTU's requires electron microscopic and or molecular examination of morphological and genotypic characteristics. Phenon W contained only reference OTU's for *V.gazogenes* UQM 2840 from the initial dataset and is described and the attributes displayed in section 3.8.6.3.

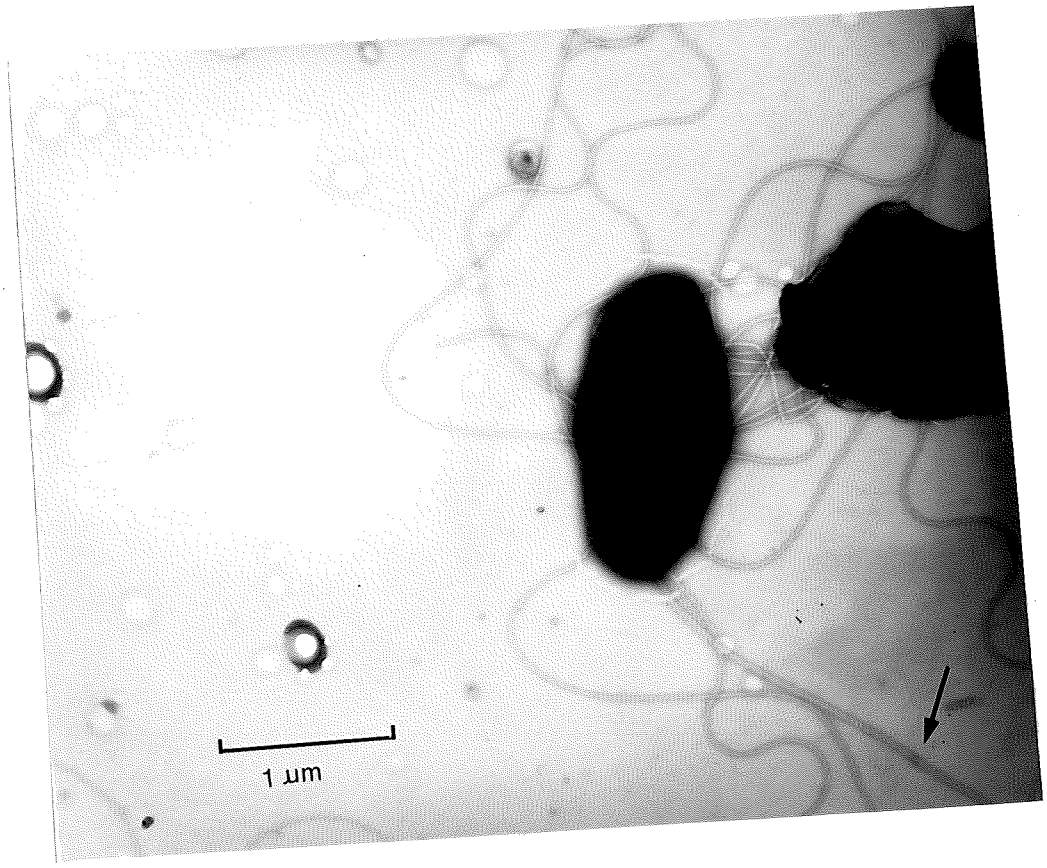


Plate 3.8: Electron Micrograph of the Red pigmented OTU Stn 2000 UQM 3256 (Phenon V), Indicating Sheathed Polar and Unsheathed Lateral Flagellation.

Phenon X, fused the reference culture for *V.hollisae* and the external fish isolate Be 12 with a $S_{j.}$ of 60 %. This phenon could be differentiated by the absence of decarboxylase reactions, the ability to grow in the temperature range 25° to 42°C. and in 0 - 3 % saline. Both OTU's were

susceptible to 10μg/ml 0/129, penicillin and polymyxin, but grew in the presence of EDDA. At 25°C. 21 carbon sources were attacked by at least one OTU from this phenon, however only d-glucosamine and citrate were used by both OTU's.

The otherwise unassociated *Photobacterium* species *P.leiognathi* UQM 2892 and *P.angustum* UQM 2893 formed a primary linkage with a S_j of 75 %. These OTU's could be distinguished from remaining phena by the absence of any ADH, ODC and LDC reactions or indole production, both OTU's produced acetoin and reduced nitrate only to nitrite, hydrolyzed DNA and produced phosphatase, and sudanophilic inclusions, but neither produced gelatinase, lecithinase, lipase, egg proteolysis, amylase, or xanthine hydrolysis. Growth occurred up to 30°C. and in between 0.5 and 3 % saline, and between pH 4.5 and 10. OTU's were susceptible to novobiocin, polymyxin, tetracycline, trimethoprim, 10 μg/ml 0/129 and haloquinol, but resistant to penicillin, streptomycin and sulphamethoxazole. Only one strain was able to utilize any of the carbon sources assayed. *P.leiognathi* was positive and *P.angustum* negative for chitin hydrolysis and SDS esterase. Apart from its separate utilization of proline, *P.angustum* differed from *P.leiognathi,* by its motility, ONPG and weak aesculin, tyrosine and thionine hydrolysis.

Phenon Y depicted as *V.costicola* comprised 5 OTU's including the type culture UQM 2888 and was fused with a similarity of 55 % prior to grouping with the aggregation of previously described phena and reference OTU's. Eighty percent or more OTU's from this phenon were positive for the characters weak cytochrome oxidase, motility, nitrate reduction, cholera-red reaction, DNA'ase, SDS esterase, growth in 0.5-3 % saline, susceptibility to novobiocin, penicillin, polymyxin, no growth 150μg/ml 0/129; and for separate utilization of glutamic acid, sucrose, maltose, lactate and pyruvate. Twenty % or less OTU's from this phenon were positive for characters including indole, acetoin, ADH, LDC, ODC; hydrolysis of ONPG, urea, casein, egg lipids, starch, serum, and xanthine; growth at 42°C., in 10 % saline, in the presence of penicillin, novobiocin, polymyxin,

trimethoprim and 150μg/ml 0/129. No OTU was able to separately utilize serine, valine, ornithine, putrescine, xylose, arabinose, rhamnose, lactose, acetate, propionate, tartarate, erythritol, benzoate or hydroxybenzoate.

Provincial OTU's from phenon Y differed from the description by Garcia *et al.,* (1987) most notably by not having been isolated from hyper-saline environments, by the absence of ADH reactions and by lesser temperature tolerances. This phenon was additionally exceptional to Garcia's *V.costicola* by the absence of OTU's which separately attacked serine, ornithine, acetate and propionate and consequently placement in this phenon may represent a gathering of weak OTU's rather than *V.costicola* i.e. an artifact rather than a taxonomic aggregate and so are designated as *V.costicola*-like.

Phenon Z, *V.ordalii* fused with a similarity of *ca* 55 %, and comprised four OTU's, represented by UQM 2906, Stn 8510 and replicates of UQM 2890. Like phenon [D,E] OTU's from phenon Z had extremely limited nutritional facility. Seventy-five percent or more OTU's from this phenon were positive for characters absent or *variable* in D and E including growth at 42°C., in *0 and 6* % saline, and by resistance to polymyxin, and tetracycline. OTU's from the phenon [D,E] were 80 % or more positive and those from phenon Z absent or variable for the characters cytochrome oxidase, ADH, hydrolysis of chitin, *DNA,* and *gelatin.*

The unassociated OTU for *Plesiomonas shigelloides* UQM 1134 differed from phenon Z by the concurrent presence of ADH, LDC and ODC, and by growth in the presence of 10 μg/ml 0/129, by its failure to grow in 6 % saline, susceptibility to tetracycline, and inability to separately utilize any of the assayed carbon sources.

3.8.6.2.2.3 Differential Potential of Collagen and Congo Red Tests.

Information from Tables 3.19.1-3 for for collagen hydrolysis shows that the character is usually positive in *V.anguillarum* and negative in *V.aestuarianus.* Other species including *V.splendidus, A.hydrophila, V.fluvialis, V.orientalis, V.diazotrophicus, V.natriegens, V.costicola* and *Photobacterium*-like species were assayed too infrequently to ascertain the

differential potential. Almost half the OTU's assigned to *V.harveyi* hydrolyzed collagen perhaps suggesting the use of this character to distinguish *V.harveyi* and *V.carchariae.*

Colony colour on congo red medium, proposed in the literature both as a virulence indicator and predictor of nitrogen fixation (Appendix 6.) was not found to distinguish between virulent and non-virulent species.

Colonies from the Batch [2.] OTU's grown on congo red medium were variably coloured amongst all species assayed except *V.cholerae, V.vulnificus* and *V.diazotrophicus;* which were coloured red. *V.diazotrophicus* however was originally described as a beneficial symbiont so these findings are contrary to uses of applying these tests i.e. red colour on congo red medium does not always indicate virulence and absence of white colouration only precludes nitrogen fixation under test conditions.

3.8.6.2.2.4 Summary of Results.

Most phena were defined by $S_{j.}$ coefficients ranging between 60 and 75 %. In order of abundance provincial OTU's were assigned to the species *V.harveyi* or *V.carchariae, V.alginolyticus, V.aestuarianus (I & 2), V.orientalis, A.hydrophila, V.fluvialis,* the genus *Photobacterium,* and the further species *V.anguillarum, V.vulnificus, V.diazotrophicus, V.natriegens, V.splendidus, V.costicola*-like, *V.campbellii, V.gazogenes, V.hollisae, V.ordalii* and *V.pelagius.*

With the exception of sub-optimally reared Benalla isolates from phenon C all unidentified imported OTU's were collated with extant species. No OTU's (in pure culture) were placed with reference OTU's from *V.cholerae, V.metschnikovii, V.damsela, V.parahaemolyticus, V.nereis, P.fischeri, P.angustum, P.leiognathi,* or *Plesiomonas shigelloides.*

More than 30 % of provincial OTU's were aggregated with reference cultures of the species *V.alginolyticus, V.harveyi* and *V.carchariae,* none were associated with the related, (Reichelt *et al.,* 1976), species *V.parahaemolyticus.* The phenon containing the type culture of *V.alginolyticus* embraced all replicated provincial OTU's, however another

reference OTU from this species, Baumann's blister isolate UQM 2675, was separated from this phenon and instead placed between replicated type cultures of *V.harveyi* which implied its initial misclassification.

The ptosis of unmatched replicates into phena holding reference OTU's from *V.harveyi* and *V.carchariae* was initially attributed to mixed cultures; however G + C determinations of two OTU's from these phena, UQM 2849 and Stn 6550, indicated that both were consistent with the type culture for *V.carchariae.* Consequent to these findings it was concluded that species overlap occurred between phenon L and phenon M. No valid phenetic segregation could be made between these species without further examination of test variability between replicates, by a program such as that of Bryant *et al.,* (1986a) and subsequent reanalysis of OTU's assigned within these phena.

Cross-batch variation also prevented differentiation between biovars of *V.fluvialis, [V.fluvialis* and *V.furnissii]* however overlap between these species was localized and not subject to intrusion by other species such as *V.alginolyticus* with similar nutritional facility.

Less than 10 provincial OTU's were placed with the archaetypal *V.anguillarum,* however *ca* 25 % of OTU's were placed into phena assigned as *V.aestuarianus, V.fluvialis,* and *V.orientalis* which would in the past have been classified as *V.anguillarum.*

Two loosely bound phena (V and C), were detected which require further taxonomic evaluation. The first contains OTU's which phenetically resembled *Photobacterium* spp. but which were exceptional by the presence of mixed flagellation. The second contained OTU's which may have been spuriously placed because of sub-optimal culture conditions.

A diagnostic schedule for gram-negative fermentative bacteria with polar flagella and comprising 65 further cross-batch replicated characters pooled from Tables 3.19.1-3 is shown in Table 3.20. The characters selected represent in similar proportions different aspects of the phenetic profiles from taxonomic types e.g. of hydrolytic enzyme activities, physiological

Table 3.20: Summary Frequency Table for the Comparatively Rapid Diagnosis of Groups Within the *Vibrionaceae*.

	V. acsndsaaphnfapcocvdnpoghlacosnhpeapeehyallaaraueeetaoenorhgo.tmlssodtugr/imlarl.zligsdiul sse srrviahepnzeasoloutagie cen12poiinhanbioigpgigsile
Swarming.	0 0 0 0 0 0 0 0 0 0 2 3 2 9 2 3 1 0 1 2 A 5 0 0 0 0 0 0 0 0
Red Pigment.	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 A 0 0 0 0 0 0
Strong catalase.	0 0 0 0 0 0 0 0 0 0 0 2 0 0 0 1 2 2 0 0 0 0 A A 0 0 0 0 A 0
Oxidase.	A A A 0 0 A A 8 A A 8 A A A A A A A A A A 2 0 A A A 8 0 A
Weak oxidase.	A A A 0 A A A A A A 8 A A A A A A A A A 6 0 0 A A A 8 A
Indole.	9 4 8 A 0 0 3 2 3 1 0 1 2 2 6 5 0 7 2 0 0 0 0 A 0 0 0 3 A
Weak indole.	A A A A 0 4 4 A 7 7 2 4 A A A 7 5 A 8 A 0 0 A 0 A 0 0 8 0
Polar flagellation.	A A A A A A A A A A A A A A A A A A A A 1 A A A A A 8 A
MRVP > 7.05.	9 A 0 0 0 0 0 0 0 4 8 5 4 0 1 1 0 0 2 0 0 8 A 0 0 0 0 0 0
MRVP > 5.15.	A A 6 A A 2 1 6 0 5 9 9 4 5 2 1 8 8 2 A 5 9 A A 0 0 8 3 0
Acetoin.	8 9 9 A A 0 4 0 0 2 5 3 5 0 1 0 0 0 2 0 0 7 A 5 A A 2 0 0
Nitrate Reduction.	A A A 0 A A A A A 9 A A A A A A 8 9 A A A A 0 A A A A A A
Nitrite Reduction.	6 3 0 0 0 6 6 6 7 9 A 9 A A 9 A 0 2 A 0 5 A 0 0 0 0 6 8 0
Cholera Red Production.	4 9 6 A 0 0 0 0 3 1 2 0 0 0 2 0 0 0 4 0 0 0 0 0 5 0 0 8 0 0
Gluconate Oxidation.	8 6 2 0 0 0 0 0 2 0 4 3 4 4 0 1 3 0 1 2 0 0 0 8 0 0 0 0 0 0 0
ADH Production.	9 0 0 A A A 8 A 3 A 2 A 0 0 0 A 5 0 6 A 5 9 0 0 0 0 2 0 A
LDC Production.	1 A 7 A A A 0 2 0 A 6 2 0 9 A 9 0 3 9 0 0 0 0 2 0 0 0 0 0 A A
ODC Production.	3 A 6 0 0 0 0 0 0 7 3 2 1 7 A 9 0 0 A 2 0 0 6 0 0 0 0 0 5 0
Gas from Glucose.	0 0 0 0 0 0 0 0 0 5 0 0 0 0 0 0 0 0 0 2 0 0 0 8 0 0 0 0 0 3 0
ONPG Hydrolysis.	A A 8 A A A 2 0 A 8 5 9 4 0 4 6 2 8 A 0 A 9 A 0 0 A 0 3 0
Urease Production.	0 0 0 0 0 0 3 8 0 0 0 1 0 2 6 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0
Aesculin Hydrolysis.	0 0 0 0 0 4 1 6 7 7 7 9 0 0 6 2 2 5 8 N 5 2 0 0 0 0 0 3 0
Weak aesculin Hydrolysis.	1 0 0 N 0 A 8 A 7 7 7 9 9 0 A 6 2 6 A N A 6 A 0 0 A 4 3 0
Aryl_sulphatase Produced.	0 0 1 0 0 0 0 0 3 0 0 0 3 0 5 6 0 2 6 0 5 2 0 0 0 0 0 0 0
Chitin hydrolysis.	A A A A 0 A 8 4 A A 3 A A A 9 8 A A 2 A A 5 N 5 A 0 2 0 0
DNA'ase.	9 A 6 A 0 A 8 A 7 8 A A 9 8 9 7 A 8 4 A A 8 A 5 A A A 5 0
Starch hydrolysis.	A 8 8 A A A 4 A 3 8 3 9 A 8 A 9 A 8 6 0 5 1 A 0 0 0 0 0 0
Xanthine hydrolysis.	0 0 0 0 0 0 1 0 0 0 0 0 2 0 0 8 0 0 2 0 0 0 0 0 0 0 0 3 0
Growth at 4oC.	A 0 1 0 0 0 0 0 0 4 0 0 1 0 0 5 2 0 A 0 0 6 0 0 N N N 0 0
Growth at 42oC.	0 A 9 A A 0 0 0 3 8 3 9 A 5 1 1 2 A 6 0 0 N A 0 0 0 0 A A
Growth at 0 % NaCl.	9 A 8 0 0 7 1 0 0 A 5 A 4 5 0 5 0 9 A 0 5 A 0 0 0 0 0 A A
Growth at 6 % NaCl.	9 6 1 A A 4 5 8 0 3 A A A A 9 8 8 2 A A 5 A A 0 0 0 6 A 0
Growth at 10 % NaCl.	0 0 0 0 0 0 0 0 0 0 0 1 6 0 1 3 2 0 2 0 0 2 A 0 0 0 2 0 0
Growth at pH 10.	9 A 1 A A 4 3 6 0 7 3 9 8 7 6 6 2 9 4 A A 4 0 0 0 0 6 0 0
Growth Novobiocin.	0 0 0 0 0 4 0 0 0 6 5 5 0 0 0 0 0 1 2 0 0 A 0 0 0 0 8 0 0
Growth Polymyxin.	1 A A 0 A 0 8 A 3 3 3 1 A 8 A 1 A A 0 0 0 6 0 0 0 0 0 3 0
Growth Tetracycline.	0 0 5 0 0 0 1 0 0 0 2 2 1 0 1 0 2 1 0 0 0 8 0 0 0 0 2 A 0
No growth 10 µg/ml 0/129.	A 9 A A A 2 A 8 A 0 0 4 6 0 4 7 5 8 A A 5 0 0 A A A 0 0 A
No growth 150 µg/ml 0/129.	A A A A A 6 A A A 1 6 9 A A A 8 A A A A A 4 A A A A A 3 A
Growth Basic Fuchsin.	6 A 4 0 0 A 8 0 0 A 5 8 7 A 9 4 0 2 0 0 0 A 0 5 A A 3 A 0
Growth Dichlorophene.	2 9 1 0 A A 6 0 0 A A 9 7 A 8 9 3 0 4 0 A A A 0 A A 8 A 0
Growth Haloquinol.	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 2 0 0 0 0 0 0 0
Growth 8_OH-quinoline.	0 1 0 0 0 0 0 0 0 5 0 1 0 0 0 0 0 0 0 0 0 8 0 0 0 0 0 5 0
Growth Methylviolet.	A A 7 0 N A A 0 3 9 A A A A A 9 0 2 A 0 5 A 5 0 0 0 8 A A
Growth l_α_Alanine.	2 4 1 N 0 0 1 8 0 8 A A A 8 8 9 A 7 8 A 5 A A 5 0 0 4 0 0
Growth dl_β_Alanine.	0 0 0 0 0 0 0 0 0 0 0 5 0 3 2 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0
Growth l_glutamic acid.	A 8 4 A 0 6 3 A 0 9 A A A A A A A A A A A A A 5 0 N A 0 0
Growth γ_Aminobutyrate.	0 0 0 0 0 0 0 0 0 0 4 7 9 5 5 1 1 0 0 2 0 5 7 5 0 0 0 0 0 0
Growth δ_Aminovalerate.	0 0 0 0 0 0 0 0 0 0 0 7 3 5 0 0 0 0 0 0 A 0 3 0 0 0 0 0 0 0
Growth Sarcosine.	0 0 0 A N 0 0 0 0 0 0 8 5 0 2 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0
Growth l_Glutamine.	A A 3 A N 0 1 5 0 A A A A A 8 9 A 8 A A 5 A A 5 N N A 0 0
Growth l_Ribose.	1 4 1 0 0 0 2 2 0 8 A 9 A A 7 6 A 5 A A 0 A A 0 0 0 4 0 0
Growth l_Xylose.	0 0 0 0 0 0 0 0 0 0 0 3 2 2 0 0 0 0 0 0 8 0 0 8 0 0 0 0 0 0 0
Growth l_Arabinose.	4 0 0 0 0 0 0 0 3 6 A A 8 8 0 4 0 0 8 0 5 7 A 5 0 0 0 0 0 0
Growth l_Sucrose.	8 4 2 A 0 0 3 8 0 A 8 A A A 8 9 7 1 A A 5 A A 5 0 0 4 0 0
Growth l_Lactose.	0 0 0 0 0 0 0 0 0 0 1 2 1 2 0 0 3 0 2 8 A 0 8 0 0 0 0 0 0 0
Growth Glucuronic acid.	0 1 0 0 0 0 1 0 0 0 8 9 7 8 9 2 0 2 4 0 0 A A 0 0 0 6 0 0
Growth Starch.	6 3 A A 0 A A A N 8 A 9 A A A 7 A 8 8 A A 7 A N N N N N N
Growth Pullulan.	1 0 0 A A 2 3 4 0 9 8 9 9 0 A 9 2 8 0 A 5 1 0 0 0 0 0 0 0 0
Growth dl_3_OH-butyrate.	0 0 0 0 0 0 1 2 0 2 7 5 2 0 0 0 0 0 0 2 0 0 4 0 0 0 0 0 0 0 0
Growth Lactate.	8 6 2 N A 0 7 3 5 9 A A A 0 A A A 9 A A A A A 5 N N A 0 0
Growth α_Ketoglutarate.	1 1 0 0 0 0 0 0 0 1 5 8 0 7 1 0 7 5 0 A A 6 A 0 0 0 0 0 0
Growth Pyruvate.	A A 0 N 0 A 7 A 3 A A A A 2 A 9 A A 6 A A A A 5 N N A 0 A
Growth Mannitol.	8 8 1 A 0 0 3 0 0 A A A A 0 8 A A 9 A 0 5 A A 0 N N A 0 0
Growth Sorbitol.	A 0 0 0 0 0 0 3 0 2 7 6 7 A 6 0 0 0 0 0 0 5 A A 0 N N N 0 0

Percent equivalents: 0, 0-4; 1, 5-14; 2, 15-24; 3, 25-34%; 4, 35-44; 5, 45-54; 6, 55-64; 7, 65-74; 8, 75-84; 9, 85-94; A, 95-100.

(N - Not Determined, *Italics* 2 or 1 OTU's.).

growth ranges and chemical tolerances, and activities towards different classes of carbon sources. In this table, percentage attribute frequencies have been rounded to correspond to 11 equally dispersed single digit numbers 0-A, except that 0 includes frequencies of 0-4 %, and A, frequencies between 95 and 100 %. Fragmented phena from *V.natriegens* and *V.orientalis* have been pooled as has data from the phena L and M which contained OTU's identified as *V.harveyi* or *V.carchariae,* but frequencies of OTU's in phenon T nominated to the second, more versatile biogroup of *V.aestuarianus* have been separately maintained. Strain data from *P.fischeri* was not shown in this table because of its aberrant appearance relative to the type culture. Because frequencies derived from 2 or less OTU's are clearly less substantive than those from larger phena, the reader has been alerted to these by placing these records in *italics.*

The characters from this table differentiate most phenotypes including biogroups of *V.aestuarianus;* however, if purely probabilistic interpretation is made the phenotypes of OTU's from *V.fluvialis* and *V.alginolyticus* may be confused. In such cases the ADH, LDC and ODC reactions of these species are stable and substantive primary differential criteria.

3.8.6.2.3 Discussion.

3.8.6.2.3.1 Replicates.

Unlike the large scale replicate analysis, which contained variably replicated OTU's from immiscible batches, (3.8.5), this more restrictive analysis had a far less complicated structure. Some loss of resolution was encountered but this was amongst the species *V.alginolyticus, V.parahaemolyticus, V.harveyi* and *V.carchariae,* and *V.fluvialis* and *V.furnissii.* The first three and the latter two of these common species have been shown by Reichelt *et al.,* (1976) and Brenner *et al.,* (1983), respectively, to be genotypically more closely related to each other than to other *Vibrio* species and so empirically vindicate the analysis procedure for the differentiation of typically dispersed *Vibrionaceae.*

In similar analysis finer resolution of taxa may be obtained by any one, or synergistically improved by a combination of the procedures which follow. Additional tests such as for decarboxylase reactions at primary screening would allow like OTU's to be segregated. This approach alone is not suitable because localized skewing effects might be selectively hidden amongst the phenetic profiles of OTU's from different batches so that prior selection criteria would seem to be validated by the resultant artifactual classification.

A program like that of Bryant *et al.,* (1986a) as qualified in 3.8.5 and perhaps aided by scalar rather than binary system for scoring strain postures may circumvent these problems. However resorting to such a program to separate these species for later ecological evaluation here might be avoided because most cross-batch variation was evident between the species *V.carchariae* and *V.harveyi.* An arbitrary sub-division of OTU's from both these phena could be made on the basis of a single character, such as urease production, absent from *V.harveyi sensu stricto* but found in most strains of *V.carchariae* (3.8.4). Using this criterion OTU's from these phena could be secondarily classified as urease positive or negative *V.carchariae/V.harveyi.* Alternatively, separate analysis of locally valid data in discrete batches into phena designated by cross-batch replicated OTU's tested by molecular methods would also increase resolution.

The separation which occurred amongst different UQM preparations of *V.natriegens* and *V.anguillarum* type cultures, seemed to have been less influenced by cross-batch test-skewing than other extrinsic factors accruing from storage in, and movement between, different culture collections and leading to phenetic or genotypic attrition of these cultures. This phenetic divergence of stored cultures has previously been reported for *V.anguillarum* ATCC 19264 by West *et al.,* (1983) and *V.pelagius* (3.8.5.3). Similar changes which occurred to a lesser extent amongst provincial OTU's may have been induced by loss of specific permeases and aggravated by inability of OTU's to cross-feed in multi-well trays or it may have resulted

from plasmid shedding as suggested in 3.8.5. These effects may be partially isolated by the addition of growth supplements such as pyridoxal hydrochloride or casamino acids to culture inoculation media. Such modifications were not considered here because consequent skewing effects might have rendered data from subsequent analyses incompatible with the *Rosetta* OTU's. However the application of the Jaccard coefficient, recoding of entire batch records as ND where discrepancies occurred between replicates, and through use of a large number of differential characters in combination with a noise-dampening or error-quenching "k-"linkage cluster procedure have been shown to produce generally acceptable results. Whether these procedures are extensible to other taxonomic groups is largely dependent upon the similarities within those groups that are used to define species, the number of internal reference OTU's used to evaluate results, the number of tests, and test bands investigated and susceptibility to intrinsic variations such as from genomic instability and control over experimental error.

3.8.6.2.3.2 Correlation of Phena with Taxa.

Most of the phena resolved unambiguously corresponded to extant species of *Vibrionaceae.* Some other taxonomically and otherwise distinct groups of OTU's were resolved, and summarized in a probabilistic system. The distribution of taxa in the dendrogram indicated that while in most cases there was a correspondance between phenetically and genotypically related species this was not always true.

The cluster procedure used did not seem to suffer from chaining or fragmentation, except perhaps for phenetically divergent type cultures, and amongst designated biogroups of *V.aestuarianus,* and strain data seemed stable to the skewing effects from a small number of mixed cultures. Similarities of phena produced here were lower than those from other environmental analyses of the *Vibrionaceae* e.g. West *et al.,* (1986) assayed OTU's over 133 characters and formed most phena into species with euclidean similarities of between 80 and 90 % and all phena were linked by a level of 60 % similarity. Here with the Jaccard coefficient the differential range

was over 60 %, and most species were defined by similarities of 70 % or greater. These differences reflect the operation of a larger number of differential tests, a more stringent similarity coefficient and also the physical preclusion of cross-feeding (and shared permeases). Phena formed with S_j coefficients of less than *ca* 65 % seemed to have been polyspecific. Phenon E was considered (because of atypical nutritional diversity) to contain more than just *Photobacterium phosphoreum,* and phenon V which associated with *P.fischeri,* contained heterogeneous OTU's some of which phenetically corresponded to no previously described species of *Vibrionaceae.* The unassigned OTU, UQM 3256, which was shown here as phenetically quite distinct from the provincial isolates of *V.gazogenes,* was compared with published reports of other red pigmented marine bacteria. There was little morphological or biochemical correspondance, with *V.psychroerythrus* of D'Aoust and Kushner (1971,1972), but a high similarity existed between UQM 3256 and *Serratia* species as described by Grimont, Grimont, Dulong de Rosnay and Sneath, (1977). The results of these latter comparisons are shown in Table 3.21 below. The strain UQM 3256 biochemically resembled *S.marcescens* and *S.marinorubra.*

Table 3.21: Characters which are Differential Between UQM 3256 and Four species of *Serratia* described by Grimont *et al.,* (1977).

	UQM 3256	*S.marcescens*	*S.marinorubra*	*S.plymuthica*	*S.liquefaciens*
Red Pigment	+	+	+	+	-
Gas from Glucose.	+	0	0	100	100
Motile.	(-)	95	91	100	95
Acetoin Barritt/O'Meara.	-	95	73	75	60
5d pH MRVP > 5.7	+	90	96	50	40
Growth pH 4.5.	+	0	0	0	-
Separate Utilization of:					
Arginine.	-	0	11	0	0
l-Arabinose.	-	0	100	100	100
β-Alanine.	-	93	0	30	15
δ-aminovalerate.	-	-	-	-	-
Sarcosine.	-	-	95	0	16
Lactose.	-	2	100	29	100
Xylose.	-	2	100	100	100
Cellobiose.	-	3	100	95	100
m-Erythritol.	-	72	95	0	0
β-Hydroxybutyrate.	+	76	0	0	0
Benzoate.	-	16	91	8	30
d & l α-Alanine, γ-aminobutyrate, inositol.	+	+	+	+	+

Original descriptions of these species lacked electron microscopic examination of flagellation of representative OTU's, consequently tentative designation of UQM 3256, because of its mixed flagellation, is as a novel *Serratia*-like sp. from the Family *Vibrionaceae.* For more precise phylogenetic placement of this OTU the 5S rRNA sequence could be determined.

Two other phena, C and Y, may have failed to correspond to extant taxa because they were denied full phenetic expression by testing in sub-optimal temperature, salinity and growth-factor conditions (3.8.6.2.2). Consequently further taxonomic evaluation of these phena could only be tentative. Phenon C tested at 37°C. may have produced a different phenetic profile more typical of an already-described species from *Vibrionaceae* assayed at 25°C. Phenon Y formed at a low similarity, but was tentatively assigned as *V.costicola*-like as recognition that it may also represent a polyspecific cluster generated through sub-optimal conditions, as specified above. These findings indicate that *Vibrionaceae* should no longer be considered as a group of bacteria with homogeneous growth temperature requirements, and for valid taxonomic diagnosis concessions must be made to cater for these requirements.

Most of the OTU's isolated phenetically resembled *V.alginolyticus, V.harveyi, V.carchariae* and ADH positive OTU's represented by *A.hydrophila* spp. and the group of species which before 1980 would have been assigned to *V.anguillarum.* To this extent work here resembles previous results of Burke and Rodgers; and Rodgers and Burke (1981).

Whether the close association of *V.carchariae,* and *V.harveyi* is phenetic or genotypic can only be ultimately proven by DNA hybridization. However 5S rRNA sequences from these species assembled by MacDonell and Colwell (1985) indicate that the greatest sequence similarity of *V.carchariae* is with *V.diazotrophicus* rather than with *V.harveyi;* which infers that the species are genetically unrelated but phenetically similar. Similar phylogenetic dis-similarity but phenotypic convergence i.e. ecological

classification is shown by the placement of the phenon for *A.hydrophila* adjacently to *Vibrio* spp.

The phenon of *A.hydrophila* shown here corresponds to the *A.hydrophila-A.punctata* group of Popoff and Veron (1976). The relative attribute frequencies used by them to separate aerogenic and anaerogenic biogroups of *A.hydrophila* were inconsistent with the frequencies obtained here i.e. since *ca* 50 % of OTU's produced gas from glucose, equal proportions of OTU's would have been expected to belong to each biogroup and so *ca* 45 % would have been expected to produce acetoin. However only 21 % of OTU's from phenon G were measured for acetoin production, *ca* half that predicted by Popoff and Veron, (1976).

One of the most common species encountered was *V.aestuarianus,* which until 1983 would have been classified as *V.anguillarum.* Phenon T, which was assigned as *V.aestuarianus* biogroup II in acknowledgment of its resemblance to Phenon E, might seem to have been segregated because of a clustering artifact; but like the two biogroups of *V.anguillarum,* this separation probably reflected differences in nutritional facility rather than clustering problems. Provincial OTU's assigned to the initial and new biogroups of this species had a low incidence of lactose utilization but this can perhaps be explained in the same terms as Reichelt *et al.,* 1976 reported for *V.vulnificus,* i.e. strains may only attack or utilize lactose, after heavy inoculation. These phena, although suitably defined (i.e. into phenetic ecotypes), for this study were not evaluated by molecular methods.

The absence of *V.parahaemolyticus* from this dataset is attributed as a sampling artifact rather than an indication of spurious clustering by chaining with a related species.

The occurrence and relative abundance of species in relation to isolation media and ecology is discussed together with results from other taxonomic analyses in sections (3.9), and (3.10).

3.8.6.2.4 Conclusion.

The unconventional analysis method used here was largely vindicated by close association of replicate OTU's among less closely related and phenetically dissimilar bacteria. Apart from experimental error at least some of the variation in the analyses was recognized as due to genomic instability and phenetic divergence of separately maintained type or reference cultures.

Provincial OTU's in this dataset which generally conformed to extant taxa were most commonly from *V.alginolyticus, V.harveyi, V.carchariae,* and *V.vulnificus,* but not from *V.cholerae,* or *V.parahaemolyticus* and also from ADH positive species including *A.hydrophila* and recently described species such as *V.aestuarianus, V.fluvialis,* and *V.orientalis* which were all earlier classified as *V.anguillarum.*

A phenon here nominated as *V.aestuarianus* biogroup II, exhibited characteristics exceptional to all previously described species of *Vibrionaceae,* as did an OTU with mixed flagellation which otherwise resembled *Serratia* spp. Unidentified imported OTU's were also generally consistent with extant taxa, but an atypical phenon from Singaporese fish was not classified because culture conditions were found here as sub-optimal.

Derived phena from this cross-batch analysis were presented in a diagnostic probability matrix with a reduced number of tests for rapid diagnosis of further isolates.

3.8.6.3 Diagnosis of Species and Phena from Batch 1, 3, 7 & 8 OTU's.

3.8.6.3.1 Experimental Design.

Data for one hundred and ninety four provincial OTU's, thirty-two tentatively or partially identified cultures and 18 type and reference cultures from Batches [3., 7. & 8.] were analyzed concurrently with the 48 *Rosetta* OTU's.

Some weakly fermentative strains which *sensu lato* could be classified as presumptive *Vibrionaceae* (2.7.2.3) were included among unidentified provincial OTU's. Intermediate results for the oxidation/fermentation reactions of these OTU's were scored as oxidative rather than as ND to improve the differential quality of the data.

Replicates of UQM 2784 and Stn 6561 were included to determine if earlier cultures (Batches [1.] & [4.] respectively) were faulty. Data from unmixed partial replicates in Batch [8a.] was substituted for doubtful data from the Batch [7.] tray tests for the OTU's: DA 11, UQM 2784, UQM 2785, UQM 2888, UQM 2890, UQM 2954, Stn 4830, 2300, 3330, 3560, 3681, 7260, 7600, 9200. Four OTU's were removed from the dataset; the Batch [1.] OTU, UQM 2778 suspected (3.8.3), then confirmed (3.8.6.2) as atypical and lacking vigour; and the mixed cultures Stn 3340 and 8190 from Batch [7.], and UQM 2771 from Batch [8.]. For continuity numerical-taxonomic microbiological literature the euclidean similarities of replicate OTU's from within- and across- batches, calculated as in (3.8.3), are shown in Table 3.22.1-2.

To test the practicality of microcomputers for the numerical analysis of comparatively large phenetic datasets a commercial program, *Microcluster*[r]. (3.8.2.4) was used to sort an upper Jaccard dissimilarity matrix, (i.e. cf. lower matrices as in Tables 3.11.1-2), formed from a specially written Fortran-77 program (Appendix A11.5). The dendrogram (Figure 3.9) which indicates OTU's examined and batch origins was plotted against a similarity scale. Phena and were assigned and attribute frequencies, (Tables 3.24.1-4) calculated as in 3.8.3.

3.8.6.3.2 Results.

3.8.6.3.2.1 Review of Replicates.

The $S_{e.}$ coefficients (3.8.6.1) of replicated cultures from this analysis are in Table 3.22.1 Under UPGMA clustering of $S_{j.}$ coefficients, the four cross-batch replicates with corresponding $S_{e.}$ coefficients of 83 % or less failed to cluster together. Because similarities for replicates of UQM 2784 and Stn 6561 were so low it is likely that the early preparations of each of these cultures was mixed. These OTU's were not included in calculations of attribute frequencies of phena H and S. The Batch [8.] OTU from *V.vulnificus* UQM 3032 clustered as a duplex between *V.cholerae* and *V.mimicus* and was separated both from its other replicate OTU, and other reference cultures of this species.

Table 3.22.1:Comparative Euclidean Similarities of OTU's Replicated Within- and Across- Batches 1,3,7 & 8.

OTU.	Accession Number.		Batch.	Euclidean Similarity % Batch. 1.	Batch. 3.	Batch. 7.	Batch. 8.
V.alginolyticus		UQM 2770	1	ND	ND	83	ND
V.alginolyticus		UQM 2770	7	*	ND	ND	ND
V.anguillarum		UQM 2771	1	94	92	90	ND
V.anguillarum		UQM 2771	1	*	90	92	ND
V.anguillarum		UQM 2771	3	*	*	87	ND
V.anguillarum		UQM 2771	7	*	*	ND	ND
V.gazogenes	Stn 180	UQM 2840	1	97	ND	ND	ND
V.gazogenes	Stn 180	UQM 2840	1	*	ND	ND	ND
V.gazogenes	Stn 170	UQM 2842	3	ND	90	ND	ND
V.gazogenes	Stn 170	UQM 2842	3	ND	*	ND	ND
V.vulnificus	Stn 1620	UQM 3032	1	ND	ND	ND	81
V.vulnificus	Stn 1620	UQM 3032	8	*	ND	ND	ND
V.nigripulchritudo		UQM 2784	1	ND	ND	*	ND
V.nigripulchritudo		UQM 2784	7,8a	67	ND	ND	ND
V.pelagius		UQM 2785	1	ND	ND	84	ND
V.pelagius		UQM 2785	7	*	ND	ND	ND
Vibrio sp.		Stn 760	3	ND	ND	95	ND
Vibrio sp.		Stn 760	7	ND	*	ND	ND
Vibrio sp.		Stn 770	3	ND	ND	92	ND
Vibrio sp.		Stn 770	7	ND	*	ND	ND
Vibrio sp.		Stn 6561	7	ND	ND	ND	76
Vibrio sp.		Stn 6561	8	ND	ND	*	ND
Vibrio sp.		Stn 6600	8	ND	ND	ND	98
Vibrio sp.		Stn 6600	8	*	ND	ND	ND
Vibrio sp.		Stn 8950	8	ND	ND	ND	97
Vibrio sp.		Stn 8950	8	*	ND	ND	ND

Table 3.22.2: Average Euclidean Similarities Within- and Across-Batches 1,3,7 & 8.

	Average Euclidean Similarity %*.			
Batch	1.	3.	7.	8.
1.	(2) 96 ± 1	(2) 91 ± 1	(4) 87 ± 4	(1) 81
3.	#	(1) 90	(2) 91 ± 3	ND
7.	#	#	ND	ND
8.	#	#	#	(2) 97 ± 0.5

(n) Number of Determinations.
* Excluding UQM 2784 and Stn 6561.
ND - Not Determined.

The replicate of *V.alginolyticus* was less versatile in the Batch [7.] than the Batch [1.] preparation.

3.8.6.3.2.2 Review of Groups.

All OTU's were positive for two characters, catalase production, and polar flagellation; and negative for eight characters, colony matt, Gram-positive, and separate utilization of n-butyric acid, hexanoate, heptanoate, and l-tartarate. These characters were therefore removed from frequency Tables 3.24.1-4.

The dendrogram (Figure 3.9), was divided into eight numbered major groups and further divided into 34 phena A - AI and 6 unassociated reference cultures, *V.costicola* UQM 2889, *V.metschnikovii*, *V.mimicus* UQM 2954, *V.nereis* UQM 2783, *V.proteolyticus* UQM 2472 and *P.damsela* UQM 2853 and the Batch [7.] replicate of *V.alginolyticus*, UQM 2770. The distributions, sizes and differential attribute frequencies of the eight groups are shown in Table 3.23. The azo-reductase inference to nitrogen fixation (Appendix 6.) occurred infrequently in three of these groups and indicated no specific taxonomic affinities.

Group 1. representing the phena A-C; contained *V.ordalii*, *P.shigelloides*, and an unidentified *Vibrio* sp., and could be distinguished by glucose fermentation, variable oxidase reactions, and by inability to utilize l-arginine and 47 other carbon sources. Group 2. contained the oxidase-negative and novobiocin sensitive species *V.metschnikovii* and *V.gazogenes*.

OTU's from Group 4. were also oxidase-negative, but these were resistant to novobiocin, non-sodium requiring and had variable ability to assimilate phenylacetic acid.

The oxidase-positive Groups, (3., 4., 5. and 6.), were exceptional by the presence of some weakly fermentative, OTU's. However in all cases these aberrant OTU's had shown typical fermentation reactions in presumptive assay and the discrepancies here are attributed to weak cultures or light inoculation. OTU's from Groups 3. and 6. attacked similar ranges of assayed carbon sources and differed mainly in sodium requirements and growth upon proline by OTU's from Group 6. and luminescence in Group 2.

Table 3.23: Percentage Frequencies of Differential Attributes Distinguishing Major Groups Formed after UPGMA Linkage Analysis of Data from Batches 1,3, 7 & 8.

Group.	1.	2.	3.	4.	5.	6.	7.	8.
Number of OTU's.	14	5	23	5	203	20	13	2
Phena:	A-C	D	E-H	I	J-AD	AE-AG	AH	AI
Attribute.								
Luminescence.	0	0	17	0	2	5	0	0
Strong Catalase.	0	0	4	60	3	0	0	0
Oxidase.	71	0	100	0	100	100	100	100
Glucose Fermentation.	100	100	95	80	99	95	0	0
Polar Flagellation.	100	100	100	100	100	100	100	100
No Sodium Requirement.	21	0	91	100	30	0	100	100
Resistance to Novobiocin.	14	0	0	100	26	0	100	100
Susceptibility to 150 µg/ml 0/129.	92	100	100	40	86	100	50	0
Starch Hydrolysis.	92	100	95	80	96	95	84	100
Separate Utilization of:								
l-Arginine.	0	0	72	20	93	60	0	100
l-Proline.	21	20	60	100	97	100	46	100
d-Glucose.	7	25	8	80	95	5	100	50
Phenylacetic acid.	0	0	0	40	0	0	0	0
Adipic acid.	0	0	0	0	3	0	0	100
Number of Carbon Sources Attacked.	28/76	42/74	32/76	52/76	63/76	31/73	37/74	41/74

Group 5. from which OTU's attacked up to *ca* 80 % of assayed carbon sources, was the most versatile of all groups. More than 90 % of OTU's from this group attacked l-arginine, l-proline and d-glucose.

Groups 7. and 8., repectively represented by the single phena AH and AI contained OTU's which were oxidase-positive and weakly fermentative or oxidative. They could be further distinguished from the residue and distinguished by the characters separate utilization of l-arginine (AH positive) and adipic acid (AI positive).

3.8.6.3.2.3 Review and Diagnosis of Phena from Groups 1-8.

The outermost OTU, *V.costicola* UQM 2888, was fused unassociated into the dendrogram, at a S_j of *ca* 30 %. This OTU was more typical of the species than was depicted in phenon Z by UQM 2888 from Figure 3.8 (Section 3.8.6.2.2). It differed in some respects from the described type culture (Garcia *et al.*, 1987) but was otherwise consistent with intrinsic variability reported simultaneously from other strains of the same species for the attributes nitrate reduction, casein hydrolysis, separate utilization of mannose, and the absence of ADH, aesculin and lecithin hydrolysis, no growth in less than 1 % saline, and by their inability to attack any other carbon sources. Variation found here might have been induced through strain attrition or sub-optimal cultivation of this halophilic OTU as suggested in 3.8.6.2.2.2.

3.8.6.3.2.3.1 Review and Diagnosis of Phena from Group 1.

The phena A, B and C fused at S_j 45 %, and respectively represented *P.shigelloides, V.ordalii,* and an unidentified *Vibrio* sp. Phenon A, was formed at a S_j of 55 % and comprised two provincial OTU's and the reference culture *P.shigelloides* UQM 1617. OTU's in phenon A exhibited greater nutritional facility than the single *P.shigelloides* assayed in (3.8.6.2). Two of three OTU's from phenon A were ADH positive and only the reference culture UQM 1617 was able to separately utilize inositol.

Phenon B formed at S_j 50 % contained two reference cultures of *V.ordalii* UQM 2890 and UQM 2906 and two provincial OTU's. No attributes were expressed by all OTU's of phenon B and none from phenon A, however nitrite reduction, ODC, SDS esterase, and separate utilization of glutamic acid, glutamine and

maltose occurred in all OTU's from phenon A but none from phenon B.

Phenon C comprised OTU's exclusively from cool temperate Australia. These had an optimum growth temperature of 15°C. and required at least 1 % saline. Phenon C is not classified further because its otherwise heterogeneous appearance infers that sub-optimal growth conditions skewed these OTU's into a primarily ecological group with secondary taxonomic structure. Individual species within phenon C may be separately determinable within this locally valid subset of the major analysis. Because no provincial OTU's were included in this phenon this was not justified here.

3.8.6.3.2.3.2 Review and Diagnosis of Phena from Group 2.

V.metschnikovii, UQM 211 was most similar to another oxidase-negative species *V.gazogenes,* (Phenon D), from which it could be readily distinguished by its production of ADH and absence of red pigmentation. Phenon D comprised only the two duplicated reference OTU's UQM 2840 and 2842 from the provincial isolates Stn 180 and 170. These OTU's, although individually corresponding to *V.gazogenes* of Baumann *et al.,* (1984), showed considerable within-species variability. On the basis that Baumann *et al.,* emended the description of this red, oxidase-negative, species based upon data from only one strain, further emendation is made herein to include additional information as follows:

Strains of *V.gazogenes* require sodium, are oxidase-negative and fermentative with and without gas. Cells have single sheathed polar flagella, are ADH and decarboxylase negative. Cells are usually red-pigmented but unpigmented mutants occur, and most cultures do not reduce nitrate or nitrite. Indole, acetoin and cholera-red are not produced. Cultures are able to hydrolyze ONPG, aesculin (weakly) and albumen, arbutin, casein, DNA, gelatin, egg yolk proteins, and starch; but not urea, agar, arylsulphate, chitin, SDS, tyrosine or xanthine. Cultures grow at 42°C. but not 4°C. Most grow in the presence of 10 % saline. No growth occurs at either pH 10 or at pH 4.5, and cultures are susceptible to the assayed concentrations of

Table 3.24.1: Attribute Frequencies (%), for UPGMA Phena A - H, and Discrete Type or Reference OTU's.

Phenon.		A	B	C	D	E		F	G	H
Species.	V.cost	P.shig	V.orda		V.gazo	V.angu	V.mimi	V.chol	V.vuln	V.sple
Number of OTU's (n).	n = 1	n = 3	n = 4	n = 7	n = 4	n = 13	n = 1	n = 5	n = 2	n = 2
Attribute.										
Colony diameter > 3 mm.	0	0	0	28	0	0/12	0	20	100	50
Colony mucoid.	0	0	0	14	100	7	0	0	0	0
Colony opaque.	0	0	50	57	75	0	0	0	0	0
Colony entire.	100	100	100	100	100	92	100	100	50	100
Colony convex.	100	100	100	100	100	92	100	100	50	100
Swarming growth.	0	0	0	28	0	23	0	0	0	0
Luminous growth.	0	0	0	0	0	0	0	40	100	0
Red pigment.	0	0	0	0	100	0	0	0	0	0
Brown pigment.	0	0	0	0	0	0	0	0	50	0
Black pigment.	0	0	0	0	0	0	0	0	0	0
Yellow/orange.	100	0/ 2	0/ 3	0/ 3	0	11/ 9	100	0/ 1	0	0/ 1
Length > 2 times width.	100	0/ 1	100/ 1	0/ 2	100	80/ 5	0	100/ 1	100/ 1	100/ 1
Strong catalase.	100	0	0	0	0	0	0	20	0	0
Oxidase.	100	66	25	100	0	100	100	100	100	100
Oxidase (Toluene).	100	66	25	100	0	100	100	100	100	100
Broth uniform turbidity.	0	100	100	100/ 6	100	92	100	100	100	100
Broth with sediment.	0	66	75	71	50	23	100	100	50	100
Broth sediment mucoid.	ND	0/ 1	0/ 1	0/ 1	100	20/ 5	ND	0/ 1	0/ 1	100/ 1
Broth growth mod. to heavy.	0	33	0	28	50	69	100	60	0	100
Pellicle.	0	0/ 2	0/ 3	0/ 6	0/ 2	66/12	100	60	0	0
Indole.	0	33	25	14	0/ 2	55/ 9	ND	100	50	0
Weak indole.	0	50/ 2	33/ 3	33/ 6	0/ 2	100	100	100	50	100
Motile.	100	100	100	100	100	100	100	100	100	100
5 day pH > 7.05.	0	0	0	0/ 6	75	100	0	80	0	0
5 day pH > 5.15.	100	0	0	0/ 6	100	100	0	80	0	0
Acetoin production.	100	0	25	0	100	69	0	80	0	0
Nitrate reduction.	100	100	100	100/ 6	25	100	100	100	100	100
Nitrite reduction.	100	100	0	57	25	38	0	80	100	50
Cholera-Red.	0	0	0	14	0	7	0	100	100	0
Gluconate oxidation.	0	0	0	0	0	92	0	20	0	50
Arginine dihydrolase.	0	66	50	28	0	61	0	20	0	100
Lysine decarboxylase.	0	100	50	28	0	0	100	80	100	0
Ornithine decarboxylase.	0	100	0	14	0	15	100	60	100	0
Glucose fermentation.	100	100	100	100	100	100	100	100	100	50
Gas from glucose.	0	0	25	14	0	0	0	0	0	0
ONPG hydrolysis.	0	33	25	28	100	100	0	100	100	100
Urea hydrolysis.	0	33	25	28	0	0	0	0	0	0
Aesculin hydrolysis.	0	0	0	42	50	0	0	0	0	0
Weak aesculin hydrolysis.	0	33	0	57	100	30	0	0	50	50
Agar hydrolysis.	0	0	0	0	0	0	0	0	0	0
Egg Albumen hydrolysis.	100	0/ 2	66/ 3	0/ 6	100	92	100	80	100	50
Arbutin hydrolysis.	0	33	50	28	100	7	0	0	50	0
Aryl-sulphatase.	0	0	0	57	0	0	100	0	0	50
Casein hydrolysis.	100	0	0	14	100	92	100	80	50	100
Chitin hydrolysis.	100	33	50	71	0/ 2	100/12	0	100	100	100
DNA hydrolysis.	0	66	50	100	100	100	100	80	100	100
Gelatin hydrolysis.	0	0	25	0	100	100	100	100	100	100
Lecithinase.	0	66	100/ 3	57	25	69	100	100	100	50
Lipase.	0	100	100	85	75	92	100	100	100	100
Esterase (SDS).	0	100	0	20/ 5	0/ 2	0/ 8	100	100	50	50
Egg proteolysis.	100	33	0	42	100	53	0	60	100	100
Phosphatase.	0	100	100	100	33/ 3	91/12	100	100	100	100
Starch hydrolysis.	0	100	100	85	100	100	0	100	100	100
Sulphide production.	0	0/ 2	0	0/ 6	0	0/12	0	0	0	0
Serum hydrolysis.	100	0/ 2	100/ 1	0/ 5	100/ 3	100/ 8	100	100	100	50

Table 3.24.1; (Cont.): Attribute Frequencies (%), for UPGMA Phena A - H, and Discrete Type or Reference OTU's.

Phenon.		A	B	C	D	E		F	G	H
Species.	V.cost	P.shig	V.orda		V.gazo	V.angu	V.mimi	V.chol	V.vuln	V.sple
Number of OTU's (n).	n = 1	n = 3	n = 4	n = 7	n = 4	n = 13	n = 1	n = 5	n = 2	n = 2
Attribute.										
Tyrosine hydrolysis.	0	33	50	28	0	0	0	0	50	50
Tyrosine pigment.	100	33	0	28	25	7	100	0	0	100
Xanthine hydrolysis.	0	0	0	0	0	0	0	0	0	0
Congo Red Decolourization.	0	0/ 1	0/ 3	0/ 3	ND	0/ 4	0	ND	0	ND
Sudanophilic inclusions.	0	0	50	0	0	23	0	40	100/ 1	0
Growth 4°C.	0	0	25	85	0	100	0	0	0	0
Growth15°C.	100	100	100	100	100	100	100	40	50	100
Growth30°C.	100	100	100	28	100	100	100	100	100	100
Growth37°C.	100	100	75	14	100	100	100	100	100	0
Growth42°C.	0	50/ 2	50/ 2	0	100	0	100	100	100	0
Growth 0 % NaCl.	0	33	50	0	0	92	100	100	100	50
Growth 0.5 % NaCl.	0	66	100	0	50	92	100	100	100	0
Growth 1.0 % NaCl.	100	100	100	85	100	100	100	100	100	100
Growth 3.0 % NaCl.	100	100	100	100	100	100	100	100	100	100
Growth 6.0 % NaCl.	100	66	100	28	100	92	0	0	0	0
Growth10 % NaCl.	100	0	0	0	75	0	0	0	0	0
GrowthpH 10.	ND	100/ 1	0/ 1	0/ 1	0	100/ 5	100	100	100/ 1	100/ 1
GrowthpH 4.5.	0	0	0	0	0	0	0	0	0	0
GrowthNovobiocin.	0	0	50	0	0	0	0	0	0	0
GrowthPenicillin.	100	100	100	100	100	100	100	100	100	100
GrowthPolymyxin.	0	0	0	28	0	53	0	100	100	0
GrowthStreptomycin.	0	100	100	71	50	100	100	100	100	50
GrowthSulphamethoxazole.	ND	100/ 2	100/ 1	80/ 5	100	100/ 8	ND	100	100/ 1	100
GrowthTetracycline.	0	0	25	14	0	0	0	0	0	50
GrowthTrimethoprim.	0	66	0	14	0	0	0	20	0	0
No growth10 μg/ml 0/129.	0	66	50	85	50	100	100	60	100	50
No growth150μg/ml0/129.	100	100	75	100	100	100	100	100	100	100
GrowthBasic fuchsin.	0	50/ 2	0/ 2	42	0	60/10	100	100	0	50
GrowthBrilliantgreen.	0	33	0	42	0	38	0	60	0	50
GrowthDichlorophene.	0	66	75	14	50	0	0	20	0	50
GrowthEDDA.	0	0	50	0	100	38	100	100	50	50
Growthon Fast yellow.	ND	0/ 1	ND	0/ 3	0/ 2	42/ 7	ND	20	ND	50
GrowthHaloquinol.	0	0	0	0	0	0	0	0	0	0
Growth8-Hydroxyquinoline.	0	0	0	0	0	0	0	0	0	0
GrowthMethylviolet.	100	100/ 2	66/ 3	33/ 6	50/ 2	50/12	0	100	0	100
GrowthPyronin-Y.	0	33	50	14	0	0	0	20	0	0
Growth0.2% SDS.	0	100	100	28	0	23	100	100	100	50
GrowthThionine.	0	50/ 2	100/ 3	0/ 6	0/ 2	0/12	0	0	50	0
Yellowon Thionine.	ND	0/ 2	66/ 3	0/ 5	ND	0/ 8	ND	0/ 4	0/ 1	0/ 1
GrowthT.T.C.	0	100	100	100	100/2	100/12	100	100	100	100
Acid from Xylose.	ND	ND	ND	ND	100/ 2	0/ 3	ND	0/ 1	ND	0/ 1
Acid from Arabinose.	ND	ND	ND	ND	100/ 2	100/ 3	ND	0/ 1	ND	0/ 1
Acid from Mannose.	ND	ND	ND	ND	100/ 2	100/ 3	ND	0/ 1	ND	0/ 1
Acid from Sucrose.	ND	ND	ND	ND	100/ 2	100/ 3	ND	100/ 1	ND	0/ 1
Acid from Maltose.	ND	ND	ND	ND	100/ 2	100/ 3	ND	100/ 1	ND	100/ 1
Acid from Cellobiose.	ND	ND	ND	ND	100/ 2	100/ 3	ND	0/ 1	ND	100/ 1
Acid from Salicin.	ND	ND	ND	ND	100/ 2	0/ 3	ND	0/ 1	ND	0/ 1
Acid from Mannitol.	ND	ND	ND	ND	100/ 2	100/ 3	ND	100/ 1	ND	100/ 1
Acid from Sorbitol.	ND	ND	ND	ND	0/ 2	0/ 3	ND	0/ 1	ND	0/ 1
Acid from Inositol.	ND	ND	ND	ND	0/ 2	0/ 3	ND	0/ 1	ND	0/ 1
Acid from Dulcitol.	ND	ND	ND	ND	0/ 2	0/ 3	ND	0/ 1	ND	0/ 1
GrowthGlycine.	0	0	0	14	50	0	0	0	0	0
Growthl-α-Alanine.	0	33	0	0	100	38	0	20	100	0
Growthd-α-Alanine.	0	33	0	14	0	69	0	40	100	0
Growthdl-β-Alanine	0	0	0	0	0	0	0	0	0	0

Table 3.24.1; (Cont.): Attribute Frequencies (%), for UPGMA Phena A - H, and Discrete Type or Reference OTU's.

Phenon.		A	B	C	D	E		F	G	H
Species.	V.cost	P.shig	V.orda		V.gazo	V.angu	V.mimi	V.chol	V.vuln	V.sple
Number of OTU's (n).	n = 1	n = 3	n = 4	n = 7	n = 4	n = 13	n = 1	n = 5	n = 2	n = 2
Attribute.										
Growth Serine.	0	0	25	0	0	61	0	40	100	50
Growth l-Leucine.	0	0	0	0	0	0	0	0	0	0
Growth Valine.	0	0	0	0	0	0	0	0	0	0
Growth l+Glutamic acid.	ND	100/ 2	0	0/ 6	100	66/12	ND	20	100/ 1	50
Growth l-Lysine.	0	0	0	0	50	0	0	0	0	0
Growth l-Arginine.	ND	0	0	0/ 6	0	84	ND	80	50	0
Growth l-Ornithine.	0	0	0	14	50	38	0	80	0	0
Growth l-Citrulline.	ND	0	0	0/ 6	0	75/12	ND	60	50	0
Growth γ-Amino-butyrate.	0	0	0	0	50	0	0	0	0	0
Growthδ-Amino-valerate.	0	0	0	0	0	0	0	0	0	0
GrowthNorvaline.	0	0/ 2	0/ 3	0/ 6	ND	0/ 9	0	0/ 4	0	0/ 1
Growthl-Proline.	0	33	50	0	25	53	0	100	100	0
GrowthPutrescine.	0	0/ 2	0/ 3	0/ 6	0/ 2	0/12	0	0	0	0
GrowthSarcosine.	0	0	0	0	0	0	0	0	0	0
Growthl-Glutamine.	0	100	0	0	100	84	0	100	100	0
Growthd-Glucosamine.	0	33	25	0	0	7	0	0	50	0
Growthn-Acetylglucosamine.	0	33	0/ 1	0/ 5	75	33/ 9	0	0	0	0
Growthd-Ribose.	ND	33	0	0/ 3	75	0/ 8	ND	0/ 1	ND	0/ 1
GrowthXylose.	0	0	0	0	50	0	0	0	0	0
Growthl-Arabinose.	0	0	0	0	75	15	0	0	0	0
Growthl-Rhamnose.	0	0	0	0	0	0	0	0	0	0
GrowthGlucose.	0	33	0	0	25	15	0	0	0	0
Growthd-Mannose.	100	0	0	0/ 6	75	46	ND	0	0/ 1	50
Growthd-Galactose.	0	0	0	0	50	7	0	0	0	0
GrowthSucrose.	ND	33	0	0/ 6	100	33/12	ND	0	0/ 1	0
GrowthTrehalose.	0	33	0	0	100	15	0	0	0	0
GrowthMaltose.	0	100	0	0	100	30	0	20	0	50
GrowthCellobiose.	0	0/ 2	100/ 1	0/ 5	75	11/ 9	0	0	0	0
GrowthLactose.	ND	0	0	0/ 6	0	0/12	ND	0	0/ 1	0
GrowthRaffinose.	0	0	0	0	0	0	0	0	0	0
GrowthGalactarate.	0	0	0	0	25	0	0	0	0	0
GrowthGalacturonicacid.	0	0/ 2	0/ 3	0/ 6	ND	0/ 9	0	0/ 4	0	0/ 1
GrowthGluconate.	0	0	25	0	0	23	0	0	0	0
GrowthGlucuronicacid.	0	0	0	0	100	0	0	0	0	0
GrowthSalicin.	ND	0	0	0/ 6	100	0/12	ND	0	0/ 1	0
GrowthStarch.	ND	50/ 2	0/ 1	0/ 4	100	37/ 8	ND	20	0/ 1	50
GrowthDextrin.	ND	100/ 1	0/ 1	0/ 1	100	75/ 4	ND	0/ 1	ND	0/ 1
GrowthPolygalacturonicacid.	0	0/ 2	0/ 3	0/ 6	ND	0/ 9	0	0/ 4	0	0/ 1
GrowthInulin.	0	0	25	0	50	0	0	0	0	0
GrowthPullulan.	ND	66	0	0/ 6	50	0/12	ND	0	0/ 1	0
GrowthFormate.	0	0	0	0	0	0	0	0	0	0
GrowthAcetate.	0	0/ 2	0/ 3	0/ 6	100/ 2	0/12	0	0	0	0
GrowthPropionate.	ND	0	0	0/ 6	0	0/12	ND	0	0/ 1	0
GrowthCaprylicacid.	0	0	0	0/ 6	0	0	0	0	0	0
GrowthNonanionicacid.	0	0	0	0	0	0	0	0	0	0
GrowthCaprate.	ND	0	0	0/ 6	0	0/12	ND	0	0/ 1	0
GrowthMalonate.	0	0	0	0	0	0	0	0	0	0
GrowthSuccinate.	0	33	25	0	100/ 2	20/10	0	0/ 4	100	0/ 1
GrowthMaleate.	ND	100/ 1	100/ 1	0/ 1	50	0/ 4	ND	0/ 1	ND	0/ 1
GrowthAdipicacid.	0	0	0	0	0	0	0	0	0	0
GrowthMalate.	ND	0/ 2	0	0/ 3	0	0/ 8	ND	0/ 1	ND	0/ 1
Growthdl-3-OH-butyrate.	0	0	25	0	25	0	0	0	0	0
Growth6-HydroxyCaproate.	0	0	0	0	0	0	0	0	0	0
GrowthLactate.	0	50/ 2	0/ 1	0/ 5	100	40/10	0	0	0	0
Growthdl-Glycericacid.	0	0	0	0	0	33/12	0	20	50	0

Table 3.24.1; (Cont.): Attribute Frequencies (%), for UPGMA Phena A - H, and Discrete Type or Reference OTU's.

Phenon.	A	B	C	D	E		F	G	H
Species.	V.cost	P.shig	V.orda		V.gazo	V.angu	V.mimi	V.chol	V.vuln	V.sple
Number of OTU's (n).	n = 1	n = 3	n = 4	n = 7	n = 4	n = 13	n = 1	n = 5	n = 2	n = 2
Attribute.										
Growth Poly-β-OH-butyrate.	0	0	0	0	0	0	0	0	0	0
GrowthCitrate.	0	0	25	0	75	15	0	0	0	0
Growthα-Ketoglutarate.	0	0	0	0	50	7	0	20	0	0
GrowthPyruvate.	ND	33	0	0/ 6	100	33/ 9	ND	100/ 4	100/ 1	0/ 1
GrowthErythritol.	ND	0/ 2	0	0/ 3	50	0/ 8	ND	0/ 1	ND	0/ 1
GrowthDulcitol.	ND	0/ 2	0	0/ 3	0	0/ 8	ND	0/ 1	ND	0/ 1
GrowthMannitol.	ND	0	0	0/ 6	50	33/12	ND	0	0/ 1	0
GrowthSorbitol.	ND	0/ 2	0/ 1	0/ 4	50	37/ 8	ND	0	0/ 1	0
GrowthInositol.	0	33	0	0	0	0	0	0	0	0
GrowthEthanol.	0	0	0	0	0/ 2	0/10	0	0/ 3	0	0/ 1
GrowthBenzoate.	0	0	0	0	0	0	0	0	0	0
GrowthHydroxybenzoate.	0	0	0	0	0	0	0	0	0	0
GrowthPhenylacetic acid.	0	0	0	0	0	0	0	0	0	0

novobiocin, tetracycline, and trimethoprim and to 150 μg/ml 0/129. Acid is produced from xylose, arabinose, mannose, sucrose, maltose, cellobiose, salicin and mannitol, but not from sorbitol, inositol or dulcitol. All cultures are separately able to utilize l-α-alanine, l+glutamic acid, l-glutamine, sucrose, trehalose, maltose, glucuronic acid, salicin, starch, acetate, succinate, lactate, and pyruvate. None are able to utilize l-arginine, citrulline, δ-aminovalerate or gluconate.

3.8.6.3.2.3.3 Review and Diagnosis of Phena from Groups 3. and 4.

Phenon E, comprised 13 OTU's, including all the type or reference cultures of *V.anguillarum.* These could be distinguished by their ability to oxidise gluconate, variable ADH reactions, growth at 4°C., absence of growth at 42°C., growth in 6 % saline and separate utilization by 75 % or more strains of l-arginine, citrulline and l-glutamine.

V.mimicus UQM 2954 was not associated with any provincial OTU's, but associated with the cluster formed by phenon F fusing with the doublet containing the Batch [8.] reference OTU for *V.vulnificus* UQM 3032 [Phenon G]. Cluster [F,G] differed from *V.mimicus* by nitrite reduction, cholera-red production, ONPG, chitin and starch hydrolysis, growth in the presence of polymyxin, and by the ability of some OTU's to separately assimilate carbon

sources. *V.mimicus* differed from cluster [F,G] in tests for arylsulphatase, and pigment production from tyrosine. OTU's from phenon G were different from phenon F by susceptibility to 10 µg/ml 0/129, and separate utilization of d and l α-alanine, l-glutamic acid, and succinate. Phena [F-J] contained species either previously described as luminescent or which were luminescent here. Phenon H contained only the type culture of *V.splendidus* and an OTU from Tasmania and is not further reviewed.

Phenon I included *P.fischeri* UQM 2889, a *Photobacterium* sp. UQM 3242 and a heterogeneous assortment of nutritionally diverse oxidase-negative OTU's, in one case with peritrichous flagellation. These *Photobacterium*-like OTU's phenetically resemble phenon V (Figure 3.8), from previous analysis (3.8.6.2); and so before assignment to species warrant separate simultaneous evaluation according to the more rigorous criteria proposed for those OTU's.

3.8.6.3.2.3.4 Review and Diagnosis of Phena from Group 5.

Phenon J formed at S_j 55 % and contained the type cultures from two almost simultaneously described species, (2.6.2.5.3) *V.orientalis* and *V.tubiashii*, four Tasmanian OTU's and one provincial OTU, Stn 450. Four of the seven OTU's hydrolyzed xanthine, however this attribute was not unique to this phenon. ATCC cultures of *V.proteolyticus*, *P.damsela*, and *V.mediterranei* (phenon S) were also able to hydrolyze xanthine. Additionally this trait was borne by provincial OTU's from Phenon K, and the unassociated phena AE and AF. Phenon J and phenon AF were also similar by containing OTU's from Tasmania which could grow at 4°C. These phena could otherwise be distinguished by occurrence in phenon J, of ONPG hydrolysis susceptibility to 0/129 at concentrations of 10µg/ml or greater and separate utilization of gluconate and pullulan but not dl-β-alanine or α-ketoglutarate.

Phenon K, (S_j 55 %), comprised replicated type cultures of *V.pelagius* UQM 2785 and a single provincial OTU, Stn 3681. Apart from variable xanthine hydrolysis OTU's, from phenon K were ADH and decarboxylase negative and susceptible to 150 µg/ml 0/129, produced arylsulphatase, and separately attacked putrescine.

Phenon L was a cohesive cluster, (S_j 80 %), represented only by OTU's isolated from Winfield copepods. It was most closely associated with the decarboxylase negative, but usually ADH positive species, *V.diazotrophicus* (Phenon M). Copepod OTU's were positive for hydrolysis of aesculin and arbutin, susceptibility to 10 µg/ml 0/129, separate utilization of d and l α-alanine, l-arginine, l-citrulline, citrate and mannitol, but differed from *V.diazotrophicus* by positive reactions for ONPG, casein, and DNA hydrolysis, growth in 10 % saline, resistance to novobiocin, separate utilization of δ-aminobutyrate, rhamnose, pullulan, and ethanol. Positive characters of *V.diazotrophicus* (Phenon M), negative in OTU's from phenon L included indole production, tyrosine hydrolysis, growth at 4°C., growth in 0.5 % saline, streptomycin, dichlorophene and separate utilization of serine and salicin. The OTU's Stn 750, 760, 770, and 780 from phenon L respectively deposited as UQM 3027-30, were examined by molecular methods to establish their taxonomic position (See Appendix 9.).

Phenon N, which contained UQM 2852, *V.hollisae,* was associated with only a single provincial OTU before fusion with *V.nereis* UQM 2783. These OTU's could be distinguished from all others by susceptibility to polymyxin and separate utilization of rhamnose, gluconate, glucuronate and pullulan. The ADH-positive strain, Stn 130 was deposited as UQM 3282 labeled *potentially V.hollisae*-like as acknowledgment to the possibility that the phenetic facility of the type culture for this species may have been adversely affected by incubation at 25°C. rather than at 37°C.

Phenon O, (S_j 70 %), with 7 OTU's was the second and major reservoir of *V.vulnificus.* This phenon was serially fused with the phena P and Q, both of which contained cultures previously designated (3.8.6.2) as *V.campbellii.* More than 85 % of OTU's from phenon [O-Q] shared attributes including casein, chitin, starch and lecithin hydrolysis, resistance to polymyxin, susceptibility to 150 µg/ml 0/129, growth in the presence of 0.2 % SDS, and separate utilization of l-glutamic acid, proline, glutamine and trehalose.

Table 3.24.2: Attribute Frequencies (%), for UPGMA Phena I - R, and Discrete Type or Reference OTU's.

Phenon.	I	J	K	L	M	N	O	P	Q	R
Species.	P.fisc	V.or/t	V.pela		V.diaz	V.holl	V.vuln	V.camb	V.camb	
Number of OTU's (n).	n = 5	n = 7	n = 3	n = 12	n = 2	n = 2	n = 7	n = 2	n = 2	n = 2
Attribute.										
Colony diameter > 3 mm.	25/ 4	0/ 6	0/ 2	0/11	0/ 1	0	0	0/ 1	0	50
Colony mucoid.	20	0	0	0	0	0	0	0	0	0
Colony opaque.	80	14	33	0	0	50	28	50	0	0
Colony entire.	100	100	100	100	100	100	100	100	100	100
Colony convex.	100	100	100	100	100	100	100	100	100	100
Swarming growth.	0	71	0	0	50	0	14	0	0	0
Luminous growth.	0	0	0	0	0	0	14	0	0	0
Red pigment.	0	0	0	0	0	0	0	0	0	0
Brown pigment.	0	0	0	0	0	0	0	0	0	0
Black pigment.	0	0	0	0	0	0	0	0	0	0
Yellow/orange.	0	0/ 6	66	0/11	0/ 1	50	0	0	0	0/ 1
Length > 2 times width.	0/ 1	100/ 1	66	90/10	ND	100/ 1	42	100/ 1	100	100/ 1
Strong catalase.	60	0	0	25	0	0	0	0	50	50
Oxidase.	0	100	100	100	100	100	100	100	100	100
Oxidase (Toluene).	0	100	100	100	100	100	100	100	100	100
Broth uniform turbidity.	100	100	100	66	100	100	100	100	100	100
Broth with sediment.	100	100	66	100	100	100	100	100	100	50
Broth sediment mucoid.	0/ 1	100/ 1	0/ 1	100/10	ND	0/ 1	0	0/ 1	50	100/ 1
Broth growth mod. to heavy.	40	100	66	91	100	100	71	100	0	100
Pellicle.	20	16/ 6	0	0/ 2	0	0/ 1	0	0	0	0
Indole.	25/ 4	66/ 3	33	0	100	0	71	0/ 1	0	100/ 1
Weak indole.	50	100/ 6	33	0/ 2	100	0/ 1	100	100	0	100
Motile.	100	100	100	100	100	100	100	100	100	100
5 day pH > 7.05.	80	0	33	0	0	0	0	0	0	0
5 day pH > 5.15.	80	0	33	0	0	50	100	100	50	0
Acetoin production.	80	0	0	0	0	0	0	0	0	0
Nitrate reduction.	60	85	100	16	100	100	100	100	100	100
Nitrite reduction.	80	42	66	16	50	0	0	0	0	100
Cholera-Red.	0	0	33	0	0	0	0	0	0	0
Gluconate oxidation.	80	0	0	0	0	50	0	0	0	0
Arginine dihydrolase.	40	85	0	0	50	50	0	50	50	100
Lysine decarboxylase.	50/ 4	14	0	0	0	0	100	0	50	0
Ornithine decarboxylase.	75/ 4	0	0	0	0	0	100	0	0	0
Glucose fermentation.	80	100	100	100	100	100	100	100	100	100
Gas from glucose.	40	0	0	0	0	0	0	0	0	0
ONPG hydrolysis.	60	14	66	100	0	0	71	0	50	50
Urea hydrolysis.	0	14	0	0	0	0	0	0	0	0
Aesculin hydrolysis.	40	57	33	100	100	0	42	50	0	100
Weak aesculin hydrolysis.	60	71	100	100	100	0	57	50	0	100
Agar hydrolysis.	0	0	0	0	0	0	0	0	0	0
Egg Albumen hydrolysis.	40	66/ 6	0	100/ 2	0	100/ 1	14	50	0	0
Arbutin hydrolysis.	100	14	33	100	100	0	57	100	0	50
Aryl-sulphatase.	0	28	100	0	0	0	14	0	0	0
Casein hydrolysis.	0	71	33	100	0	0	100	100	100	50
Chitin hydrolysis.	0/ 4	71	100	16	0	100	100	100	100	100
DNA hydrolysis.	40	100	100	100	0	100	100	100	100	100
Gelatin hydrolysis.	20	100	0	100	50	100	100	100	100	100
Lecithinase.	60	100/ 6	66	100/ 2	100	50	100	100	100	100
Lipase.	75/ 4	100/ 6	33	100	100	50	100	100	100	100
Esterase (SDS).	0	66/ 6	100	0/ 2	50	100/ 1	100	100	50	100
Egg proteolysis.	40	14	100	91	50	100	85	100	100	100
Phosphatase.	100/ 4	85	100/ 2	33/ 9	100	100	100/ 6	100	100	100
Starch hydrolysis.	80	100	66	100	100	50	85	100	100	100
Sulphide production.	50/ 4	0	0	0	0	0	0/ 6	0	0/ 1	0
Serum hydrolysis.	100/ 2	100/ 1	50/ 2	90/10	ND	0/ 1	100/ 6	ND	ND	100/ 1

Table 3.24.2; (Cont.): Attribute Frequencies (%), for UPGMA Phena I - R, and Discrete Type or Reference OTU's.

Phenon.	I	J	K	L	M	N	O	P	Q	R
Species.	P.fisc	V.or/t	V.pela		V.diaz	V.holl	V.vuln	V.cmib	V.cmib	
Number of OTU's (n).	n = 5	n = 7	n = 3	n = 12	n = 2	n = 2	n = 7	n = 2	n = 2	n = 2
Attribute.										
Tyrosine hydrolysis.	20	71	33	0	100	100	42	100	50	50
Tyrosine pigment.	0	85	66	0	50	100	57	100	50	100
Xanthine hydrolysis.	0	57	33	0	0	0	0	0	0	0
Congo Red Decolourization.	0/ 4	0/ 6	0/ 1	0/ 2	50	0/ 1	ND	0/ 1	ND	0/ 1
Sudanophilic inclusions.	0/ 4	0	0	0	0	0	28	50	0	0
Growth 4°C.	40	57	0	0	100	0	0	0	50	0
Growth15°C.	80	100	100	100	100	50	100	100	100	100
Growth30°C.	100	71	100	100	100	100	100	100	100	100
Growth37°C.	100	42	100	100	100	100	100	50	50	50
Growth42°C.	100/ 2	16/ 6	33	100/10	ND	100/ 1	100	0/ 1	0	0/ 1
Growth 0 % NaCl.	100	0	33	0	100	0	100	50	0	100
Growth 0.5 % NaCl.	100/ 3	42	100	16	100	50	100	100	100	100
Growth 1.0 % NaCl.	100	100	100	100	100	100	100	100	100	100
Growth 3.0 % NaCl.	100	100	100	100	100	100	100	100	100	100
Growth 6.0 % NaCl.	100	85	100	100	100	100	0	50	100	100
Growth10 % NaCl.	0	14	0	100	0	50	0	0	50	0
GrowthpH 10.	50/ 4	66/ 3	100/ 1	0/10	ND	0/ 1	85	0/ 1	50	100
GrowthpH 4.5.	60	0	0	0	0	0	0	0	0	0
GrowthNovobiocin.	100	14	33	100	50	0	0	0	0	0
GrowthPenicillin.	100	100	100	100	100	100	85	50	50	100
GrowthPolymyxin.	20	28	0	0	0	0	100	100	100	0
GrowthStreptomycin.	100	85	66	0	100	100	100	100	100	100
GrowthSulphamethoxazole.	100/ 2	0/ 2	100/ 1	0/10	ND	100/ 1	100	100/ 1	100	100/ 1
GrowthTetracycline.	60	14	0	0	0	0	0	0	0	0
GrowthTrimethoprim.	40	0	33	0	50	0	85	0	50	0
No growth10 µg/ml 0/129.	0	100	0	100	100	100	71	100	50	100
No growth150µg/ml0/129.	40	100	100	100	100	100	100	100	100	100
GrowthBasic fuchsin.	100/ 2	20/ 5	66	20/10	0	0	14	0/ 1	0	50
GrowthBrilliantgreen.	80	0	0	0	0	0	0	0	0	0
GrowthDichlorophene.	100/ 3	71	33	16	100	50	0	50	50	100
GrowthEDDA.	100	0	33	16	0	0	28	0	100	100
Growthon Fast yellow.	66/ 3	ND	0/ 1	ND	ND	ND	100	100/ 1	100	100/ 1
GrowthHaloquinol.	20	0	0	0	0	0	0	0	0	0
Growth8-Hydroxyquinoline.	0	0	0	0	0	0	0	0	0	0
GrowthMethylviolet.	100	16/ 6	66	100/ 2	0	0/ 1	14	50	0	50
GrowthPyronin-Y.	100	0	0	0	0	0	0	0	0	0
Growth0.2% SDS.	100	42	100	0	50	50	100	100	100	100
GrowthThionine.	100	0/ 6	0	0/ 2	0	0/ 1	0	0	0	100
Yellowon Thionine.	50/ 4	16/ 6	ND	0/ 2	0	0/ 1	ND	0/ 1	ND	100/ 1
GrowthT.T.C.	100	100/ 6	100	100/ 4	100	100	100	100	100	100
Acid from Xylose.	100/ 1	ND	100/ 1	ND	ND	ND	0	0/ 1	0	0/ 1
Acid from Arabinose.	100/ 1	ND	0/ 1	ND	ND	ND	0	0/ 1	0	0/ 1
Acid from Mannose.	100/ 1	ND	100/ 1	ND	ND	ND	100	100/ 1	100	100/ 1
Acid from Sucrose.	100/ 1	ND	100/ 1	ND	ND	ND	14	100/ 1	0	0/ 1
Acid from Maltose.	100/ 1	ND	100/ 1	ND	ND	ND	100	100/ 1	100	100/ 1
Acid from Cellobiose.	100/ 1	ND	100/ 1	ND	ND	ND	71	100/ 1	50	100/ 1
Acid from Salicin.	100/ 1	ND	100/ 1	ND	ND	ND	28	100/ 1	0	0/ 1
Acid from Mannitol.	100/ 1	ND	100/ 1	ND	ND	ND	100	100/ 1	50	100/ 1
Acid from Sorbitol.	100/ 1	ND	100/ 1	ND	ND	ND	14	0/ 1	0	0/ 1
Acid from Inositol.	0/ 1	ND	0/ 1	ND	ND	ND	0	0/ 1	0	0/ 1
Acid from Dulcitol.	0/ 1	ND	0/ 1	ND	ND	ND	14	0/ 1	0	0/ 1
GrowthGlycine.	40	0	66	0	0	100	0	50	50	50
Growthl-α-Alanine.	100	28	100	100	100	100	71	100	100	100
Growthd-α-Alanine.	100	57	100	100	100	100	42	100	50	100
Growthdl-β-Alanine	0	0	0	0	0	0	0/ 5	0	0	0

Table 3.24.2; (Cont.): Attribute Frequencies (%), for UPGMA Phena I - R, and Discrete Type or Reference OTU's.

Phenon.	I	J	K	L	M	N	O	P	Q	R
Species.	P.fisc	V.or/t	V.pela		V.diaz	V.holl	V.vuln	V.camb	V.camb	
Number of OTU's (n).	n = 5	n = 7	n = 3	n = 12	n = 2	n = 2	n = 7	n = 2	n = 2	n = 2
Attribute.										
Growth Serine.	80	0	100	0	100	100	14	100	100	50
Growth l-Leucine.	0	0	0	0	0	50	0	0	0	0
Growth Valine.	0	0	0	0	0	0	14	0	0	0
Growth l+Glutamic acid.	80	57	100/ 1	100	100	100	100	100	100	100
Growth l-Lysine.	20	28	33	16	50	100	14	0	50	50
Growth l-Arginine.	20	57	100/ 2	91	100	100	42	100	100	100
Growth l-Ornithine.	20	0	100	0	0	50	14	0	0	50
Growth l-Citrulline.	0	57	100	100	100	50	71	100	50	50
Growth ɣ-Amino-butyrate.	60	14	66	91	50	0	0	0	0	0
Growthδ-Amino-valerate.	20	0	0	0	0	0	0	0	0	0
GrowthNorvaline.	0/ 4	0/ 6	0/ 2	0/ 2	0	0/ 1	ND	0/ 1	ND	0/ 1
Growthl-Proline.	100	57	100	100	100	100	85	100	100	100
GrowthPutrescine.	40	0/ 6	100	0/ 2	100	0/ 1	0	0	0	50
GrowthSarcosine.	0	0	0	0	0	0	0	0	0	0
Growthl-Glutamine.	80	42	100	100	100	100	85	100	100	100
Growthd-Glucosamine.	100	57	100	100	100	100	85	100	0	0
Growthn-Acetylglucosamine	100	100/ 4	0	100	100	50	71	100	50	0/ 1
Growthd-Ribose.	100	28	0/ 1	100	100	100	71	100	100	50
GrowthXylose.	100	14	0	0	100	0	0	0	0	0
Growthl-Arabinose.	100	14	0	100	100	50	0	0	0	50
Growthl-Rhamnose.	80	14	0	100	0	100	0	0	0	0
GrowthGlucose.	80	85	66	100	100	100	100	100	100	50
Growthd-Mannose.	100	28	100	0	0	50	100	50	100	100
Growthd-Galactose.	80	85	66	100	100	100	85	50	50	50
GrowthSucrose.	80	85	0/ 1	100	50	100	0	100	50	100
GrowthTrehalose.	100	42	33	0	50	100	100	100	100	50
GrowthMaltose.	100	85	66	100	100	100	100	100	100	100
GrowthCellobiose.	100/ 4	100/ 4	0	100	100	0/ 1	57	100	100	100/ 1
GrowthLactose.	40	42	50/ 2	33	100	0	14	0	0	50
GrowthRaffinose.	40	0	0	0	0	0	0	0	0	0
GrowthGalactarate.	80	0	0	0	50	50	14	0	0	0
GrowthGalacturonicacid.	75/ 4	0/ 6	0/ 2	0/ 2	0	0/ 1	ND	0/ 1	ND	0/ 1
GrowthGluconate.	100	100	33	100	100	100	100	100	0	100
GrowthGlucuronicacid.	100	42	50/ 2	100	50	100	14	0	0	50
GrowthSalicin.	100	0	0/ 1	0/11	100	0	28	50	0	50
GrowthStarch.	50/ 2	100/ 4	100/ 1	100	100/ 1	100/ 1	85	100	100	100
GrowthDextrin.	100/ 1	100/ 1	100/ 1	100/10	ND	100/ 1	100	100/ 1	100	100/ 1
GrowthPolygalacturonicacid.	0/ 4	0/ 6	0/ 2	100/ 2	0	0/ 1	ND	0/ 1	ND	0/ 1
GrowthInulin.	20	0	0	0	0	50	0	0	0	0
GrowthPullulan.	0	85	0/ 1	100	0	100	85	50	100	100
GrowthFormate.	0	14	0	66	0	0	0	50	0	0
GrowthAcetate.	100	16/ 6	0	100/ 2	100	100/ 1	0	0	0	0
GrowthPropionate.	20	0	0/ 1	16	50	0	0	0	0	0
GrowthCaprylicacid.	0	0	0/ 2	0	0	0	0	0	0	0
GrowthNonanionicacid.	20	0	0	0	0	0	0	0	0	0
GrowthCaprate.	0	0	50/ 2	8	0	0	0	0	0	0
GrowthMalonate.	40	0	0	0	0	100	0	0	0	0
GrowthSuccinate.	100/ 4	57	100/ 2	100	100	100	ND	0/ 1	ND	0/ 1
GrowthMaleate.	ND	100/ 1	0/ 1	100/10	ND	0/ 1	0	0/ 1	0	0/ 1
GrowthAdipicacid.	0	0	0	0	0	50	0	0	0	0
GrowthMalate.	100/ 4	14	100/ 1	100	100	50	57	0	100	50
Growthdl-3-OH-butyrate.	0/ 4	0	0	0	0	100	0	0	0	0
Growth6-HydroxyCaproate.	0	0	0	0	0	0	0	0	0	0
GrowthLactate.	100	100/ 6	100	100/10	100	100	100	100	100	100
Growthdl-Glycericacid.	80	42	33	16	0	50	85	100	50	50

Table 3.24.2; (Cont.): Attribute Frequencies (%), for UPGMA Phena I - R, and Discrete Type or Reference OTU's.

Phenon.	I	J	K	L	M	N	O	P	Q	R
Species.	P.fisc	V.or/t	V.pela		V.diaz	V.holl	V.vuln	V.camb	V.camb	
Number of OTU's (n).	n = 5	n = 7	n = 3	n = 12	n = 2	n = 2	n = 7	n = 2	n = 2	n = 2
Attribute.										
Growth Poly-β-OH-butyrate.	0	0	0	0	0	0	0	0	0	0
GrowthCitrate.	80	85	33	100	100	50	71	50	100	0
Growthα-Ketoglutarate.	80	0	33	0	0	0	100	50	100	50
GrowthPyruvate.	100	100	100/ 1	100	100	100	100/ 3	100/ 1	100	100
GrowthErythritol.	0/ 3	0	0/ 1	0	0	0	0	0	0	0
GrowthDulcitol.	0/ 3	0	0/ 1	0	0	0	0/ 6	0	0	0
GrowthMannitol.	100	71	0/ 1	100	100	50	100	100	50	0
GrowthSorbitol.	100/ 4	0/ 1	0/ 1	0/10	ND	0/ 1	0	0/ 1	0	0/ 1
GrowthInositol.	0/ 4	0	0	0	0	0	0	0	0	0
GrowthEthanol.	25/ 4	14	0/ 2	100	0	100	ND	0/ 1	ND	0/ 1
GrowthBenzoate.	20	0	0	0	0	0	0	0	0	0
GrowthHydroxybenzoate.	20	0	0	0	0	0	0	0	0	0
GrowthPhenylacetic acid.	40	0	0	0	0	0	0	0	0	0

Phenon O was exceptional by the presence of both LDC and ODC, the facility of most strains for ONPG hydrolysis, to grow in the absence of saline, but not at 4°C. and to separately utilize galactose, but not sucrose. Phenon O differed from Phenon G, (also designated to *V.vulnificus),* principally by its greater nutritional facility; OTU's from these two phena attacked 32 and 12 carbon sources respectively.

Phenon R was a doublet fused with a $S_{j.}$ of 70 % containing a non-xanthinolytic OTU, (Stn 6690 UQM 3236), designated earlier (Phenon O, Figure 5.3.8; 3.8.6.2) as *V.orientalis.* Significant attributes from this phenon not also expressed in phenon J were for growth in the presence of EDDA and variable utilization of glycine.

Phenon S comprised four pure OTU's, ($S_{j.}$ 65 %), including the type culture of *V.mediterranei,* UQM 3076. This phenon apart from being entirely xanthinolytic, showed variable ADH and decarboxylation reactions. Variation within this phenon was consistent with the description of Pujalte and Garay, (1986) except that no OTU could separately assimilated m-inositol, all hydrolyzed gelatin, and all but the type culture hydrolyzed chitin. Further attributes which emend the description for *V.mediterranei* include ONPG, casein, DNA and starch hydrolysis, susceptibility to 150 μg/ml 0/129, and

growth of all strains in the range 1-6 % saline and at temperatures between 25°C. and 37°C.; and attack of d- & l-α-alanine, ornithine, putrescine and n-acetylglucosamine.

Phenon T included *V.natriegens* UQM 2782 and three provincial OTU's fused at (S_j *ca* 67 %). All were ADH negative and all but one also ODC and LDC negative. No strains grew at 4°C. or in less than 0.5 % saline. Seventy-five % or more OTU's from this phenon were positive for characters including arbutin, casein, DNA, gelatin, and starch hydrolysis, and separate utilization of putrescine, arabinose, rhamnose, salicin, starch, pullulan, dl-3-hydroxybutyrate and ethanol.

Phenon U, (S_j 75 %) comprised four OTU's including the Batch [7.] replicate OTU for *V.nigripulchritudo* UQM 2784. This OTU had attributes, (presence of ADH, and separate utilization of rhamnose and absence of black intracellular pigmentation), even more aberrant to the original description than the Batch [1.] replicate OTU. These properties are more consistent with "Group F," *[V.fluvialis I & II],* of Lee *et al.,* (1978) and indicate that UQM 2784 can no longer be considered a valid preparation of *V.nigripulchritudo* ATTC 27043.

Phena V, W & X were fused at a S_j of 75%. Each phenon was composed by OTU's from discrete batches, except for phenon W in which a single OTU from Batch [3.] was placed concurrently with OTU's from Batch [7.] Phenon V was exclusively represented by OTU's from Batch [1.], and included type cultures of both biovars of *V.fluvialis.* It would seem then, that phena V, W and X each represent locally valid (i.e. batch-driven) depictions potentially of both biovars of *V.fluvialis* rather than separate species.

The description of *V.fluvialis* (Lee *et al.,* 1981) relied on OTU's characterized in a single batch; this analysis provides opportunity to amend the description of *V.fluvialis* according to a stable set of differential criteria characteristic of both biovars of this species: Most strains do not swarm on solid media, all are oxidase-positive, produce catalase and

ferment glucose; gas production was not observed. All strains reduce nitrate to nitrate and most can reduce nitrite to gas. Most strains produce ADH but never LDC or ODC, all strains hydrolyze ONPG, DNA, lecithin, and egg lipids, most hydrolyze, casein, chitin, gelatin, SDS, starch, and serum. Most strains grow at 42°C. but not at 4°C. and in the presence of 0 but not 10 % saline and at pH 10 but not pH 4.5 and are sensitive to novobiocin, 10 µg/ml 0/129, and to pyronin-Y. All or most strains can separately assimilate glycine, d- & l-α-alanine, serine, l+glutamic acid, l-arginine, l-ornithine, l-citrulline, ɣ-aminobutyrate, glutamine, d-glucosamine, n-acetylglucosamine, l-proline, d-ribose, l-arabinose, glucose, gluconate, glucuronic acid, salicin, starch, pullulan, acetate, lactate, citrate, pyruvate, and mannitol. No growth occurs on lactose, inulin, or adipic acid and aliphatic acids.

OTU's from phenon U were otherwise in accord with the above emended description of biovars of *V.fluvialis* except for their resistance to tetracycline, and separate utilization on dl-β-alanine. No OTU's from phenon U were positive for growth in the presence of thionine, and separate utilization of ɣ-aminobutyrate, as expressed by both biovars of *V.fluvialis.* These OTU's are tentatively designated as a new biogroup, III, of *V.fluvialis* and two OTU's from this group Stn 5800 and 5810 were deposited as UQM 3275 and UQM 3354 respectively.

The phena Y and Z each contained *Rosetta* OTU's, (3.8.3), assigned to *A.hydrophila.* OTU's were resistant to 150 µg/ml 0/129 and produced LDC and ADH, but less than half aerogenically fermented glucose. OTU's from phenon Y had greater nutritional facility than those from phenon Z. These biogroups could be differentiated by attributes exclusively present in phenon Y including aesculin and arbutin hydrolysis and separate utilization of salicin.

OTU's representing *V.proteolyticus* and *P.damsela* fused at S_j 75 %, before joining with Phenon Z, *[A.hydrophila].* They could be differentiated from

Table 3.24.3: Attribute Frequencies (%), for UPGMA Phena S - Z, and Discrete Type or Reference OTU's.

Phenon.	S	T	U	V	W	X	Y	Z		
Species.	V.medi	V.natr		V.fluv				A.hydr	V.prot	P.dams
Number of OTU's (n).	n = 4	n = 4	n = 4	n = 6	n = 9	n = 12	n = 9	n = 3	n = 1	n = 1
Attribute.										
Colony diameter > 3 mm.	25	0/ 3	75	0	0/ 5	75	0/ 7	0/ 2	ND	0
Colony mucoid.	0	0	0	0	0	0	11	0	0	0
Colony opaque.	0	100	0	0	11	0	22	0	0	0
Colony entire.	100	75	100	100	88	83	88	100	0	100
Colony convex.	100	75	100	100	88	83	100	100	0	100
Swarming growth.	50	50	25	0	11	33	0	0	100	0
Luminous growth.	0	0	0	0	0	0	0	0	0	0
Red pigment.	0	0	0	0	0	0	0	0	0	0
Brown pigment.	0	0	0	0	11	0	0	0	0	0
Black pigment.	0	0	0	0	0	0	0	0	0	0
Yellow/orange.	50/ 2	0/ 3	100/ 1	0	0/ 2	0/ 1	25/ 4	0	0	0
Length > 2 times width.	50/ 2	0/ 3	100/ 1	66	100/ 1	ND	50/ 4	50/ 2	ND	ND
Strong catalase.	0	0	0	16	0	0	0	0	0	0
Oxidase.	100	100	100	100	100	100	100	100	100	100
Oxidase (Toluene).	100	100	100	100	100	100	100	100	100	100
Broth uniform turbidity.	100	75	100	100	75/ 8	100/11	100	100	100	100
Broth with sediment.	100	100	75	100	100/ 8	81/11	100	100	100	100
Broth sediment mucoid.	0/ 2	33/ 3	ND	0	100/ 1	ND	0/ 2	0/ 2	ND	ND
Broth growth mod. to heavy.	50	100	100	83	88	100/11	100/ 8	100	100	100
Pellicle.	0/ 3	0/ 3	25	0	14/ 7	18/11	0	33	0	0
Indole.	66/ 3	0	25	0	0/ 1	0/11	0/ 2	50/ 2	0	100
Weak indole.	100/ 3	33/ 3	25	0	100/ 7	72/11	77	66	100	ND
Motile.	100	100	50	83	100/ 8	100/11	100	100	100	100
5 day pH > 7.05.	0	0	50	33	12/ 8	100/11	100	0	0	0
5 day pH > 5.15.	0	25	50	83	50/ 8	100/11	100	33	0	100
Acetoin production.	0	0	0	50	25/ 8	9/11	100	0	0	0
Nitrate reduction.	100	100	100	100	100/ 8	100/11	100	100	100	100
Nitrite reduction.	50	100	75	83	100/ 8	90/11	88	66	100	0
Cholera-Red.	25	0	0	0	0	0	0	0	0	0
Gluconate oxidation.	0	0	0	16	33	0/11	66	33	0	0
Arginine dihydrolase.	50	0	100	100	100	90/11	100	100	100	100
Lysine decarboxylase.	25	25	0	0	0	0/11	88	100	100	100
Ornithine decarboxylase.	0	25	0	0	0	0/11	0/ 8	0	0	0
Glucose fermentation.	100	100	100	100	100	100/11	100	100	100	100
Gas from glucose.	0	0	25	0	0	0/11	11	66	0	0
ONPG hydrolysis.	100	25	25	100	100/ 8	100/11	100	66	0	0
Urea hydrolysis.	0	0	0	0	0	0/11	0	0	0	100
Aesculin hydrolysis.	50	75	100	83	100	75	100	0	0	0
Weak aesculin hydrolysis.	75	75	100	83	100	83	100	0	100	0
Agar hydrolysis.	0	0	0	0	0	0	0	0	0	0
Egg Albumen hydrolysis.	100/ 3	0/ 3	100	100	100	91	100	0	100	100
Arbutin hydrolysis.	50	100	100	66	100	83	100	0	0	0
Aryl-sulphatase.	25	0	25	0	0	0	0	0	0	0
Casein hydrolysis.	100	100	25	66	100	100	88	100	100	100
Chitin hydrolysis.	75	75	100	100	88	100	100	100	100	100
DNA hydrolysis.	100	100	75	100	100	100	100	100	100	100
Gelatin hydrolysis.	100	75	0	100	88	83	100	100	100	100
Lecithinase.	100	66/ 3	75	100	100	100	100	100	100	100
Lipase.	100/ 3	100/ 3	75	100	100/ 6	100	100/ 7	100	100	100
Esterase (SDS).	75	100	100	50	88	91	44	66	100	100
Egg proteolysis.	100	75	100	100	77	100	88	100	0	100
Phosphatase.	100	100/ 3	100	100/ 4	100	100	100	100/ 2	100	100
Starch hydrolysis.	100	75	100	83	100	100	100	66	100	100
Sulphide production.	25	25	0	ND	25/ 8	0	28/ 7	0/ 2	0	0
Serum hydrolysis.	50/ 2	0/ 1	100	100/ 5	100/ 1	91	100/ 4	ND	ND	ND

Table 3.24.3; (Cont.): Attribute Frequencies (%), for UPGMA Phena S - Z, and Discrete Type or Reference OTU's.

Phenon.	S	T	U	V	W	X	Y	Z		
Species.	V.medi	V.natr		V.fluv				A.hydr	V.prot	P.dams
Number of OTU's (n).	n = 4	n = 4	n = 4	n = 6	n = 9	n = 12	n = 9	n = 3	n = 1	n = 1
Attribute.										
Tyrosine hydrolysis.	25	100	75	100	33	66	77	100	100	100
Tyrosine pigment.	0	100	100	100	0	0	11	33	100	100
Xanthine hydrolysis.	100	0	0	0	0	0	0	0	100	100
Congo Red Decolourization.	0/ 2	ND	0/ 3	ND	33/ 6	0/ 9	0/ 6	ND	0	0
Sudanophilic inclusions.	0	0	0/ 1	0	0	50/ 2	0	33	0	0
Growth 4°C.	0	0	0	0	11	0	66	0	0	0
Growth15°C.	100	100	100	100	100	100	100	100	100	100
Growth30°C.	100	100	100	100	100	100	100	100	100	100
Growth37°C.	100	100	100	100	77	100	100	100	100	100
Growth42°C.	50	100/ 3	100	83	33/ 3	100	100/ 4	100/ 2	ND	ND
Growth 0 % NaCl.	0	0	0	100	100	91	100	100	100	0
Growth 0.5 % NaCl.	50/ 2	100/ 3	100	100	100/ 2	100	100/ 7	100	100	100
Growth 1.0 % NaCl.	100	100	100	100	100	100	100	100	100	100
Growth 3.0 % NaCl.	100	100	100	100	100	100	100	100	100	100
Growth 6.0 % NaCl.	100	100	100	100	100	100	22	66	100	100
Growth10 % NaCl.	0	0	50	16	0	0	0	0	100	0
GrowthpH 10.	0/ 3	50	ND	100	75/ 8	100/ 9	100/ 6	100/ 2	ND	ND
GrowthpH 4.5.	0	0	0	0	0	0	0	0	0	0
GrowthNovobiocin.	0	0	50	66	66	83	77	100	0	0
GrowthPenicillin.	100	100	100	100	100	100	100	100	100	100
GrowthPolymyxin.	50	25	0	0	55	0	77	0	100	100
GrowthStreptomycin.	100	100	100	100	100	83	100	100	100	100
GrowthSulphamethoxazole.	0/ 1	100/ 3	100/ 3	100	100/ 1	100	100/ 2	100/ 2	ND	ND
GrowthTetracycline.	0	0	100	0	0	0	0	0	0	0
GrowthTrimethoprim.	0	100	25	66	33	0	100	100	100	0
No growth10 µg/ml0/129.	75	25	0	0	33	8	0	0	0	100
No growth150µg/ml0/129.	100	100	100	100	77	100	0	0	0	100
GrowthBasic fuchsin.	33/ 3	33/ 3	100	100	100/ 1	100	100/ 4	100/ 2	ND	ND
GrowthBrilliantgreen.	0	50	25	100	88	100	100	66	100	0
GrowthDichlorophene.	50/ 2	100/ 3	0	100	100/ 2	0	71/ 7	100	100	100
GrowthEDDA.	0	50	25	33	100	100	88	33	0	0
Growthon Fast yellow.	0/ 2	66/ 3	ND	100	0/ 7	0/ 2	50/ 4	100/ 2	ND	ND
GrowthHaloquinol.	0	0	0	0	0	0	0	0	0	0
Growth8-Hydroxyquinoline.	0	0	0	0	0	0	0	33	0	0
GrowthMethylviolet.	33/ 3	66/ 3	100	100	50/ 8	100	100	100	100	100
GrowthPyronin-Y.	25	0	0	0	0	8	100	100	0	0
Growth0.2% SDS.	100	100	100	100	100	100	100	100	100	100
GrowthThionine.	100/ 3	33/ 3	0	100	100/ 8	100	66	0	100	100
Yellowon Thionine.	33/ 3	0/ 1	0/ 3	ND	100/ 8	91	14/ 7	0/ 1	0	0
GrowthT.T.C.	100/ 3	100	100	100	100	100	100	100	100	100
Acid from Xylose.	ND	0/ 1	ND	ND	ND	ND	0/ 2	ND	ND	ND
Acid from Arabinose.	ND	100/ 1	ND	ND	ND	ND	0/ 2	ND	ND	ND
Acid from Mannose.	ND	0/ 1	ND	ND	ND	ND	100/ 2	ND	ND	ND
Acid from Sucrose.	ND	100/ 1	ND	ND	ND	ND	100/ 2	ND	ND	ND
Acid from Maltose.	ND	100/ 1	ND	ND	ND	ND	100/ 2	ND	ND	ND
Acid from Cellobiose.	ND	100/ 1	ND	ND	ND	ND	0/ 2	ND	ND	ND
Acid from Salicin.	ND	100/ 1	ND	ND	ND	ND	100/ 2	ND	ND	ND
Acid from Mannitol.	ND	0/ 1	ND	ND	ND	ND	100/ 2	ND	ND	ND
Acid from Sorbitol.	ND	0/ 1	ND	ND	ND	ND	100/ 2	ND	ND	ND
Acid from Inositol.	ND	0/ 1	ND	0/ 2	ND	ND	0/ 2	ND	ND	ND
Acid from Dulcitol.	ND	0/ 1	ND	ND	ND	ND	0/ 2	ND	ND	ND
GrowthGlycine.	75	100	100	100	100	100	100	33	100	100
Growthl-α-Alanine.	100	100	100	100	100	100	100	100	100	100
Growthd-α-Alanine.	100	75	100	100	100	100	100	100	100	100
Growthdl-β-Alanine	0	33/ 3	100	0/ 1	0	0	42/ 7	0/ 2	100	0

Table 3.24.3; (Cont.): Attribute Frequencies (%), for UPGMA Phena S - Z, and Discrete Type or Reference OTU's.

Phenon.	S	T	U	V	W	X	Y	Z		
Species.	V.medi	V.natr		V.fluv				A.hydr	V.prot	P.dams
Number of OTU's (n).	n = 4	n = 4	n = 4	n = 6	n = 9	n = 12	n = 9	n = 3	n = 1	n = 1
Attribute.										
Growth Serine.	75	75	100	100	100	100	100	100	100	100
Growth l-Leucine.	0	50	33/ 3	66	66	0	33	33	0	100
Growth Valine.	0	50	0	50	0	0	11	0	0	0
Growth l+Glutamic acid.	100/ 3	100	100/ 3	100	100	100	100/ 7	100	100	0
Growth l-Lysine.	0	0	25	33	33	25	0	0	0	100
Growth l-Arginine.	75	100	100	100	100	91	100	100	100	100
Growth l-Ornithine.	100	75	100	100	88	100	22	0	0	0
Growth l-Citrulline.	75	75	100	83	88	91	66	0	100	100
Growth ɣ-Amino-butyrate.	25	25	0	100	100	100	100	33	0	0
Growthδ-Amino-valerate.	0	50	0	16	0	0	0	0	0	0
GrowthNorvaline.	0/ 3	0/ 1	0	ND	0/ 8	0	0/ 7	0/ 1	0	0
Growthl-Proline.	100	100	100	100	100	100	100	100	100	100
GrowthPutrescine.	100/ 3	100/ 3	100	83	100/ 8	83	66	33	100	100
GrowthSarcosine.	0	50	0	83	0	0	0	0	0	0
Growthl-Glutamine.	100	100	100	100	100	100	100	100	100	100
Growthd-Glucosamine.	50	25	50	83	100	100	100	100	100	100
Growthn-Acetylglucosamine	100	100	100	100	100	100	100	100	100	100
Growthd-Ribose.	66/ 3	75	66/ 3	100	100	80/ 5	100/ 7	100	100	100
GrowthXylose.	0	0	0	16	0	0	0	0	0	0
Growthl-Arabinose.	0	75	100	100	100	100	0	0	0	0
Growthl-Rhamnose.	0	100	100	50	44	0	0	33	0	0
GrowthGlucose.	75	100	75	100	100	100	100	100	100	0
Growthd-Mannose.	100	50	100	100	88	91	100	100	100	100
Growthd-Galactose.	100	100	100	100	100	91	100	100	0	100
GrowthSucrose.	75	75	25	100	100	100	100	66	0	100
GrowthTrehalose.	75	100	100	100	100	100	100	100	100	100
GrowthMaltose.	75	100	100	100	100	100	100	100	100	100
GrowthCellobiose.	66/ 3	75	0	50	100	16	0/ 4	100	100	100
GrowthLactose.	50	0	0/ 3	0	0	0	0/ 7	33	0	0
GrowthRaffinose.	25	50	75	50	0	0	0	0	0	0
GrowthGalactarate.	0/ 3	25	0	33	0	0	0	0	0	0
GrowthGalacturonicacid.	0/ 3	0/ 1	0	ND	62/ 8	100	0/ 7	0/ 1	0	0
GrowthGluconate.	75	75	75	100	100	100	100	100	100	100
GrowthGlucuronicacid.	50	75	100	100	88	83	11	0	0	0
GrowthSalicin.	33/ 3	75	100	100	100/ 8	91	100/ 8	0	0	0
GrowthStarch.	100/ 3	100	100/ 3	100	100	100	100	100	100	100
GrowthDextrin.	100/ 1	100/ 3	ND	100	100/ 1	ND	100/ 2	100/ 2	ND	ND
GrowthPolygalacturonicacid.	0/ 3	0/ 1	0	ND	0/ 8	0	0/ 7	0/ 1	0	0
GrowthInulin.	0	0	0	0	0	0	0	0	0	0
GrowthPullulan.	50	100	100	100	100	91	88	66	100	100
GrowthFormate.	0	25	0	33	55	0	22	0	0	100
GrowthAcetate.	66/ 3	100/ 3	0	100	100/ 8	33	77	66	100	100
GrowthPropionate.	0/ 3	25	0/ 3	0	88	0	14/ 7	0	100	0
GrowthCaprylicacid.	0	0	33/ 3	0	0	0	0	0	0	0
GrowthNonanionicacid.	0	0	0	0	0	0	0	0	0	0
GrowthCaprate.	0/ 3	25	25	16	88	16	88	66	100	100
GrowthMalonate.	0/ 3	25	0	33	11	0	0	0	0	0
GrowthSuccinate.	100	50/ 2	100	ND	100	100	100/ 7	100/ 1	100	100
GrowthMaleate.	0/ 1	50/ 2	ND	0/ 1	100/ 1	ND	0/ 1	0/ 1	ND	ND
GrowthAdipicacid.	0	25	0	0	0	0	0	0	0	0
GrowthMalate.	33/ 3	100	ND	100	88	ND	87/ 8	66	100	100
Growthdl-3-OH-butyrate.	50	75	25	33	88	0/10	55	0	0	0
Growth6-HydroxyCaproate.	0	0	0	0	0	0	0	0	0	0
GrowthLactate.	100	100	100	100	100	100	100	100	100	100
Growthdl-Glycericacid.	100	75	100	100	100	100	100	100	100	100

Table 3.24.3; (Cont.): Attribute Frequencies (%), for UPGMA Phena S - Z, and Discrete Type or Reference OTU's.

Phenon.	S	T	U	V	W	X	Y	Z		
Species.	V.medi	V.natr		V.fluv				A.hydr	V.prot	P.dams
Number of OTU's (n).	n = 4	n = 4	n = 4	n = 6	n = 9	n = 12	n = 9	n = 3	n = 1	n = 1
Attribute.										
Growth Poly-β-OH-butyrate.	0	0	0	0	22	0	0	0	0	100
GrowthCitrate.	75	100	75	100	100	100	66	100	100	100
Growthα-Ketoglutarate.	0	50	0	100	100	8	11	66	100	100
GrowthPyruvate.	100/ 3	75	100/ 3	100	100	91	100/ 7	100	100	100
GrowthErythritol.	0/ 3	0/ 2	ND	ND	0	ND	0/ 5	0/ 2	0	0
GrowthDulcitol.	0/ 3	0/ 3	ND	ND	0	ND	0/ 5	0/ 2	0	0
GrowthMannitol.	75	75	100	100	100	91	100	100	100	100
GrowthSorbitol.	50/ 2	0/ 3	0/ 3	33	100/ 6	58	0/ 2	50/ 2	100	100
GrowthInositol.	0	0/ 2	0	0/ 2	0	0	0/ 7	0/ 2	0	0
GrowthEthanol.	0	100/ 2	0	ND	100	8	0/ 7	100/ 1	0	0
GrowthBenzoate.	0	0	0	0	0	0	0	0	0	0
GrowthHydroxybenzoate.	0	75	0	100	88	0	22	0	0	0
GrowthPhenylacetic acid.	0	0	0	0	0	0	0	0	0	0

phenon Z by egg albumen and xanthine hydrolysis, sensitivity to novobiocin, resistance to polymyxin and thionine, and by separate assimilation of citrulline. *V.proteolyticus* differed from *P.damsela* by swarming, growth in 10 % saline, resistance to 150 μg/ml 0/129 and separate utilization of dl-β-alanine. *P.damsela* further differed from *V.proteolyticus* by production of indole, urease and separate attack of lysine, formate and poly-β-hydroxybutyrate.

Phenon AA is a problematical aggregate of OTU's fused at S_j *ca* 75 %. The OTU Stn 7450 from this phenon, after comparison with earlier preparations was found to be mixed and so was removed from computation of attribute frequencies. Two other OTU's from this phenon Stn 6710 and Stn 6590 were earlier assigned, (Figure 3.8, Phenon M; 3.8.6.2), to the species *V.carchariae/-V.harveyi*. Attribute frequencies of the five pure Batch [8.] OTU's from this phenon were relatively consistent and most agree with phenon AB but with aberrations. Consequently this phenon is classified as *V.carchariae/-V.harveyi* but no further comparative review is made here because construction of this phenon may have been may affected by mixed cultures.

Phenon AB, ($S_{j.}$ 70 %), fused Batch [1.] type cultures of *V.parahaemolyticus* and *V.harveyi* with 14 provincial OTU's from Batch [1.] including Stn 6550 earlier assigned, (3.8.6.2), to *V.carchariae*], then Batches [3.] and [7.] Characters which differentiated this phenon from all others included presence of both ODC and LDC, and hydrolysis of chitin, DNA, gelatin, lecithin, and starch, growth at 37°C. and in 6 % saline, susceptibility to novobiocin and resistance to polymyxin, and separate utilization of glycine, l-α-alanine, serine, l-glutamic acid, proline, glutamine, ribose, sucrose, trehalose starch and pullulan.

Phenon AC, comprised a triplet of provincial OTU's, ($S_{j.}$ of 75 %), which were most similar to those from both neighbouring phena but could be distinguished by several characters including sulphide production, separate utilization of lysine, putrescine, formate, arabinose, ethanol and mannitol and by the absence of egg proteolysis, arylsulphatase, and growth in the presence of 10 % saline. Two OTU's Stn 4710 and 4740 were deposited as UQM 3368 and 3369 respectively.

The largest phenon; AD, ($S_{j.}$ of 75 %), contained 76 OTU's including the type culture for *V.alginolyticus* UQM 2770 assayed in Batch [1.], but not the UQM 2770 Batch [7.] replicate. Attributes with a frequency of greater than 90 % in phenon AD but absent in the Batch [7.] replicate of UQM 2770 included ODC, separate utilization of glycine, d-α-alanine, serine, l+glutamic acid, l-arginine, l-ornithine, d-glucosamine, d-ribose, maltose, gluconate, pullulan, succinate, dl-glyceric acid and citrate. The Batch [7.] replicate was also resistant to novobiocin. These results suggest that an attenuated strain used in Batch [7.] was developed in storage ampoules of UQM 2770.

Apart from an absence of luminescence and variable presence of gluconate oxidation and acetoin production, phenon AD differed from *V.parahaemolyticus, V.harveyi* and *V.carchariae* (Phenon AB) by the characters (more than 80 % positive); growth at 42°C. and in the presence of brilliant green, separate utilization of l-leucine, l-ornithine, l-

Table 3.24.4: Attribute Frequencies (%), for UPGMA Phena AA - AI, and Discrete Type or Reference OTU's.

Phenon.	AA	AB	AC	AD		AE	AF	AG	AH	AI
Species.		V.pa/ha/ca		V.algi	V.algi	V.sp	V.sp	V.sp	S.putr	
Number of OTU's (n).	n = 5	n = 16	n = 3	n = 76	n = 1	n = 8	n = 3	n = 9	n = 13	n = 2
Attribute.										
Colony diameter > 3 mm.	100	6	0	64/67	ND	37	100	88	20/ 5	0
Colony mucoid.	0	12	0	2	0	0	33	0	15	50
Colony opaque.	0	31	0	10	0	12	0	11	0	50
Colony entire.	60	87	100	52	0	100	66	55	76	100
Colony convex.	80	93	66	50	0	100	100	55	92	100
Swarming growth.	20	6	33	67	0	50	0	22	15	0
Luminous growth.	0	25	0	0	0	0	0	11	0	0
Red pigment.	0	0	0	0	0	0	0	0	0	0
Brown pigment.	0	0	0	0	0	0	33	0	7	0
Black pigment.	0	0	33	0	0	0	0	0	0	0
Yellow/orange.	ND	0/15	0/ 1	12/31	0	ND	ND	ND	0/ 1	0
Length > 2 times width.	ND	50/14	ND	76/25	ND	ND	ND	ND	ND	ND
Strong catalase.	0	6	0	0	0	0	0	0	0	0
Oxidase.	100	100	100	100	100	100	100	100	100	100
Oxidase (Toluene).	100	100	100	100	100	100	100	100	100	100
Broth uniform turbidity.	100	100	0/ 2	93	100	100	100	100	100	100
Broth with sediment.	80	100	100/ 2	94	100	100	66	88	100	50
Broth sediment mucoid.	ND	21/14	ND	47/23	ND	ND	ND	ND	ND	ND
Broth growth mod. to heavy.	100	81	66	97	100	100	100	100	92	0
Pellicle.	0	36/11	0/ 2	29/55	100	12	33	0	50/12	0
Indole.	0	46/15	100/ 2	9/61	ND	0	0	11	0/12	0
Weak indole.	100	100/11	100	96/61	100	100	100	100	0/12	0
Motile.	100	100	100	98	100	100	100	100	100	100
5 day pH > 7.05.	0	0	33	68	0	0	0	0	100	100
5 day pH > 5.15.	0	6	33	71	0	0	0	0	100	50
Acetoin production.	0	0	0	57	100	0	0	11	0	0
Nitrate reduction.	100	87	100	96	100	100	100	100	84	100
Nitrite reduction.	100	81	100	97	100	100	100	100	100	100
Cholera-Red.	0	0	0	0	0	0	0	0	0	0
Gluconate oxidation.	0	0	0	69	0	0	0	0	69	0
Arginine dihydrolase.	20	18	0	5/75	0	87	66	0	0	0
Lysine decarboxylase.	100	100	100	98	100	0	0	100	66/ 3	ND
Ornithine decarboxylase.	100	100	100	92	0	0	0	100	100/ 4	ND
Glucose fermentation.	100	100	100	98	100	100	66	100	0	0
Gas from glucose.	0	0	0	1	0	0	0	44	0	0
ONPG hydrolysis.	20	68	66	19	0	62	33	66	0	0
Urea hydrolysis.	20	43	66	3	0	0	33	100	0	0
Aesculin hydrolysis.	0	56	66	11	0	37	0	66	0	0
Weak aesculin hydrolysis.	40	93	100	22	100	75	0	55	15	50
Agar hydrolysis.	20	0	0	0	0	0	0	0	0	0
Egg Albumen hydrolysis.	80	18/11	100	95/65	100	100	100	66	92	100
Arbutin hydrolysis.	40	75	66	10	0	25	100	88	7	50
Aryl-sulphatase.	100	43	0	34	0	100	0	22	23	0
Casein hydrolysis.	80	100	100	89	100	100	100	88	100	50
Chitin hydrolysis.	100	100/15	100	97/75	100	100	100	100	84	100
DNA hydrolysis.	100	100	100	100	100	100	100	88	100	50
Gelatin hydrolysis.	80	93	66	97	100	100	33	88	92	100
Lecithinase.	100	100/13	100	93/32	100	75	100	100	92	100
Lipase.	100	100/15	ND	91/71	100	100	100	55	100/10	100
Esterase (SDS).	40	87	100	96	100	50	100	100	38	50
Egg proteolysis.	100	93	0	72	100	87	100	100	53	50
Phosphatase.	100	100	100	100	100	100	33	100	100	100
Starch hydrolysis.	100	100	100	98	100	100	100	88	84	100
Sulphide production.	0	14/ 7	100	8/71	0	0	0	0	100	0
Serum hydrolysis.	100	100/12	ND	96/61	ND	50	66	100	33/ 3	ND

Table 3.24.4; (Cont.): Attribute Frequencies (%), for UPGMA Phena AA - AI, and Discrete Type or Reference OTU's.

Phenon.	AA	AB	AC	AD		AE	AF	AG	AH	AI
Species.		V.pa/ha/ca		V.algi	V.algi	V.sp	V.sp	V.sp	S.putr	
Number of OTU's (n).	n = 5	n = 16	n = 3	n = 76	n = 1	n = 8	n = 3	n = 9	n = 13	n = 2
Attribute.										
Tyrosine hydrolysis.	20	68	66	76	100	87	33	88	69	50
Tyrosine pigment.	80	68	0	30	100	87	66	33	76	0
Xanthine hydrolysis.	40	0	0	0	0	37	33	0	0	0
Congo Red Decolourization.	0	0/ 2	66	3/27	0	0/ 3	0	0/ 4	10/10	0
Sudanophilic inclusions.	ND	0	0	8/61	0	40/ 5	ND	25/ 4	8/12	0
Growth 4°C.	0	6	0	14	0	37	0	0	38	50
Growth15°C.	100	100	100	100	100	100	100	100	92	100
Growth30°C.	100	100	100	100	100	87	100	100	100	100
Growth37°C.	100	100	66	98	100	0	66	100	100	100
Growth42°C.	20	46/15	0/ 1	98/62	ND	0	0	0	40/ 5	ND
Growth 0 % NaCl.	0	12	0	1	0	0	0	0	100	100
Growth 0.5 % NaCl.	80	100/15	ND	90/65	100	0	0	77	88/ 9	100
Growth 1.0 % NaCl.	100	100	100	100	100	87	100	100	100	100
Growth 3.0 % NaCl.	100	100	100	100	100	100	100	100	100	100
Growth 6.0 % NaCl.	100	100	100	100	100	87	33	77	53	100
Growth10 % NaCl.	0	0	0	65	0	0	0	0	7	0
GrowthpH 10.	ND	73/15	100	65/40	ND	100/ 1	ND	ND	20/ 5	ND
GrowthpH 4.5.	0	0	0	0	0	0	0	0	0	0
GrowthNovobiocin.	20	6	0	0	100	0	0	0	100	100
GrowthPenicillin.	80	100	100	100	100	100	100	100	100	100
GrowthPolymyxin.	80	100	100	61	100	12	66	66	69	0
GrowthStreptomycin.	100	87	100	96	100	87	100	100	100	100
GrowthSulphamethoxazole.	100	100/14	ND	100/59	ND	100	66	100	100/ 3	ND
GrowthTetracycline.	0	0	0	7	0	0	0	11	30	0
GrowthTrimethoprim.	20	87	100	69	100	0	0	22	84	0
No growth10 µg/ml 0/129.	0	12	66	21	0	75	100	0	0	0
No growth150µg/ml0/129.	80	87	100	92	100	100	100	100	53	0
GrowthBasic fuchsin.	100	60/15	ND	79/62	ND	100	66	100	50/ 4	0
GrowthBrilliantgreen.	100	18	0	84	0	62	33	66	23	0
GrowthDichlorophene.	20	93/15	ND	55/65	100	0	33	22	62/ 8	100
GrowthEDDA.	80	56	0	17/75	100	0	66	33	76	50
Growthon Fast yellow.	ND	90/10	0	2/34	ND	0/ 5	ND	0/ 4	0/ 7	ND
GrowthHaloquinol.	0	0	0	0	0	0	0	0	0	0
Growth8-Hydroxyquinoline.	0	0	0	0	0	0	0	0	0	0
GrowthMethylviolet.	100	72/11	33	100/55	0	50	33	100	76	0
GrowthPyronin-Y.	80	6	66	60	100	42/ 7	0	77	76	100
Growth0.2% SDS.	100	100	100	98	100	100	100	100	61	50
GrowthThionine.	100	18/11	66	89/55	100	62	0	77	69	0
Yellowon Thionine.	80	0/ 2	66	75/54	0	50	0	66	46	0
GrowthT.T.C.	100	100	100	100	100	100	100	100	100	100
Acid from Xylose.	ND	0/ 4	ND	100/ 1	ND	ND	ND	ND	ND	ND
Acid from Arabinose.	ND	0/ 7	ND	100/ 2	ND	ND	ND	ND	ND	ND
Acid from Mannose.	ND	100/ 4	ND	100/ 1	ND	ND	ND	ND	ND	ND
Acid from Sucrose.	ND	50/ 4	ND	100/ 1	ND	ND	ND	ND	ND	ND
Acid from Maltose.	ND	100/ 4	ND	100/ 1	ND	ND	ND	ND	ND	ND
Acid from Cellobiose.	ND	100/ 4	ND	100/ 1	ND	ND	ND	ND	ND	ND
Acid from Salicin.	ND	100/ 4	ND	0/ 1	ND	ND	ND	ND	ND	ND
Acid from Mannitol.	ND	100/ 4	ND	100/ 1	ND	ND	ND	ND	ND	ND
Acid from Sorbitol.	ND	100/ 4	ND	100/ 1	ND	ND	ND	ND	ND	ND
Acid from Inositol.	ND	0/ 4	ND	0/ 1	ND	ND	ND	ND	ND	ND
Acid from Dulcitol.	ND	0/ 4	ND	0/ 1	ND	ND	ND	ND	ND	ND
GrowthGlycine.	40	100	100	98	0	87	66	100	23	0
Growthl-α-Alanine.	100	100	100	100	100	50	66	100	76	100
Growthd-α-Alanine.	100	100	100	100	0	100	66	100	7	100
Growthdl-β-Alanine	0	0/12	0	8/75	0	0	0	0	0	100

Table 3.24.4; (Cont.): Attribute Frequencies (%), for UPGMA Phena AA - AI, and Discrete Type or Reference OTU's.

Phenon.	AA	AB	AC	AD		AE	AF	AG	AH	AI
Species.		V.pa/ha/ca		V.algi	V.algi	V.sp	V.sp	V.sp	S.putr	
Number of OTU's (n).	n = 5	n = 16	n = 3	n = 76	n = 1	n = 8	n = 3	n = 9	n = 13	n = 2
Attribute.										
Growth Serine.	100	75	100	100	0	100	66	100	100	50
Growth l-Leucine.	0	6	100	86	100	0	0	0	53	0
Growth Valine.	0	0	0	1	0	0	0	0	23	0
Growth l+Glutamic acid.	100	100	66	100/54	0	100	100	100/ 5	76	100
Growth l-Lysine.	0	43	100	23	0	0	0	0	0	100
Growth l-Arginine.	80	93	66	100	0	37	100	66	0	100
Growth l-Ornithine.	20	37	66	94	0	50	66	88	30	0
Growth l-Citrulline.	0	43	33	86	0	50	0	44	15	0
Growth ɣ-Amino-butyrate.	0	12	33	13	0	0	0	0	15	0
Growth6-Amino-valerate.	0	0	0	2	0	0	0	0	0	0
GrowthNorvaline.	0	0/ 2	0	0/54	0	0	0	0	0	0
Growthl-Proline.	100	100	66	100	100	100	100	100	46	100
GrowthPutrescine.	0	9/11	100	44/56	0	0	0	0	100	100
GrowthSarcosine.	0	12	0	0	0	0	0	0	0	0
Growthl-Glutamine.	100	100	100	100	100	87	100	100	100	100
Growthd-Glucosamine.	20	56	66	92	0	25	33	0	84	0
Growthn-Acetylglucosamine.	100	62	100	98	ND	37	0	33	92	ND
Growthd-Ribose.	100	100	100	100/67	0	ND	ND	60/ 5	10/10	100
GrowthXylose.	0	6	0	0	0	0	0	0	0	0
Growthl-Arabinose.	0	25	100	46	0	0	0	0	84	50
Growthl-Rhamnose.	0	25	33	13	0	0	0	0	0	0
GrowthGlucose.	100	93	100	98	100	12	0	0	100	50
Growthd-Mannose.	100	75	100	89	0	50	66	44	0	0
Growthd-Galactose.	100	93	100	82/75	0	0	0	55	15	100
GrowthSucrose.	20	93	100	80/75	0	12	0	11	0	50
GrowthTrehalose.	100	87	100	98	100	12	0	0	0	100
GrowthMaltose.	100	100	33	100	0	25	0	44	92	100
GrowthCellobiose.	80	93	100	71/71	ND	12	0	55	0/ 3	100/ 1
GrowthLactose.	0	6	0	1/73	0	0	0	0	0	0
GrowthRaffinose.	0	6	0	1	0	0	0	0	0	0
GrowthGalactarate.	0	31	0	1	0	0	0	0	7	50
GrowthGalacturonicacid.	0	0/ 2	0	1/54	0	0	0	0	0	0
GrowthGluconate.	100	100	100	98	0	12	0	66	0	100
GrowthGlucuronicacid.	100	93	66	22	0	0	0	22	23	100
GrowthSalicin.	0	20/15	66	1/73	0	0	0	11	0	0
GrowthStarch.	100	100	100	98/73	ND	0	0	66	75/12	100
GrowthDextrin.	ND	100/14	ND	100/22	ND	ND	ND	ND	ND	ND
GrowthPolygalacturonicacid.	0	50/ 2	0	0/54	0	0	0	0	0	0
GrowthInulin.	0	0	0	1	0	0	0	0	0	0
GrowthPullulan.	40	100	100	97	0	0	0	22	0	100
GrowthFormate.	0	0	100	27	0	0	0	0	7	0
GrowthAcetate.	0	27/11	100	47/55	0	0	0	0	76	100
GrowthPropionate.	0	0	0	22/74	0	0	0	0	76	100
GrowthCaprylicacid.	0	0	0	0	0	0	0	0	0	0
GrowthNonanionicacid.	0	0	0	0	0	0	0	0	0	0
GrowthCaprate.	0	0	33	17/74	0	0	0	0	92	50
GrowthMalonate.	0	12	0	3	0	0	0	0	7	50
GrowthSuccinate.	100	100/ 7	100	100/75	0	100	100	100	100	50
GrowthMaleate.	ND	50/10	ND	68/22	ND	ND	ND	ND	ND	ND
GrowthAdipicacid.	0	18	0	1	0	0	0	0	0	100
GrowthMalate.	ND	100	66	89/37	0	ND	ND	ND	100/10	100
Growthdl-3-OH-butyrate.	0	0	0	5	0	0	0	0/ 8	15	100
Growth6-HydroxyCaproate.	0	6	0	0	0	0	0	0	0	100
GrowthLactate.	80	100	100	100	100	87	0	77	92	100
Growthdl-Glycericacid.	100	43	100	98	0	12	66	0	92	100

Table 3.24.4; (Cont.): Attribute Frequencies (%), for UPGMA Phena AA - AI, and Discrete Type or Reference OTU's.

Phenon.	AA	AB	AC	AD		AE	AF	AG	AH	AI
Species.		V.pa/ha/ca		V.algi	V.algi	V.sp	V.sp	V.sp	S.putr	
Number of OTU's (n).	n = 5	n = 16	n = 3	n = 76	n = 1	n = 8	n = 3	n = 9	n = 13	n = 2
Attribute.										
Growth Poly-β-OH-butyrate.	0	0	33	3	0	0	0	0	0	50
GrowthCitrate.	80	93	100	97	0	25	0	66	7	100
Growthα-Ketoglutarate.	0	56	100	17	0	0	0	0	23	50
GrowthPyruvate.	100	100	100	94/73	100	100	66	100	100	50
GrowthErythritol.	ND	8/12	0	0/36	0	ND	ND	ND	0/10	0
GrowthDulcitol.	ND	0/14	0	5/36	0	ND	ND	ND	0/10	0
GrowthMannitol.	100	100	100	100	100	0	0	33	0	0
GrowthSorbitol.	60	35/14	ND	3/60	ND	0	0	0	0/ 3	ND
GrowthInositol.	0	0/15	0	1	0	0	0	0	0	0
GrowthEthanol.	20	14/ 7	100	81/75	0	0	0	0	7	100
GrowthBenzoate.	0	0	0	0	0	0	0	0	0	0
GrowthHydroxybenzoate.	0	18	0	1	0	0	0	0	0	0
GrowthPhenylacetic acid.	0	0	0	0	0	0	0	0	0	0

citrulline, and d-glucosamine. OTU's from phenon AD were variable for only two characters more than 90 % positive in phenon AB, these were egg proteolysis and separate utilization of d-glucuronic acid.

3.8.6.3.2.3.5 Review and Diagnosis of Phena from Group 6.

Group 6., [AE,AF,AG] comprised 20 OTU's, all from Batch [8.] and fused at $S_{j.}$ *ca* 60 %. The constituent phena AE, AF and AG in turn contained 8, 3, and 9 OTU's which respectively were bound by $S_{j.}$ coefficients of *ca* 75 %, 65 % and 75 %. All but one weak OTU from Group 6. fermented glucose, and all were susceptible to 150 μg/ml 0/129, were sodium requiring and were able to grow in the presence of basic fuchsin. Phena AE and AF both contained OTU's which hydrolyzed xanthine, and produced ADH, phenon AE as was previously noted produced arylsulphatase. Additional characters which differentiate these phena from other xanthinolytic phena were abstracted from Table 3.24.2-4 and shown in Table 3.25. Both AE and AF attacked *ca* 50 % less carbon sources than those from all other groups of xanthinolytic phena.

Phena AE and AF could represent new taxonomic groups or secondary formations of existing species induced through sub-optimal culture conditions to aggregate around a nucleus of seeming nutritionally limited OTU's. The

greatest correspondence of Phenon AE is with *V.orientalis,* (ADH positive and xanthinolytic species with taxonomic precedence over *V.tubiashii).* The status of phenon AF cannot be resolved here in isolation.

Table 3.25: Differential Characters Amongst Xanthinolytic OTU's.

Phenon:	J	K	S			AA	AE	AF
	V.or/tu	*V.pela*	*V.medi*	*V.prot*	*V.dams*			
Number of OTU's (n).	(7)	(3)	(4)	(1)	(1)	(5)	(8)	(6)
Batches.	3,7	1,7	3,7,8	7	7	8	8	8
Xanthine hydrolysis	57	33	100	100	100	40	37	33
ADH	85	0	50	100	100	20	87	66
LDC	14	0	25	100	100	100	0	0
ODC	0	0	0	0	0	100	0	0
ONPG hydrolysis	14	66	100	0	0	20	62	33
Urea hydrolysis	14	0	0	0	100	20	0	33
Arbutin hydrolysis	14	33	50	0	0	40	25	100
Arylsulphatase	28	100	25	0	0	100	100	0
Growth 4°C.	57	0	0	0	0	0	37	0
Growth 0 % NaCl.	0	33	0	100	0	0	0	0
Growth10 % NaCl.	14	0	0	100	0	0	0	0
Growth10 % NaCl.	14	0	0	100	0	0	0	0
No growth10 µg/ml0/129.	100	0	75	0	100	0	75	100
No growth150 µg/ml0/129.	100	100	100	0	100	80	100	100
Growthon, dl-β-alanine.	0	0	0	100	0	0	0	0
Putrescine	0/6	100	100/3	100	100	0	0	0
Gluconate	85	33	75	100	100	100	12	0
Pullulan.	85	0/1	50	100	100	40	0	0
α-Ketoglutarate.	0	33	0	100	100	0	0	0
NumberC sourcesattacked.	39	32	40	34	36	32	24	15

From previous assignments of replicated phenon AF OTU's, (Figure 3.8; 3.8.6.2.2), Stn 6561 was unclustered, and Stn 6500 assigned to a biogroup of *V.aestuarianus* (phenon E). This infers that phenon AF is polyspecific and comprises both a biogroup of sometimes xanthinolytic *V.aestuarianus* and a further *V.aestuarianus*-like taxon. A representative OTU of *V.aestuarianus* from this latter phenon Stn 6610 was deposited as UQM 3358, for later examination.

All OTU's from phenon AG, produced ODC, LDC, and urease and four also produced gas from glucose. More than 85 % of OTU's hydrolyzed arbutin, casein, chitin, gelatin, starch and tyrosine; grew between 15°C. and 37°C., in 1 % and 3 % saline; were susceptible to 150 µg/ml 0/129 and novobiocin, and were separately able to utilize glycine, d- & l- α-alanine, serine, l-

glutamic acid, l-proline, l-glutamine, succinate and pyruvate but not l-leucine, l-lysine, γ-aminobutyrate, δ-aminovalerate, or putrescine. This phenon most closely resembles *V.carchariae* of Grimes *et al.,* (1984) however OTU's from this analysis were not as versatile as others assayed earlier (3.8.4) and were otherwise exceptional by production of gas from glucose and by tyrosine hydrolysis.

3.8.6.3.2.3.6 Review and Diagnosis of Phena from Groups 7. and 8.

The phenon AH, (S_j *ca* 55 %), (Group 7.), exclusively comprised weakly fermentative OTU's, which produced sulphide and ODC. Only half the OTU's were susceptible to 150 μg/ml 0/129. More than 90 % of OTU's produced a 5 d MRVP pH greater than 7.05, and hydrolyzed egg albumen casein, chitin, DNA, gelatin, and lecithin, grew in 0-6 % saline, and were able to separately utilize the carbon sources serine, putrescine, glutamine, n-acetylglucosamine, glucose, maltose, caprate, succinate, malate, lactate, dl-glyceric acid and pyruvate. This phenetic profile is most consistent with *Shewanella putrefaciens (Alteromonas putrefaciens),* as depicted by Lennette, Ballows, Hausler and Truant (1980).

Phenon AI, (S_j *ca* 70 %), (Group 8), comprised 2 tentatively identified *Alteromonas* OTU's. This non-sodium requiring species could be differentiated from the other weakly fermentative, (or oxidative) phenon AH, by resistance to 0/129 and by its greater nutritional facility. This included growth upon dl-β-alanine, l-lysine, arginine, d-galactose, trehalose, gluconate, glucuronate, pullulan, heptanoate, adipic acid, dl-3-hydroxybutyrate, 6-hydroxycaproate, citrate and ethanol. Because of its low sodium requirement, and range of exo-enzymes this phenon does not conform to any previously described species of *Alteromonas* or *Pseudomonas.* As no provincial OTU's were associated with this phenon no further classification than as an unidentified *Alteromonas* sp. or *Pseudomonas* sp. was justifiable.

3.8.6.3.2.4 Review of Phena.

Previously unidentified provincial OTU's clustered into 20 phena with named reference OTU's from the *Vibrionaceae* or grouped into five unreferenced taxa. In order of abundance these OTU's were assigned to the taxa *V.alginolyticus, V.fluvialis/-V.furnisii [V.fluvialis* Biovars I and II], *S.putrefaciens, A.hydrophila,* Phenon L, *V.carchariae, V.orientalis, V.parahaemolyticus/-V.harveyi/-V.carchariae, V.mediterranei, V.fluvialis* biogroup 3, Phenon AC, *V.natriegens, V.vulnificus, Photobacterium*-like, *V.campbellii, V.gazogenes, V.aestuarianus, V.anguillarum, Plesiomonas shigelloides, V.diazotrophicus, V.hollisae, V.pelagius* and *V.ordalii.* No provincial or other OTU's were found to associate with type or reference OTU's from the six species *V.costicola, P.damsela, V.metschnikovii, V.mimicus, V.nereis* and *V.proteolyticus.*

Amongst OTU's placed into phena were three groups of related or phenetically similar species *V.carchariae/-V.harveyi/-V.parahaemolyticus, V.fluvialis/-V.furnissii,* and *V.orientalis/-V.tubiashii,* which were not always separately resolved.

Apart from artifactual species fusions some phena attributed to the same species were described as fragmented. As in the analysis of the previous dataset some phena, comprised principally by Tasmanian OTU's, appeared to have been affected by super-optimal incubation temperature, and to have been drawn primarily into ecological groups with secondary taxonomic structure. This was seen by the designation of the heterogeneous phenon C as polyspecific, and of the phena J and AE to *V.orientalis.*

Fragmentation amongst other phena occurred for different reasons. It reflected the obvious i.e. genuine phenetic differences independent of batch origins of constituent OTU's. Such differences were indicated by separate biogroups of *Aeromonas hydrophila,* occurring in phena Y and Z apart from the anaerogenic OTU UQM 2769. Similarly the OTU Stn 6690 from phenon R earlier designated, (3.8.6.2), as a non-xanthinolytic *V.orientalis* UQM 3236

was here presented as distinct from that species. This was consistent with its relatively late placement into phenon O of Figure 3.8. Fragmentation also occurred more subtley as an artifact of cluster procedure e.g. the three phena used to describe *V.fluvialis* biovars were placed as consecutive batch-originated components to the same minor hierarchy. Similarly the phena P and Q both with OTU's earlier attributed as *V.campbellii* did not form primary associations but were successively fused with the phenon assigned as *V.vulnificus.* Similarly fragmentation could occur as an artifact of phenetic attenuation in ampouled cultures as from replicate cultures of *V.alginolyticus* UQM 2770 and of *V.vulnificus* UQM 3032.

One distinctive but atypical phenon, L, was also subjected to comparative molecular examination to determine its taxonomic status. Other atypical clusters were deposited in the UQM collection for future work and not further investigated here because they contained too few OTU's or because of their association with potentially skewing mixed cultures.

The occurrence of other xanthinolytic species from the *Vibrionaceae* apart from *V.tubiashii* (Hada *et. al.,* 1984) was confirmed here (from 3.8.4) for *V.orientalis,* and shown for an associated biogroup of *V.aestuarianus,* and all OTU's assigned to *V.mediterranei,* and for type cultures of *P.damsela, V.proteolyticus* and also from OTU's in phenon AA designated to *V.carchariae/-V.harveyi.* Other traits rare amongst the *Vibrionaceae* but expressed uniformly amongst OTU's from atypical phena included aryl sulphatase from *V.mimicus, V.pelagius* and phena AA and AE, sulphide production by all OTU's from phenon AC, urea hydrolysis from *P.damsela* and phenon AG, assigned as *V.carchariae;* and separate attack of rhamnose by *V.natriegens, V.hollisae,* the new biogroup, [III], of *V.fluvialis,* and another, phenon L unlike any previously described species. Derived phena correlated with extant species were usually consistent with the traditional ALO patterns attributed to those species, however some phena had ALO deviant OTU's which necessitated a more broadly based classificatory system.

3.8.6.3.3 **Discussion.**

This analysis by *Microcluster* appeared to have been more prone to the effects of noise, as demonstrated by cluster fragmentation, than the one described in section (3.8.6.2) undertaken using *SAS*. This was probably related to the absence of a "k-" linkage damping-option on the microcomputer program. Despite this problem the presence of reference and replicate cultures in the analysis largely indicated where phenon integrity could be relied upon and it was found that most imported and provincial OTU's could be correlated to previously described species.

While in some instances fragmentation was attributed to genuine phenon differences other noises which contributed to artifactual fragmentation included lost phenetic facility of stored replicated cultures, across-and within-batch test-variation, and due to cultivation of cool water strains at super-optimal temperatures, and from the inclusion of some mixed cultures in the dataset. For taxonomic purposes fragments of the same or related species would at best be expected to be placed serially as components within the same hierarchy. This was shown here for OTU's assigned to *V.fluvialis* and *V.campbellii* but not for other fragmented species.

While this fragmentation probably resulted in a lower final similarity between all OTU's than would have been obtained with a program with "k"-linkage facility such as *SAS*, the relative stringency of *Microcluster* induced within-species biogeographical variations to prominence.

In addition to specific requirements of groups of environmental *Vibrionaceae* such as was shown for those from Benalla (3.8.6.2) for moderately high incubation temperature, some locally adapted population variants (from Tasmania) were shown here to prefer cooler temperatures. These features are complimentary with indications of other specific growth requirements of environmental *Vibrionaceae* particularly by species from *Aeromonas* which are non-sodium requiring and from *Shewanella benthica* for which growth is enhanced by increased hydrostatic pressures.

The species *V.orientalis* and *V.tubiashii* while not concurrently placed in Batch [6.] (3.8.4) were phenetically quite similar and shown as such for both pairs of replicate OTU's from 3.8.5. No separate resolution of these species occurred in this phenetically more stringent analysis, i.e. in conditions unaffected by improperly purified water; but it was reaffirmed that both species are ADH positive and xanthinolytic suggesting that rather than for two similar species to exhibit such a rare combination of attributes that these are the same species. Yang *et al.,* (1983) did not investigate xanthine hydrolysis when *V.orientalis* was described, and Hada *et al.,* (1984) did not undertake DNA hybridization between-strains subsequently designated as *V.tubiashii* with *V.orientalis;* obviously such work is required. If *V.orientalis* and *V.tubiashii* are synonymous, the culture with priority according to rule 18b of the *Bacteriological Code* (Lapage, Sneath, Lessel, Skerman, Seeliger and Clark, 1976) is the type culture for *V.orientalis* ATCC 33934 which was effectively published and then validated before *V.tubiashii,* despite the earlier deposition of ATCC 1909. The name *V.orientalis,* has tentatively been adopted for all provincial OTU's designated in this study to the shared phenotype.

Consequent to findings about the phenetic and molecular (See Appendix 9.) character of OTU's from phenon L, the proposal for *V.zobellii sp. nov.* (After C.E. ZoBell in recognition of his foundation work in marine microbiology and bacterial micro-ecology.) with Stn 750, UQM 3027, as the type culture will be made. The species has a mole % G + C (Appendix 9.) in the range 42.4-45.6 and less than 30 % homology with type cultures from the species *V.campbellii, V.pelagius, V.natriegens,* and *V.nereis.*

More than 90 % of strains of *V.zobellii* are positive for the characters cytochrome oxidase, polar flagellation, anaerogenic glucose fermentation, β-galactosidase, hydrolysis of aesculin, arbutin, casein, DNA, gelatin, starch, egg protein and lipid, growth between 15 and 42°C., growth in 1 to 10 % saline, resistance to 10 μg/ml novobiocin, 15 unit/ml penicillin, separate utilization of d and l α-alanine, l-glutamic acid, l-arginine, γ-

aminobutyrate, l-proline, l-glutamine, d-glucosamine, n-acetylglucosamine, d-ribose, l-arabinose, l-rhamnose, d-glucose, mannose, sucrose, maltose, cellobiose, gluconate, glucuronate, starch, pullulan, succinate, maleate, malate, lactate, citrate, pyruvate, mannitol, and ethanol. Ninety percent or more OTU's of *V.zobellii* are negative for the differential characters, weak indole, acetoin, agar hydrolysis, aryl-sulphatase, casein hydrolysis, sulphide production, sudanophilic inclusions, growth at 4°C., resistance to 5 μg/ml polymyxin, 10 μg/ml streptomycin, 5 μg/ml sulphamethoxazole, 10 μg/ml tetracycline, 10 μg/ml trimethoprim, and 10 μg/ml 0/129, 0.0013 % brilliant green, 0.0005 % haloquinol, 0.002 % pyronin-Y, 0.2 % SDS, or could separately utilize glycine, dl-β-alanine, serine, leucine, valine, l-ornithine, δ-aminovalerate, sarcosine, xylose, d-mannose, trehalose, raffinose, galactarate, salicin, dl-hydroxybutyrate, poly-β-hydroxybutyrate, α-ketoglutarate, erythritol, dulcitol, sorbitol, inositol, benzoate, hydroxybenzoate, or phenylacetic acid. Specific aspects of *V.zobellii* relating to its isolation and ecology are discussed in sections of (3.10). The species not separately resolved here, *V.fluvialis/-V.furnissii;* and *V.harveyi/-V.parahaemolyticus* and some *V.carchariae* were also found closely affiliated in the previous analysis (3.8.6.2) indicating that the placements were not random and that genuine phenetic similarities existed between these groups. Except for *V.carchariae* these groups of species were found similar by DNA hybridization and so their presentation as overlapping species under the *SAS* protocol was not unexpected. Once these species are segregated from other *Vibrionaceae,* a second classificatory process may be invoked upon these groups according to a few cross-batch stable attributes as suggested in 3.8.6.2 or in accord with differential characters from the fragmented OTU's in phenon AG assigned to *V.carchariae.*

Some comparatively rare traits were found to define atypical phena and relatively recently described species. Before observation here of xanthine hydrolysis in *V.mediterranei* it was defined (Pujalte and Garay, 1986) principally by comparison with published phenotypes and without acknowledgment of uncertainties associated with such procedure (See 3.8.1).

Other uniformly rare traits in phena included urease and gas production in OTU's designated to *V.carchariae* in phenon AG; sulphide production by all OTU's placed in phenon AC, and in *Shewanella putrefaciens,* and aryl-sulphatase production by all OTU's from *V.carchariae/-V.harveyi* in phenon AA and phenon AE, tentatively designated as a biogroup of *V.orientalis.* Whether these traits have classificatory significance awaits appropriate molecular evaluation of UQM deposited reference cultures.

The results of the azoreductase inference to nitrogen fixation indicated little differential taxonomic value, i.e. that this is a rare but broadly distributed feature, (such as also found by West *et al.* 1985), with ecological rather than taxonomic significance.

The relative frequencies of species identified among provincial OTU's reflects, as previously indicated (3.8.6.2), both isolation media and ecological origins of these OTU's. These are described and discussed in section 3.12.

3.8.6.4 Comparative Evaluation Taxa of Deduced by Principal Component Procedure.

3.8.6.4.1 Experimental Design.

A principle component analysis by *SAS*$^{r.}$ (A11.6.5.1-2) upon a separately compiled euclidean distance matrix (See MacDonell and Schwartz *et al.,* 1986) composed by abstracted data from the 96 OTU's in Batches [1. & 3.], was undertaken to independently evaluate phena formed by cluster analysis (3.8.6.3). Output vectors, (Disk_2 in the file PRNCOM.EUC of Appendix 13), were plotted into Figure 3.10 as *Sun Ray* glyphs (2.9.3.7) using the STSC-*Statgraphics*$^{r.}$ program (3.8.2.4). Constituent vectors rays number anti-clockwise from 1 to 10 from the 3 o'clock position. The center of each ray is a zero point which permits both positive (outer) and negative (inner) vector co-ordinates to be plotted. Resultant glyphs were grouped according to phena from UPGMA cluster analysis (3.8.6.3) and examined for consistency.

3.8.6.4.2 Comparative Review of Glyphs.

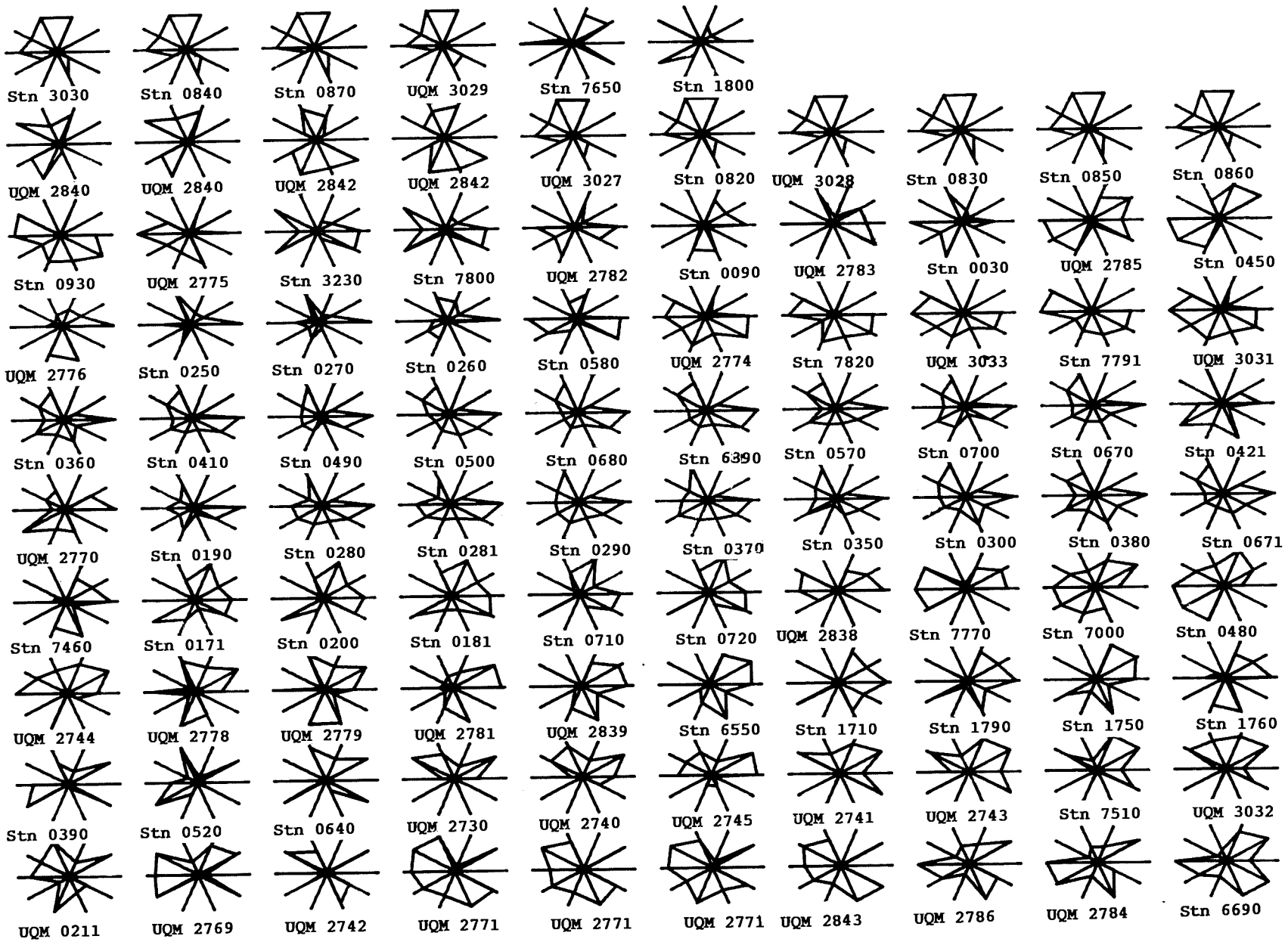
The ten principal component vectors summarized *ca* 92 % of the information available from the euclidean distance matrix. Almost 80 % of this information was contained by the first four vectors and the remaining six vectors each subtracted 3 % or less from the residue. However the first four vectors alone did not resolve differences between the glyphs of OTU's from the species of *V.anguillarum* (UQM 2771 and UQM 2843) and those from *V.fluvialis* UQM 2774, 3031 & 3033; Stn 930, 7280, & 7791 and *V.furnissii* UQM 2775. This was probably related to the lower relative abundance of *V.anguillarum* compared to other ODC and LDC positive species.

Phenetic variation encountered between the replicated OTU's from *V.gazogenes* in cluster analysis was confirmed here; replicate glyphs of UQM 2842 were almost fully positive for vectors 9 and 10 and fully negative in glyphs from UQM 2840. Glyphs from other oxidase-negative OTU's from the *Photobacterium* sp. Stn 1800, and *V.metschnikovii* UQM 211 were quite distinct from each other and from those of all other OTU's.

Comparison of the *Rosetta* glyph from the OTU UQM 3032 indicates that it is more similar to others assigned to *V.vulnificus* than to *V.cholerae* UQM 2742. Glyphs from the OTU's UQM 2778 and UQM 2784 previously designated and discounted (3.8.6.2.2.2) as atypical are also confirmed as such here. The OTU UQM 2744 which by cluster analysis was placed intermediately between *V.vulnificus* and *V.campbellii* was shown here to produce a glyph quite distinct from all other OTU's.

Glyphs from the provincial OTU's Stn 450 and Stn 6690 which were separately designated to *V.orientalis/-tubiashii* groups were quite distinct, which was consistent with placement in Figure 3.9 but not with placement to cluster [N,O] from Figure 3.8. These differences might be attributable to the operation of euclidean rather than Jaccard coefficients and may ultimately only be resolved by molecular methods or concurrent phenetic analysis.

Figure 3.10: Relative Sun-Ray Plots (glyphs), for 96 Batch [1. & 3.] OTU's.


Stn 3030
Stn 0840
Stn 0870
UQM 3029
Stn 7650
Stn 1800
UQM 2840
UQM 2840
UQM 2842
UQM 2842
UQM 3027
Stn 0820
UQM 3028
Stn 0830
Stn 0850
Stn 0860
Stn 0930
UQM 2775
Stn 3230
Stn 7800
UQM 2782
Stn 0090
UQM 2783
Stn 0030
UQM 2785
Stn 0450
UQM 2776
Stn 0250
Stn 0270
Stn 0260
Stn 0580
UQM 2774
Stn 7820
UQM 3033
Stn 7791
UQM 3031
Stn 0360
Stn 0410
Stn 0490
Stn 0500
Stn 0680
Stn 6390
Stn 0570
Stn 0700
Stn 0670
Stn 0421
UQM 2770
Stn 0190
Stn 0280
Stn 0281
Stn 0290
Stn 0370
Stn 0350
Stn 0300
Stn 0380
Stn 0671
Stn 7460
Stn 0171
Stn 0200
Stn 0181
Stn 0710
Stn 0720
UQM 2838
Stn 7770
Stn 7000
Stn 0480
UQM 2744
UQM 2778
UQM 2779
UQM 2781
UQM 2839
Stn 6550
Stn 1710
Stn 1790
Stn 1750
Stn 1760
Stn 0390
Stn 0520
Stn 0640
UQM 2730
UQM 2740
UQM 2745
UQM 2741
UQM 2743
Stn 7510
UQM 3032
UQM 0211
UQM 2769
UQM 2742
UQM 2771
UQM 2771
UQM 2771
UQM 2843
UQM 2786
UQM 2784
Stn 6690

OTU's assigned as *A.hydrophila* i.e. Stn 3230 and 7800, UQM 2838 and Stn 7700 and UQM 2769, comprised three discrete groups in cluster analysis; however, glyphs from these OTU's if examined in the sequence as listed above demonstrate progressions of different component vectors consistent with normal infra-specific variation.

The glyphs from Stn 7000 which associated with both *V.campbellii* and *V.vulnificus* in cluster analyses was most like that of Stn 480, which in cluster analysis was placed with *V.mediterranei* UQM 3076. The weak OTU's Stn 520, 390 and 640 present in three phena from Group 1. in Figure 3.9 produced glyphs with similar predominant vectors, but to different degrees.

The glyph from Stn 7650 which was placed variably with type cultures of *V.nereis, V.pelagius* and *A.hydrophila* UQM 2769 in successive cluster analyses, can be seen here to be quite distinct from all other OTU's. Glyphs from the OTU's which clustered in phenon L, UQM 3027-3030, Stn 820, 830, 840, 850, 860, 870, (Figure 3.9) and proposed as *V.zobellii* were also similar by this method however no glyphic similarity occurred between these OTU's and another decarboxylase-negative species *V.pelagius* UQM 2785 which was loosely associated with this phenon in cluster analysis.

The glyph from the OTU Stn 580 corresponded more closely to that of *V.natriegens* UQM 2782 than Stn 6390 which here was most similar to the group of species represented by *V.parahaemolyticus, V.harveyi, V.alginolyticus* and *V.campbellii.*

Unidentified OTU's formed glyphs which could be assigned into four groups the first containing the OTU's UQM 2839, Stn 6550, 1710, 1790, 1750, 1760, 171, 200, 181, 190, 710 and 720 and 7460; the second, Stn 130, 250, 260, 270 and the third the OTU's Stn 360, 410, 490, 500, 680, 690, 6390, 0570, 700, 670, 421, 190, 280, 281, 290, 370, 350, 300, 380 and 671.

The OTU's from the first group formed a progression of glyphs most consistent with the species *V.campbellii,* V.harveyi and *V.parahaemolyticus.*

However none of the unidentified OTU's from this group developed fourth vectors to the extent of *V.campbellii,* and all except two had glyphs which most closely resembled *V.harveyi* UQM 2781. The exceptional OTU's Stn 190 and 7460 most closely resembled *V.parahaemolyticus.* Glyphs from the second and third groups were generally pleomorphic but characterized by the presence of a third vector which was fully negative or very nearly so. This feature was shared with the type culture for *V.alginolyticus* but not by the species *V.parahaemolyticus, V.harveyi* or *V.campbellii.*

Only three OTU's from this principal component analysis were not placed consistently with the two dimensional grouping by cluster analysis. These were the already mentioned OTU UQM 2744, the unclustered OTU Stn 471, found here to resemble *V.alginolyticus,* and Stn 6390 placed here with *V.harveyi* rather than with *V.natriegens* as by cluster analysis.

3.8.6.4.3 Discussion.

The reduction of information into fewer vectors, and the expression of these vectors as multi-dimensioned glyphs removes the constraints of two dimensioned cluster analysis and facilitates rapid visual comparison of secondary similarities between-phenotypes.

The correspondance of glyphs generated through principal component analysis with taxa assigned after interpretation of dendrograms also endorsed the application of average linkage cluster analysis to diagnose unknowns. The three discrepant assignments could have been produced as a result of the multivariate analysis being upon a euclidean rather than upon a Jaccard distance matrix or, perhaps because of the increased sensitivity of this method; OTU's which were loosely associated with phena in different cluster analyses formed principal component glyphs distinct from all other taxa. The absorption of missing values through the assembly of a similarity matrix resulted in OTU's being expressed relative to each other rather than in terms of the most differential characters. However attribute frequencies are as accessible by this method as by cluster analysis. This analysis

compared to normal row by row principal component procedure and only lacked information about the relative contributions of different bands of differential and correlated tests.

The large scale implementation of such multivariate technique is clearly dependent upon available computer power, but typical multivariate analyses with 100 OTU's or more will become possible on 32 bit personal computers as programs become available.

3.8.6.5 Derivation of a Diagnostic Probability Matrix..

3.8.6.5.1 Experimental Design.

Information from derived phena in Tables 3.24.1-4 was examined and attributes from fragmented phena identified as *A.hydrophila, V.vulnificus, V.campbellii,* and *V.fluvialis* biovars I and II, were respectively pooled. Data from the two phena, including phenon R, which were assigned as *V.orientalis* were presented separately. Attributes from the unassociated Batch [1.] reference cultures of *V.metschnikovii* UQM 211, and *V.nereis* UQM 2783 shown in Table 3.12.1-2 and so not duplicated in Tables 3.24.1-4 were also included for comparative examination. Phenetic information from derived taxa in (3.8.6.2) was summarized in a probability matrix initially formulated using the characters from Table 3.20, but revised to better resolve taxa.

Little new differential information was contributed by the strain results for the following characters: Presence of strong oxidase, aesculin hydrolysis, growth in the presence of basic fuchsin, haloquinol, δ-aminovalerate, sarcosine, galacturonate, 6-hydroxycaproate and sorbitol. These were deleted and replaced in the matrix, Table 3.26, by more differential attribute frequencies for casein hydrolysis, gelatin hydrolysis, sulphide production, growth in the presence of pyronin-Y, thionine, streptomycin, sulphamethoxazole, and separate attack of glycine, serine, leucine, ornithine, proline, citrulline, putrescine, mannose, galactose, rhamnose, cellobiose and maleate so that altogether 80 characters were represented.

Table 3.26: Probability Matrix for Rapid Identification of *Vibrionaceae* and Related Bacteria.

Taxon:	*V. cholerae*	*V. cholerae*	*V. cholerae*	*V. mimicus*	*V. cholerae*	*V. mimicus*	*V. cholerae*	*V. vulnificus*	*V. cholerae*	*V. cholerae*	*V. nereis*	*V. cholerae*	*V. vulnificus*	*V. cholerae*	*V. vulnificus*	*V. parahaemolyticus*	*V. vulnificus*	*V. parahaemolyticus*	*V. vulnificus*	*V. vulnificus*	*V. vulnificus*	*V. vulnificus*	*V. parahaemolyticus*	*V. parahaemolyticus*	*V. parahaemolyticus*	*V. alginolyticus*	*V. cholerae*	*V. fluvialis*	*V. fluvialis*	*Plesiomonas shigelloides*	*Aeromonas hydrophila*	*Pleiomonas shigelloides*	*Aeromonas hydrophila*
	cholerae	*cholerae*	*cholerae*	*mimicus*	*cholerae*	*mimicus*	*cholerae*	*vulnificus*	*cholerae*	*cholerae*	*nereis*	*cholerae*	*vulnificus*	*cholerae*	*vulnificus*	*parahaemolyticus*	*vulnificus*	*parahaemolyticus*	*vulnificus*	*vulnificus*	*vulnificus*	*vulnificus*	*parahaemolyticus*	*parahaemolyticus*	*parahaemolyticus*	*alginolyticus*	*cholerae*	*fluvialis*	*fluvialis*	*shigelloides*	*hydrophila*	*shigelloides*	*hydrophila*
	cholerae	*cholerae*	*cholerae*	*mimicus*	*cholerae*	*mimicus*	*cholerae*	*vulnificus*	*cholerae*	*cholerae*	*nereis*	*cholerae*	*vulnificus*	*cholerae*	*vulnificus*	*parahaemolyticus*	*vulnificus*	*parahaemolyticus*	*vulnificus*	*vulnificus*	*vulnificus*	*vulnificus*	*parahaemolyticus*	*parahaemolyticus*	*parahaemolyticus*	*alginolyticus*	*cholerae*	*fluvialis*	*fluvialis*	*shigelloides*	*hydrophila*	*shigelloides*	*hydrophila*
	cholerae	*cholerae*	*cholerae*	*mimicus*	*cholerae*	*mimicus*	*cholerae*	*vulnificus*	*cholerae*	*cholerae*	*nereis*	*cholerae*	*vulnificus*	*cholerae*	*vulnificus*	*parahaemolyticus*	*vulnificus*	*parahaemolyticus*	*vulnificus*	*vulnificus*	*vulnificus*	*vulnificus*	*parahaemolyticus*	*parahaemolyticus*	*parahaemolyticus*	*alginolyticus*	*cholerae*	*fluvialis*	*fluvialis*	*shigelloides*	*hydrophila*	*shigelloides*	*hydrophila*
	cholerae	*cholerae*	*cholerae*	*mimicus*	*cholerae*	*mimicus*	*cholerae*	*vulnificus*	*cholerae*	*cholerae*	*nereis*	*cholerae*	*vulnificus*	*cholerae*	*vulnificus*	*parahaemolyticus*	*vulnificus*	*parahaemolyticus*	*vulnificus*	*vulnificus*	*vulnificus*	*vulnificus*	*parahaemolyticus*	*parahaemolyticus*	*parahaemolyticus*	*alginolyticus*	*cholerae*	*fluvialis*	*fluvialis*	*shigelloides*	*hydrophila*	*shigelloides*	*hydrophila*
	cholerae	*cholerae*	*cholerae*	*mimicus*	*cholerae*	*mimicus*	*cholerae*	*vulnificus*	*cholerae*	*cholerae*	*nereis*	*cholerae*	*vulnificus*	*cholerae*	*vulnificus*	*parahaemolyticus*	*vulnificus*	*parahaemolyticus*	*vulnificus*	*vulnificus*	*vulnificus*	*vulnificus*	*parahaemolyticus*	*parahaemolyticus*	*parahaemolyticus*	*alginolyticus*	*cholerae*	*fluvialis*	*fluvialis*	*shigelloides*	*hydrophila*	*shigelloides*	*hydrophila*
	cholerae	*cholerae*	*cholerae*	*mimicus*	*cholerae*	*mimicus*	*cholerae*	*vulnificus*	*cholerae*	*cholerae*	*nereis*	*cholerae*	*vulnificus*	*cholerae*	*vulnificus*	*parahaemolyticus*	*vulnificus*	*parahaemolyticus*	*vulnificus*	*vulnificus*	*vulnificus*	*vulnificus*	*parahaemolyticus*	*parahaemolyticus*	*parahaemolyticus*	*alginolyticus*	*cholerae*	*fluvialis*	*fluvialis*	*shigelloides*	*hydrophila*	*shigelloides*	*hydrophila*
	cholerae	*cholerae*	*cholerae*	*mimicus*	*cholerae*	*mimicus*	*cholerae*	*vulnificus*	*cholerae*	*cholerae*	*nereis*	*cholerae*	*vulnificus*	*cholerae*	*vulnificus*	*parahaemolyticus*	*vulnificus*	*parahaemolyticus*	*vulnificus*	*vulnificus*	*vulnificus*	*vulnificus*	*parahaemolyticus*	*parahaemolyticus*	*parahaemolyticus*	*alginolyticus*	*cholerae*	*fluvialis*	*fluvialis*	*shigelloides*	*hydrophila*	*shigelloides*	*hydrophila*
Phenon:														*AB*	*AF*	*R*							*AA*	.	*AC*			*2*	*3*				*AI*
Number of OTU's:	1	4	4	1	13	1	5	9	4	2	1	7	8	3	2	3	12	2	2	4	4	5	16	3	76	9	27	4	1	1	5	13	12	3	2
Attribute:																																			
Swarming growth.	*0*	0	0	*0*	2	*0*	0	1	0	*0*	*A*	7	5	0	*0*	0	0	*5*	*0*	5	5	2	1	3	7	2	2	3	*A*	*0*	0	2	0	0	*0*
Red pigment.	*0*	0	A	*0*	0	*0*	0	0	0	*0*	*0*	0	0	0	*0*	0	0	*0*	*0*	0	0	0	0	0	0	0	0	0	*0*	*0*	0	0	0	0	*0*
Strong catalase.	*A*	0	0	*0*	0	*0*	2	0	3	*0*	*0*	0	0	0	*5*	0	3	*0*	*0*	0	0	0	6	0	0	0	0	0	*0*	*0*	6	0	0	0	*0*
Weak oxidase.	*A*	3	0	*0*	A	*A*	A	A	A	*A*	*A*	A	A	A	*A*	A	A	*A*	*A*	A	A	A	A	A	A	A	A	A	*A*	*A*	0	A	A	7	*A*
Indole.	*0*	3	*0*	*A*	6	N	A	7	0	*0*	*0*	7	0	0	*A*	3	0	*A*	*0*	6	0	0	5	*A*	1	1	0	3	*0*	*A*	3	0	3	3	*0*
Weak indole.	*0*	3	*0*	*A*	A	*A*	A	9	5	*A*	*A*	A	A	A	*A*	3	*0*	*A*	*0*	A	3	A	A	A	A	A	8	3	*A*	N	6	0	8	*5*	*0*
5 day pH > 7.05.	*0*	0	8	*0*	A	*0*	8	0	0	*0*	*0*	0	0	0	*0*	3	0	*0*	*0*	0	0	0	0	3	7	0	6	5	*0*	*0*	8	A	8	0	*A*
5 day pH > 5.15.	*A*	0	A	*A*	A	*0*	8	8	8	*0*	*A*	0	0	0	*0*	3	0	*0*	*5*	0	3	0	6	3	7	0	8	5	*0*	*A*	8	A	9	0	*5*
Acetoin production.	*A*	3	A	*A*	7	*0*	8	0	0	*0*	*0*	0	0	0	*0*	0	0	*0*	*0*	0	0	0	0	0	6	1	6	0	*0*	*0*	8	0	9	0	*0*
Nitrate reduction.	*A*	A	3	*0*	A	*A*	A	A	A	*A*	*A*	9	A	A	*A*	A	2	*A*	*A*	A	A	A	9	A	A	A	A	A	*A*	*A*	6	8	A	A	*A*
Nitrite reduction.	*A*	0	3	*0*	4	*0*	8	2	0	*5*	*0*	4	A	A	*A*	7	2	*5*	*0*	5	A	A	8	A	A	A	9	8	*A*	*0*	8	A	9	A	*A*
Cholera Red.	*0*	0	0	*A*	1	*0*	A	2	0	*0*	*0*	0	0	0	*0*	3	0	*0*	*0*	3	0	0	0	0	0	0	0	0	*0*	*0*	0	0	0	0	*0*
Gluconate oxidation.	*0*	0	0	*A*	9	*0*	2	0	0	*5*	*0*	0	0	0	*0*	0	0	*0*	*5*	0	0	0	0	0	7	0	2	0	*0*	*0*	8	7	6	0	*0*
Arginine dihydrolase.	*0*	5	0	*A*	6	*0*	2	0	5	*A*	*A*	9	9	7	*A*	0	0	*5*	*5*	5	0	2	2	0	1	0	A	A	*A*	*A*	4	0	A	7	*0*
Lysine decarboxylase.	*0*	5	0	*A*	0	*A*	8	A	3	*0*	*0*	1	0	0	*0*	0	0	*0*	*0*	3	3	A	A	A	A	A	0	0	*A*	*A*	5	7	9	A	N
Ornithine decarboxylase.	*0*	0	0	*0*	1	*A*	6	A	0	*0*	*0*	0	0	0	*0*	0	0	*0*	*0*	0	3	A	A	A	9	A	0	0	*0*	*0*	8	A	0	A	N
Glucose fermentation.	*A*	A	A	*A*	A	*A*	A	A	A	*5*	*A*	A	A	7	*A*	A	A	*A*	*A*	A	A	A	A	A	A	A	A	A	*A*	*A*	8	0	A	A	*0*
Gas from glucose.	*0*	3	0	*0*	0	*0*	0	0	0	*0*	*0*	0	0	0	*0*	0	0	*0*	*0*	0	0	0	0	0	0	4	0	3	*0*	*0*	4	0	3	0	*0*
ONPG hydrolysis.	*0*	3	A	*0*	A	*0*	A	8	3	*A*	*0*	1	7	3	*5*	7	A	*0*	*0*	A	3	2	7	7	2	7	A	3	*0*	*0*	6	0	9	3	*0*
Urea hydrolysis.	*0*	3	0	*0*	0	*0*	0	0	0	*0*	*0*	1	0	3	*0*	0	0	*0*	*0*	0	0	2	4	7	0	A	0	0	*0*	*A*	0	0	0	3	*0*
Weak aesculin hydrolysis.	*0*	0	A	N	3	*0*	0	6	3	*5*	N	7	8	0	*A*	A	A	*A*	*0*	8	8	4	9	A	2	6	9	A	*A*	*0*	6	2	8	3	*5*
Aryl-sulphatase.	*0*	0	0	*0*	0	*A*	0	1	0	*5*	*0*	3	A	0	*0*	A	0	*0*	*0*	3	0	A	4	0	3	2	0	3	*0*	*0*	0	2	0	0	*0*
Casein hydrolysis.	*A*	0	A	*A*	9	*A*	8	9	A	*A*	*A*	7	A	A	*5*	3	A	*A*	*0*	A	3	8	A	7	9	A	9	3	*A*	*A*	0	A	9	0	*5*
Chitin hydrolysis.	*A*	5	*0*	*A*	A	*0*	A	A	A	*A*	*A*	7	A	A	*A*	A	2	*0*	*A*	8	8	A	A	A	A	A	A	A	*A*	*A*	0	8	A	3	*A*
DNA hydrolysis.	*0*	5	A	*A*	A	*A*	8	A	A	*A*	*A*	A	A	A	*A*	A	A	*0*	*A*	A	A	A	A	A	A	9	A	8	*A*	*A*	4	A	A	6	*5*
Gelatin hydrolysis.	*0*	3	A	*A*	A	*A*	A	A	A	*A*	*A*	A	A	3	*A*	0	A	*A*	*A*	A	0	8	7	7	A	A	A	8	*A*	*A*	2	9	A	0	*A*
Starch hydrolysis.	*0*	A	A	*A*	A	*0*	A	9	A	*A*	*0*	A	A	A	*A*	7	A	*A*	*5*	A	8	A	A	A	A	8	A	A	*A*	*A*	8	8	9	A	*A*
Sulphide production.	*0*	0	0	*0*	0	*0*	0	0	0	*0*	*0*	0	0	0	*0*	0	0	*0*	*0*	3	3	0	1	A	A	0	1	0	*0*	*0*	5	A	2	*0*	*0*
Xanthine hydrolysis.	*0*	0	0	*0*	0	*0*	0	0	0	*0*	*0*	6	4	3	*0*	3	0	*0*	*0*	A	0	4	0	0	0	0	0	0	*A*	*A*	0	0	0	0	*0*
Growth 4oC.	*0*	3	0	*0*	A	*0*	0	0	3	*0*	*0*	6	4	0	*0*	0	0	*A*	*0*	0	0	0	1	0	1	0	2	0	*0*	*0*	4	4	5	0	*5*
Growth 30oC.	*A*	A	A	*A*	A	*A*	A	A	A	*A*	*A*	7	8	A	*A*	A	A	*A*	*A*	A	A	A	A	A	A	A	A	A	*A*	*A*	A	A	A	A	*A*
Growth 37oC.	*A*	8	A	*A*	A	*A*	A	A	5	*0*	*A*	4	0	7	*5*	A	A	*A*	*A*	A	A	A	A	7	A	A	9	A	*A*	*A*	A	A	A	A	*A*
Growth 42oC.	*0*	*5*	A	*A*	0	*A*	A	A	0	*0*	*0*	1	0	0	*0*	3	A	N	A	5	A	2	5	*0*	A	0	9	A	N	N	*A*	4	A	*5*	N
Growth 0 % NaCl.	*0*	5	0	*0*	9	*A*	A	A	3	*5*	*0*	0	0	0	*A*	3	0	*A*	*0*	0	0	0	1	0	1	0	A	0	*A*	*0*	A	A	A	3	*A*
Growth 6.0 % NaCl.	*A*	A	A	*A*	9	*0*	0	0	8	*0*	*A*	9	9	3	*A*	A	A	*A*	*A*	A	A	A	A	A	A	A	8	A	A	*A*	A	5	3	6	*A*
Growth 10 % NaCl.	*A*	0	8	*0*	0	*0*	0	0	3	*0*	*0*	1	0	0	*0*	0	A	*0*	*5*	0	0	0	0	0	7	0	2	5	*A*	*0*	0	7	0	0	*0*
Growth pH 10.	N	*0*	0	*A*	A	*A*	A	9	3	*A*	*A*	7	*A*	N	*A*	*A*	0	N	*0*	0	5	N	7	A	7	N	9	N	N	N	5	2	A	*A*	N

Growth Novobiocin.	*0*	5	0	*0*	0	*0*	0	0	0	*0*	*0*	1	0	0	*0*	3	A	*5*	*0*	0	0	2	6	0	0	0	7	5	*0*	*0*	A	A	8	0	*A*
Growth Polymyxin.	*0*	0	0	*0*	5	*0*	A	A	A	*0*	*0*	3	1	7	*0*	0	0	*0*	*0*	5	3	8	A	A	6	7	2	0	*A*	*A*	2	7	6	0	*0*
Growth Streptomycin	*0*	A	5	*A*	A	*A*	A	A	A	*5*	*A*	9	9	7	*A*	7	0	*0*	*A*	A	A	A	9	A	A	A	9	A	*A*	N	A	A	A	0	*A*
Growth Sulphamethoxazole.	N	A	A	A	A	*N*	A	9	A	*A*	*A*	0	A	7	*A*	A	0	*0*	*A*	0	3	A	A	N	A	A	A	A	N	N	A	A	A	N	N
Growth Tetracycline.	*0*	3	0	*0*	0	*0*	0	0	0	*5*	*0*	1	0	0	*0*	0	0	*0*	*0*	0	0	0	0	0	1	1	0	A	*0*	*0*	6	3	0	0	*0*
No growth 10 μg/ml 0/129.	*0*	5	5	*A*	A	*A*	6	8	8	*5*	*A*	A	8	A	*A*	0	A	*A*	*A*	8	3	0	1	7	2	0	1	0	*0*	*A*	0	0	0	7	*0*
No growth 150μg/ml 0/129.	*A*	8	A	*A*	A	*A*	A	A	A	*A*	*A*	A	A	A	*A*	A	A	*A*	*A*	A	A	8	9	A	9	A	9	A	*0*	*A*	4	5	0	A	*0*
Growth Dichlorophene.	*0*	8	5	*0*	0	*0*	2	0	5	*5*	*0*	7	0	3	*A*	3	2	*A*	*5*	*5*	*A*	2	9	N	6	2	4	0	*A*	*A*	A	6	8	7	*A*
Growth Methylviolet.	*A*	7	*5*	*0*	5	*0*	A	1	3	*A*	*0*	2	5	3	*5*	7	*A*	*0*	*0*	3	7	A	7	3	A	A	9	A	*A*	*A*	A	8	A	*A*	*0*
Growth Pyronin-Y.	*0*	5	0	*0*	0	*0*	2	0	0	*0*	*0*	0	4	0	*0*	0	0	*0*	*0*	3	0	8	6	7	6	8	0	0	*0*	*0*	A	8	A	3	*A*
Growth Thionine.	*0*	A	0	*0*	0	*0*	0	1	0	*0*	*0*	0	6	0	*A*	0	0	*0*	*0*	A	3	A	2	7	9	8	A	0	*A*	*A*	A	5	5	5	*0*
Growth Glycine.	*0*	0	5	*0*	0	*0*	0	0	5	*0*	*A*	0	9	7	*5*	7	0	*0*	*A*	8	A	4	A	A	A	A	A	A	*A*	*A*	4	2	9	0	*0*
Growth l-α-Alanine.	*0*	0	A	N	4	*0*	2	8	A	*0*	*A*	3	5	7	*A*	A	A	*A*	*A*	A	A	A	A	A	A	A	A	A	*A*	*A*	A	8	A	3	*A*
Growth dl-β-Alanine	*0*	0	0	*0*	0	*0*	0	0	0	*0*	*0*	0	0	0	*0*	0	0	*0*	*0*	0	3	0	0	0	1	0	0	A	*A*	*0*	0	0	3	0	*A*
Growth Serine.	*0*	3	0	N	6	*0*	4	3	A	*0*	*A*	0	A	7	*5*	A	0	*0*	*A*	8	8	A	8	8	A	8	A	A	*A*	*A*	8	A	A	0	*5*
Growth Leucine.	*0*	0	0	*0*	0	*0*	0	0	0	*0*	*A*	0	0	0	*0*	0	0	*0*	*5*	0	5	0	1	A	9	0	4	3	*0*	*A*	0	5	0	0	*0*
Growth l+Glutamic acid.	N	0	A	*A*	7	N	2	A	A	*5*	*A*	6	A	A	*A*	*A*	A	*A*	*A*	A	A	A	A	7	A	A	A	A	*A*	*0*	8	8	A	*A*	*A*
Growth l-Citrulline.	N	0	0	*0*	8	N	6	7	8	*0*	*A*	6	5	0	*5*	A	A	*A*	*5*	8	8	0	4	3	1	4	9	A	*A*	*A*	0	2	5	0	*0*
Growth ɣ-Amino-butyrate.	*0*	0	5	*0*	0	*0*	0	0	0	*0*	*0*	1	0	0	*0*	7	9	*5*	*0*	3	3	0	1	3	1	0	A	0	*0*	*0*	6	2	8	0	*0*
Growth Ornithine.	*0*	0	5	*A*	4	*0*	0	1	0	*0*	*A*	0	5	7	*5*	A	0	*0*	*5*	A	8	2	4	4	9	9	A	A	*0*	*0*	2	3	2	0	*0*
Growth Proline.	*0*	5	3	*0*	5	*0*	0	9	A	*0*	*A*	6	A	A	*A*	A	A	*A*	*A*	A	A	A	A	A	A	A	A	A	*A*	*A*	A	5	A	3	*A*
Growth Putrescine.	*0*	0	*0*	*0*	0	*0*	0	0	0	*0*	*A*	0	0	0	*5*	A	0	*A*	*0*	A	A	0	1	A	4	0	9	A	*A*	*A*	4	A	6	*0*	*A*
Growth l-Glutamine.	*0*	0	A	*A*	8	*0*	A	9	A	*0*	*A*	4	9	A	*A*	A	A	*A*	*A*	A	A	A	A	A	A	A	A	A	*A*	*A*	8	A	A	A	*A*
Growth d-Ribose.	N	0	8	*0*	0	N	*0*	7	A	*0*	*A*	3	N	N	*5*	*0*	A	*A*	*A*	7	8	A	A	A	A	6	A	7	*A*	*A*	A	1	A	3	*A*
Growth Xylose.	*0*	0	5	*0*	0	*0*	0	0	0	*0*	*0*	1	0	0	*0*	0	0	*A*	*0*	0	0	0	6	0	0	0	0	0	*0*	*0*	A	0	0	0	*0*
Growth l-Arabinose.	*0*	0	8	*0*	2	*0*	0	0	0	*0*	*0*	1	0	0	*5*	0	A	*A*	*5*	0	8	0	3	A	5	0	A	A	*0*	*0*	A	8	0	0	*5*
Growth l-Rhamnose.	*0*	0	0	*A*	0	*0*	0	0	0	*0*	*0*	1	0	0	*0*	0	A	*0*	*A*	0	A	0	3	3	1	0	A	3	*0*	*0*	8	0	1	0	*0*
Growth d-Mannose.	*A*	0	8	*A*	5	N	0	A	8	*5*	*0*	3	5	7	*A*	A	0	*0*	*5*	A	5	A	8	A	9	4	9	A	*A*	*A*	A	0	A	0	*0*
Growth d-Galactose.	*0*	0	5	*0*	1	*0*	0	7	5	*0*	*0*	9	0	0	*5*	7	A	*A*	*A*	A	A	A	9	A	8	6	A	A	*0*	*A*	8	2	A	0	*A*
Growth Sucrose.	N	0	A	*A*	3	N	0	0	8	*0*	*A*	9	7	0	*A*	*0*	A	*5*	*A*	8	8	2	9	A	8	1	A	3	*0*	*A*	8	0	9	3	*5*
Growth Cellobiose.	*0*	A	8	N	1	0	0	4	A	*0*	*0*	A	1	0	*A*	A	A	*A*	*0*	7	8	8	9	A	A	6	5	0	*A*	*A*	A	0	4	0	*A*
Growth Lactose.	N	0	0	*0*	0	N	0	1	0	*0*	*A*	4	0	0	*5*	*5*	3	*A*	*0*	5	0	0	6	0	1	0	0	0	*0*	*0*	4	0	1	0	*0*
Growth Glucuronate.	*0*	0	N	*0*	0	*0*	0	1	0	*0*	*0*	4	0	0	*5*	4	A	*A*	*A*	5	8	A	A	7	7	2	9	A	*0*	*0*	A	2	1	0	*A*
Growth Salicin.	N	0	A	*0*	0	N	0	2	3	*0*	*0*	0	0	0	*5*	0	0	*A*	*0*	3	8	0	2	7	1	1	A	A	*0*	*0*	A	0	7	0	*0*
Growth Starch.	N	*0*	A	*A*	4	N	2	8	A	*5*	*A*	A	0	0	*A*	*A*	A	*A*	*A*	A	A	A	A	A	A	7	A	A	*A*	*A*	*5*	8	A	*5*	*A*
Growth Pullulin.	N	0	5	*A*	0	N	0	8	8	*0*	*A*	A	0	0	*A*	*0*	A	*0*	*A*	5	A	4	A	A	A	2	A	A	*A*	*A*	0	0	8	7	*A*
Growth Maleate.	N	A	5	*0*	0	*0*	0	0	0	*0*	*0*	1	N	N	*0*	0	A	*A*	*0*	0	5	N	5	N	7	N	*5*	N	N	N	N	N	*0*	A	N
Growth dl-3-OH-butyrate.	*0*	3	3	*0*	0	*0*	0	0	0	*0*	*0*	0	0	0	*0*	0	0	*0*	*A*	5	8	0	0	0	1	0	4	3	*0*	*0*	0	2	4	0	*A*
Growth Lactate.	*0*	*0*	A	N	4	*0*	0	9	A	*0*	*A*	A	1	0	*A*	A	A	*A*	*A*	A	A	8	A	A	A	8	A	A	*A*	*A*	A	9	A	*5*	*A*
Growth α-Ketoglutarate.	*0*	0	5	*0*	7	*0*	2	9	8	*0*	*A*	0	0	0	*5*	3	0	*0*	*0*	0	5	0	6	A	2	0	6	0	*A*	*A*	8	2	3	0	*5*
Growth Pyruvate.	N	0	A	N	3	N	A	A	A	*0*	*A*	A	A	7	*A*	*A*	A	*A*	*A*	A	8	A	A	A	9	A	A	A	*A*	*A*	A	A	A	3	*5*
Growth Mannitol.	N	0	5	*A*	3	N	0	9	8	*0*	*0*	7	0	0	*0*	0	A	*A*	*5*	8	8	A	A	A	A	3	A	A	*A*	*A*	A	0	A	0	*0*
Growth Ethanol.	*0*	0	*0*	*0*	0	*0*	0	*0*	*0*	*0*	N	1	0	0	*0*	0	A	*0*	*A*	0	*A*	2	1	A	8	0	5	0	*0*	*0*	3	1	3	0	*A*

Percent equivalents: 0, 0-4; 1, 5-14; 2, 15-24; 3, 25-34; 4, 35-44; 5, 45-54; 6, 55-64; 7, 65-74; 8, 75-84; 9, 85-94; A, 95-100.
(N = Not Determined, *Italics* indicate 2 or 1 OTU's.).

Taxon attribute frequencies were condensed into single digit scores formulated as in Table 3.20. Eleven of the 35 taxa were represented by 2 or less OTU's. These are shown in italics to indicate the relative uncertainty of their associated frequencies. The percent conformity to taxa can be obtained by successively multiplying tabulated probabilities of test results from matches and non-matches (10 - tabulated probability), reading tabulated "0" as 0.5 and "A" values as 9.5, multiplying by 100, and dividing the resultant score by that of the tabulated taxon profile.

3.8.6.5.2 Results.

Few characters had uniform differential value across all taxa, rather different groups of taxa were identified by different bands of characters. Most OTU's could be identified by comparison with taxon attribute scores of 9 or greater. However three groups of phena; from *V.campbellii* and *V.vulnificus,* two phena attributed to biogroups of *V.orientalis* and the phenetically heterogeneous group of OTU's depicted by the phena AA, AB, AC, AD and AG and including the species *V.harveyi, V.parahaemolyticus, V.alginolyticus* and *V.carchariae;* and also the species *V.orientalis* and *V.aestuarianus* can best be resolved within those respective groups by reference to relative probabilities of less uniform attribute scores over the entire range of tests.

3.8.6.5.3 Discussion.

Differences in the number and distribution of characters used to probabilistically resolve phena in this analysis compared with those used to separate phena from Batches [1., 2., 4. and 5.] may to an extent reflect the inability of *Microcluster* to compensate for the effects of noisy data as shown by taxon-fragmentation. However by pooling fragmented phena deduced as from the same taxa these effects should have been minimized. Differences in differential characters in the two identification matrices can also be attributed to the presence of distinct groups of taxa in each analysis. Species which were common to both analyses but which were phenetically distinct may represent different taxon-phenotypes selected in different

biogeographic conditions, or reflect selection according to different isolation media and enrichment protocols 3.8.7.

Amongst the 80 differential characters used here and after equating different decarboxylation test methods, and Tween 80 hydrolysis with SDS esterase, 37 are common to the eighty two characters (including glucose fermentation) of Bryant *et al.,* (1986b). This difference in the use of differential traits probably reflects the need to depict different species, however it may also indicate that phenetic classification within the *Vibrionaceae* is not necessarily restricted to a narrowly defined set of characters. Instead, and consistent with the concept of stability, it seems that equally valid classifications can be derived from sufficient different characters which canvass the same bands and facets of each OTU's phenetic profile.

These different diagnostic schemes also offer predictive opportunities for cross-testing of identifications and have very much more facility for the *Vibrionaceae* identification than commercial schemes such as evaluated in (Appendix 8.).

3.8.7 Review of Numerical Analyses.

3.8.7.1 Phenetic Classification of the *Vibrionaceae*.

Of 728 presumptive provincial *Vibrionaceae* isolates, 391 were phenetically examined and in most cases these correlated with extant species as shown below. The data suggest, that the species *V.orientalis* and *V.tubiashii* are synonymous; and the name *V.orientalis* has taxonomic priority. A new species *V.zobellii* was described from copepods, and phenetically distinct new biogroups of *V.aestuarianus, V.orientalis, A.hydrophila,* and *V.fluvialis* were also described and descriptions of *V.gazogenes* and *V.fluvialis sensu stricto* were emended. One aerogenically fermentative, red-pigmented, oxidase-negative provincial OTU, UQM 3256, which resembled two *Serratia* spp. was shown by electron microscopy to have mixed flagellation. The taxonomic position of this OTU remains uncertain, but in the absence of molecular information its tentative designation has been to the *Vibrionaceae*.

In order of abundance the taxa represented by provincial OTU's were as follow: *V.alginolyticus,* (87); *V.carchariae/-V.harveyi,* (51); *V.fluvialis/-V.furnissii,* (45); *V.parahaemolyticus,* (25); *Aeromonas hydrophila,* (21); *V.aestuarianus* I & II, (18); *Shewanella putrefaciens,* (17); *V.carchariae,* (15); *V.orientalis* (14); *Photobacterium* and *Photobacterium*-like spp., (11); *V.fluvialis/-V.furnissii*-like, (10); *V.zobellii,* (10); *V.vulnificus* (9); *V.carchariae/-V.harveyi/-V.parahaemolyticus,* (7); *V.natriegens*-like, (5); *V.anguillarum,* (5); *V.diazotrophicus,* (5); *V.natriegens,* (4); *V.costicola*-like (3); *V.diazotrophicus*-like, (3); *V.mediterranei,* (3); *Vibrio* sp. Phenon AC, (3); *V.campbellii,* (2); *V.gazogenes,* (2); *V.ordalii,* (2); *V.pelagius* (2); *V.splendidus,* (2); *Plesiomonas shigelloides,* (2); *V.alginolyticus/-V.carchariae/-V.harveyi,* (1); *V.hollisae,* (1); *V.anguillarum*-like, (1); *Vibrio* sp. Stn 421 (1); *Vibrio* sp. Stn 390 (1); *Vibrio* sp. Stn 7790 (1); *Vibrio* sp. Stn 7680 (1); and (1) *Serratia*-like sp. These OTU's represent the first substantiative Australian identifications of the species *V.aestuarianus, V.carchariae, V.hollisae, V.mediterranei, V.orientalis,* and *V.ordalii*.

Classification of 95 OTU's of tentatively and partially identified cultures (Table 3.5) revealed many taxa which were shared, and some e.g. *V.cholerae,* which were distinct to taxa found in provincial OTU's. Others such as from Singaporese fish did not correspond to any extant species of *Vibrionaceae.* The culture UQM 2675, designated as *V.alginolyticus* by Baumann *et al.,* (1971) was found here to associate more closely with the type culture of *V.harveyi.* Similarly two cultures designated as sucrose negative *V.cholerae,* UQM 2729 and UQM 2730, were here classified as *V.vulnificus.*

In some cases the classification of imported OTU's was impeded by sub-optimal conditions of temperature and salinity. For example, OTU's from Tasmania were separated from provincial OTU's into a heterogeneous phenon as an ecological group with secondary taxonomic structure.

Two groups of species which were not separately resolved in the present work i.e. some [*V.alginolyticus /- V.carchariae /- V.harveyi /- V.parahaem-olyticus*], and [*V.fluvialis /- V.furnissii*] have been reported in the literature as similar by DNA hybridization, with the exception of *V.carchariae* for which such data was not available. However phylogenetically significant differences of 5S rRNA have been reported in the literature. The differentiation of *V.carchariae* from *V.harveyi* can be improved as indicated in (3.8.6.2.3) or the problem may be skirted by describing such strains as urease positive or negative *V.carchariae/-V.harveyi.*

Collagen hydrolysis is a potentially useful criterion for the differentiation of *V.carchariae* and *V.harveyi,* but results are difficult to read. The presence of anoxic azo-reductases against the dye congo red may have some value, in the inference of nitrogen fixation, however this activity was not uniformly distributed within the several taxa in which it was expressed. Little differential taxonomic (or pathogenicity/-nitrogen fixation) information was indicated by red or white, (or orange), colonies on congo red agar medium.

Some attributes relatively uncommon in the *Vibrionaceae* were often expressed in taxa as follow: Xanthine hydrolysis, in *V.orientalis* and *V.mediterranei;* urea hydrolysis in *V.carchariae;* arylsulphatase production in the [Batches 1., 3., 7. & 8.] phena AA and AE; and sulphide production by *Shewanella putrefaciens,* and all OTU's from phenon AC. These and 77 further characters were used in a probability matrix, for 35 taxa generated by UPGMA analysis of data from Batches [1., 3., 7. & 8]. Because differential features from this matrix were assembled from cross-batch data they are intrinsically more stable to skewing effects which operate when reference and unknown cultures are assayed at different times than tabulated single batch data. The differential characters from this probability matrix differed from those used by Bryant *et al.,* (1986) and so supported Sneath's (1957) concept of stability (of relative interrelationships) which underlkies the operation of all numerical taxonomic sampling. Additionally this probabilistic classification scheme provides an independent means for phenetic evaluation of identifications produced by other schemes or vice-versa.

For future work, and to serve as an empirical measure of the validity of the numerical analysis technique representative cultures of provincial and imported OTU's remain stored under paraffin oil or have been deposited at UQM with accession numbers as listed in (Appendix 14.).

3.8.7.2 Numerical Analysis Methods.

Numerical analyses were undertaken in a progression initiated by the definition of a diagnostic *Rosetta,* and comparative review of cluster protocols and missing character assimilation procedures. Using the chosen best method, (UPGMA sorting of a Jaccard matrix), a review of miscibility for 277 within and cross-batch and partial and complete replicates was undertaken. Similar UPGMA cross-batch analyses respectively with 287 and 288 type, provincial and reference OTU's were undertaken on mainframe- and personal- computers and these addressed the taxonomic position of strains. Partial secondary-testing, and evaluation of diagnoses from above was undertaken by mainframe-operated principal-component analysis.

The need to examine OTU's in batches introduced complications related to cross-batch miscibility. Specifically these problems were attributed to phenetic attrition i.e. lost enzymic and nutritional facility in OTU's and aggravated by a preclusion of OTU's cross-feeding or sharing extracellular permeases in multi-well trays; and skewing of test results by slightly different conditions of culture or media, e.g. from impure distilled water. These problems were partly overcome by replicating strains within- and across- batches to allow data standardization. This standardization was undertaken in two stages. The first to remove unreliable tests from individual data sets, and then by cross-correlation of results between datasets to remove consistently skewed results.

One theoretical basis for this standardization was that of stability i.e. if enough comparative tests (samples) are made, the sampled similarity coefficient between two OTU's should approach the global similarity coefficient. The corollary from this is that if data are balanced then it shouldn't matter what tests are conducted, only that the sample is sufficiently large. The second basis was the consideration of each character locus as potentially able to hold three, rather than one bit of information i.e. if a test was unreliable in one batch or not tested, coding a missing value to act as a space marker allows assimilation of information from residual batches. The splitting of positive and negative missing character data takes consideration of skewing and so minimizes information removal. Two cross-batch replicate pairs partially consistent for an attribute between batches, e.g. both positive in one batch and one positive and one negative in the other, indicate, (in the absence of individual strain mutations), that in the second batch that all positive results are consistent but negative results are inconsistent, because conditions are some how more stringent; so only negative results in the second batch need be coded as missing.

Three methods for assimilation of missing character data were compared. The first was used in concert with a program, (*SAS* average linkage, with scalar

data), which sorted OTU characters on a row by row basis and placed OTU's into phena according to whether individual attributes were greater or less than the row-average, by substituting row-averages for missing character data (Anderberg, 1973) no local program assignments can be made. The second (Wishart 1986, *Clustan,* ESS) scored missing characters as equivalent to all other characters compared, (including other missing characters), and so caused artificially high similarities. The third method, used to assemble similarity matrices eliminated missing characters from comparisons between OTU's, character by character, so that coefficients were formed only from uniformly present data.

The sorting methods used in the definition of the *Rosetta,* average linkage, CP and ESS analyses, were all inappropriate for the sorting of large data sets. The latter two because the programs operating these protocols physically lacked facility to treat many OTU's and the third because of the time involved in manually assigning each missing character to row averages.

Comparison of four sorting protocols used to cluster similarity matrices, from Batch [6.] data assembled from euclidean and Jaccard coefficients, was on the basis of species and replicate OTU stability within phena, and indicated that UPGMA, (k-linkage list length of 8), sorting the Jaccard matrix was the most appropriate. Comparison of 277 completely and partially replicated OTU's under this protocol indicated that most species were discernible either as discrete phena or sub-phena. This inferred that taxa were sometimes fragmented but otherwise presented in a highly structured fashion. Sufficient non-overlapping taxonomic space existed in most cases for the resolution of local pockets of taxa each with intrinsic local hierarchies.

Nutritionally diverse species such as *V.natriegens, V.fluvialis, V.furnissii, V.harveyi* and *V.parahaemolyticus* were less subject to the effects of partial replication, (*ca* 70 characters), when compared by the Jaccard coefficient, than phenetically less versatile species such as

V.cholerae and *V.vulnificus* i.e. the minimum sample size required to accurately estimate the global similarity between two OTU's differs between species depending on nutritional facility. It was further deduced that cross-batch miscibility between replicates was affected by data from other mixed cultures and partial replicates; and also replicate data from Batch [6.] which was locally valid but skewed by media prepared in impure distilled water.

Residual batches were divided into two subsets each of *ca* 240 OTU's (Batches [2., 4. & 5.] and Batches [3., 7. & 8.]), and clustered with the *Rosetta* by UPGMA sorting of Jaccard matrices. The second dataset was analyzed by personal computer driven program lacking "k-linkage list" facility. Despite differences in final fusion similarity which indicated that the microcomputer analysis was more stringent, under both protocols the structure of the data was less complicated than that of the previous replicate analysis. Fragmented species typically reflected genuine differences in phenetic character rather than batch-driven artifacts.

The comparative review of the microcomputer UPGMA analysis by a different (principal component) procedure indicated that taxa were generally correspondent, and that differences were related to the greater sensitivity of principal component generated glyphs in showing differences, particularly of OTU's fused at the margins of clusters, and to its derivation from a euclidean rather than Jaccard similarity matrix.

A feature of the pooled-batch analyses was the inability to always resolve the closely related or phenetically similar taxa mentioned in (3.8.7.1). It was proposed that resolution might be synergistically improved by refinements at all procedural stages i.e. culture storage, testing procedure, data scoring and preliminary examination. Specific alterations to test procedure including addition of growth factor to all inoculum suspensions as undertaken in Batch [5a.], incubation of all OTU's, including those from different areas, in media with temperatures and salinities

relatively close to optimum, preparation of organic acids at lower effective concentrations to reduce toxicity and compensate for lesser volatilization and loss of these in confined multi-well trays, scoring of batch results according to a scalar rather than binary system,, and more objectively deleting positive or negative components of batch character records by adapting a program such as that of Bryant *et al.,* (1986a) to accept more than 2 replicates of an OTU and to accept such scalar data. Further improvement to analyses by slight increases in similarity coefficients may also be possible by adapting Wishart's method for incorporation of missing values as suggested in (3.8.3.3.3).

Generally, it was indicated from numerical analyses of *Vibrionaceae* that UPGMA protocols with "k-"linkage list facility and clustering Jaccard matrices have greater stability to the effects of procedural noise than other cluster procedures sorting these or euclidean matrices. Improved resolution of this technique may be possible by adaptation of Wishart's (1986) method of missing value treatment. The novel graphic depiction of principal component vectors provided an effective means to evaluate phena formed from cluster analysis and probably similar analyses will soon comprise a greater proportion of primary analyses by desk top computers.

3.9 Review of Taxa Recovered under Different Isolation Media Protocols.

3.9.1 Experimental Design.

The relative abundances of 391 provincial OTU's from 31 taxa, and 4 single unidentified OTU's under different isolation protocols, and identified in sections (3.8.3-6) were compared in order that in future, specific protocols might be applied for more efficient recovery of target species.

The data (Table 3.27) was aggregated into 8 media groups Simidu, ATW-Simidu, BTB-Simidu; TCBS, ATW-TCBS, BTB-TCBS; SENA and EDDA. The two latter small groups, comprised isolates taken from these media directly and after primary enrichment as indicated in Table 3.9.

3.9.2 Results.

No single protocol or medium recovered all taxa. However one taxon representing the species *V.fluvialis* and *V.furnissii* was recovered from all media groups. Three taxa *V.alginolyticus* and *V.carchariae/-harveyi*, *Shewanella putrefaciens* and *A.hydrophila* were recovered from all but one media group; and three representing three species, *V.carchariae*, *V.parahaemolyticus* and *V.carchariae/-V.harveyi/-V.parahaemolyticus* were recovered from all but three.

Direct isolations onto Simidu and TCBS media recovered the greatest numbers of strains and taxa. Amongst common taxa, (ten or more isolates); only one *V.zobellii*, was recovered from one medium, (Simidu); alone. *V.orientalis* was isolated predominantly from TCBS, and from Simidu only after enrichment. Six taxa, *V.costicola*-like, *V.gazogenes*, *V.ordalii*, *P.shigelloides*, *V.hollisae* and *V.natriegens*-like were isolated from Simidu but not directly from TCBS. Five taxa, *V.mediterranei*, *V.pelagius*, *V.diazotrophicus*, *V.anguillarum* and *V.fluvialis/-furnissii*-like were isolated from TCBS but not directly from Simidu. Infrequently isolated taxa, (nine or less strains), in order of abundance were isolated from Simidu medium (10), ATW-TCBS (10), TCBS (9), All SENA (6), EDDA (4), ATW-Simidu (3), BTB-Simidu (2), and BTB-TCBS (1). The greatest proportion of different taxa *ca* 50 % was

Table 3.27: Numbers of Taxa and Proportions Obtained, (%) under Different Isolation Protocols.

	Number of Cultures from Isolation Media, (%).								
Taxon.	**Simidu**	**ATW Simidu**	**BTB Simidu**	**TCBS**	**ATW TCBS**	**BTB TCBS**	**ALL SEWA**	**ALL EDDA**	**Phenon Total.**
V.alginolyticus	14 (18).	17 (37).	8 (9).	8 (10).	21 (56).	15 (17).	4 (9).	0	**87**
V.carchariae/-V.harveyi	10 (13).	1 (2).	1 (2).	14 (18).	3 (5).	2 (5).	20 (45).	0	**51**
V.fluvialis/-V.furnissii	6 (8).	8 (16).	8 (27).	3 (4).	6 (16).	10 (27).	2 (5).	2 (12).	**45**
V.parahaemolyticus	7 (9).	7 (14).	3 (10).	5 (7).	3 (8).	0	0	0	**25**
A.hydrophila	2 (3).	4 (8).	3 (10).	5 (7).	2 (5).	2 (5).	0	3 (18).	**21**
V.aestuarianus	5 (6).	0	0	6 (8).	0	0	7 (16).	0	**18**
S.putrefaciens	2 (3).	6 (12).	3 (10).	1 (1).	3 (8).	0	1 (2).	1 (6).	**17**
V.carchariae	1 (1).	0	0	4 (5).	6 (16).	1 (3)	3 (7).	0	**15**
V.orientalis	0	3 (6).	0	10 (13)	0	0	1 (2).	0	**14**
Photobacterium-like	1 (1).	3 (6).	0	0	0	1 (3)	0	6 (35).	**11**
V.fluvialis/-furnissii-like	0	0	2 (7).	1 (1).	2 (5).	5 (14).	0	0	**10**
V.zobellii	10 (13).	0	0	0	0	0	0	0	**10**
V.vulnificus	2 (3).	0	0	4 (5).	3 (8).	0	1 (2).	0	**9**
V.carc./-V.harv./-V.para.	3 (4).	0	0	3 (4).	1 (3).	0	0	0	**7**
V.natriegens-like	4 (5).	0	0	0	1 (3).	0	0	0	**5**
V.anguillarum	0	0	0	4 (5).	0	0	0	1	**5**
V.diazotrophicus	0	0	0	2 (3).	1 (3).	1 (3).	0	1 (6).	**5**
V.natriegens	1 (1).	1 (2).	1 (3).	1 (1).	0	0	0	0	**4**
V.costicola-like	1 (1).	0	0	0	0	0	2 (4).	0	**3**
V.diazotrophicus-like	0	1 (2).	0	0	1 (3).	0	1 (2).	0	**3**
V.mediterranei	0	0	0	2 (2).	0	0	1 (2).	0	**3**
Phenon AC [Batches 1,3,7, & 8]	0	1 (1).	0	0	2 (5).	0	0	0	**3**
V.campbellii	1 (1).	0	0	1 (1).	0	0	0	0	**2**
V.gazogenes	2 (3).	0	0	0	0	0	0	0	**2**
V.ordalii	1 (1).	0	0	0	0	0	0	1 (6).	**2**
V.pelagius	0	0	1 (3).	1 (1).	0	0	0	0	**2**
V.splendidus	0	0	0	0	0	0	0	2 (12).	**2**
P.shigelloides	1 (1).	0	0	0	1 (3).	0	0	0	**2**
V.hollisae	1 (1).	0	0	0	0	0	0	0	**1**
V.anguillarum-like	0	0	0	0	1 (3).	0	0	0	**1**
Serratia-like	0	0	0	0	1 (3).	0	0	0	**1**
V.algi/-V.carc/-V.harv.	0	0	0	0	0	0	1 (2).	0	**1**
Unidentified	Stn 390 Stn 421	-	-	Stn 7680 Stn 7790	-	-	-	-	**4**
Totals:	77	52	30	77	57	37	44	17	Total 391
Number Taxa:	20	11	9	17	16	8	11	8	
Proportion, (%).	(26).	(21).	(30).	(22).	(28).	(21).	(25).	(47).	

obtained from EDDA based protocols however this observation is from only a small pooled sample. From a larger sample it is indicated that protocols involving BTB-teepol broths were selective against less common taxa, (with the exception of *V.fluvialis/furnissii*-like), and any taxon isolated after enrichment in this medium was also recovered by direct isolation onto Simidu or TCBS media i.e. it was redundant. Taxa from ATW-Simidu protocols were also obtained directly on Simidu and TCBS media, with the exception of the

V.diazotrophicus-like species which was also isolated from SENA and ATW-TCBS. Only one taxon *V.alginolyticus/-V.carchariae/-V.harveyi* was unique to SENA protocols and the constituent species from this taxon, and all others were also isolated under Simidu, TCBS and ATW-TCBS protocols. Non-parallel EDDA based protocols were similarly redundant with exception for the isolation of *V.splendidus*. *V.alginolyticus* was not recovered from any EDDA based protocol.

3.9.3 Discussion.

It is directly indicated that six isolation protocols including three involving SENA are redundant to 24 h enrichment ATW and isolation from TCBS, and direct isolation, (48 h), from Simidu and TCBS media. Less clear is whether EDDA based protocols which were not conducted in parallel are also redundant, or if with more isolations a plateau of different recoverable species would have been reached. It would seem from (3.7.3.1-2) that Simidu supports growth of *V.splendidus* UQM 2786 and possibly also wild strains of this species and absence of *V.splendidus* reflected its absence from sampled material rather than any intrinsic inhibitory qualities of this medium. Earlier reports (2.10.2) about the inhibition of *V.anguillarum* by TCBS are not supported by these results.

For non-specific isolations of *Vibrionaceae* considerable time savings would accrue, and so allow more and different material to be examined, if only the three protocols from above, (Simidu, TCBS, ATW-TCBS), were used. Non-fermentative selective media with EDDA, perhaps used in combination with anaerobic incubation, may in future prove useful for the isolation of specific ecological groups (See Pham *et al.*, 1985), or where overgrowth on media by ubiquitous species such as *V.alginolyticus* occurs. Any such second application of this medium should be in the context of its inhibition to other non-siderophore producing strains or species.

3.10.1 Ecology of *Vibrionaceae Associated* with *Red-Spot* Disease.

3.10.1 Experimental Design.

In accord with the general experimental design (3.2), taxa deduced in (3.8.3-6) were tabulated according to their originating niches and comparatively examined (3.10.2) descriptively and after numerical analyses, for the existence of flora associated with specific abiotic and biotic niches.

Evaluation was made of the pathogenicity (3.10.3) of selected *Vibrio* strains towards juvenile wild fish of a species known to be susceptible to RSD. Further work (3.10.4) was conducted to investigate the comparative associative behavior of *Vibrio* spp. towards naupliar larvae of a copepod fish parasite.

An attempt was made on the basis of these investigations to deduce aspects of *Vibrionaceae* ecology which might be relevant to the pathogenesis of RSD.

3.10.2 Ecological Distribution of Deduced *Vibrionaceae* Taxa.

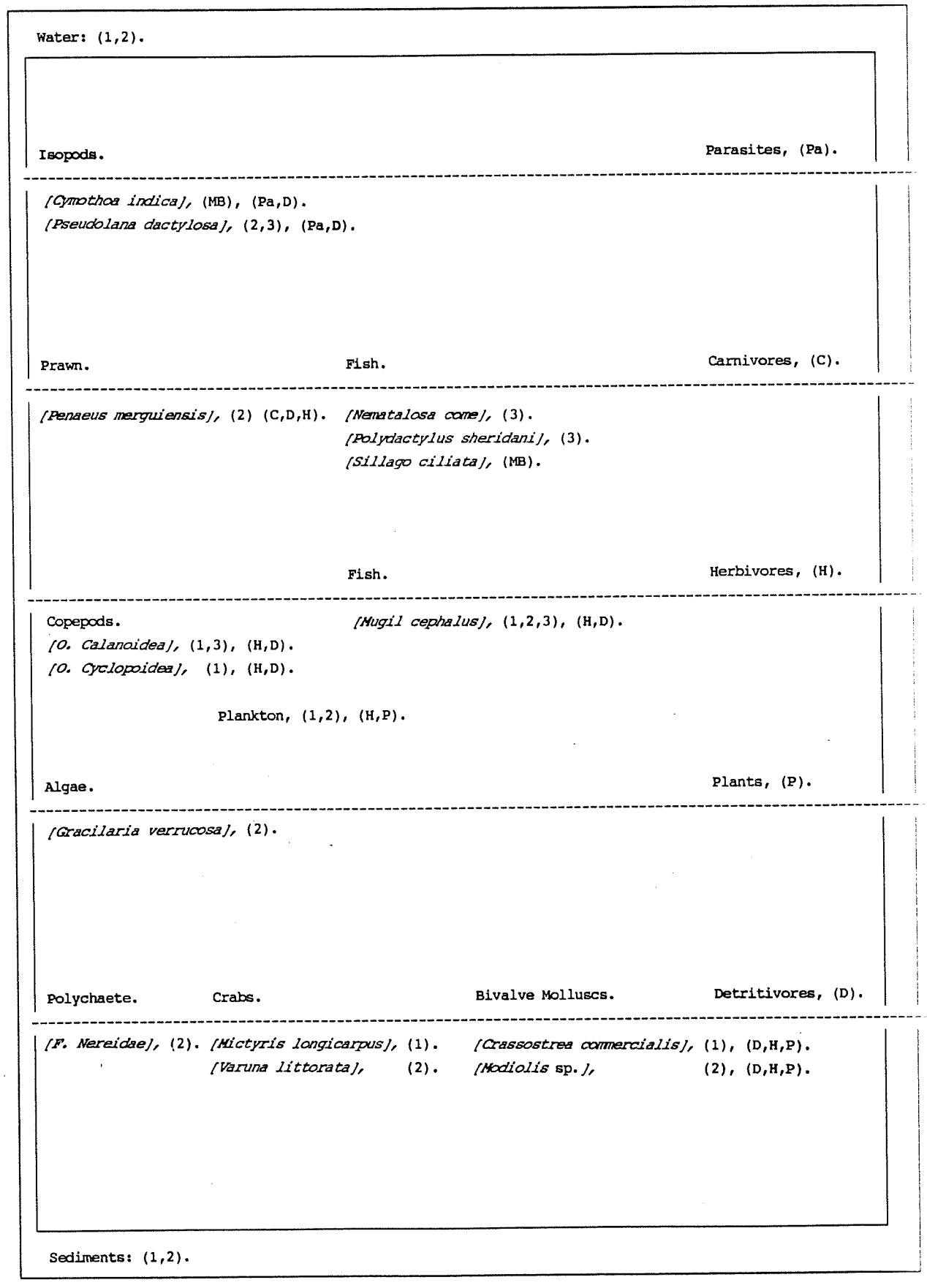
3.10.2.1 Experimental Design.

Environmental material from biotic and abiotic components of aestuarine and brackish-water communities habituated by RSD susceptible fish was examined to determine its *Vibrionaceae* flora on the basis of the existence of varying grades of symbioses between *Vibrionaceae* and multicellular species and the unproven, but inferred potential for vectored transmission as indicated in sections (2.4.4.0-8, 2.5.0-3). Environmental material from Sites [1.] and [2.] canvassed the same trophic levels; but these were depicted locally by different aestuarine or brackish-water species. With the exception of the three whiting from Moreton Bay, fish were all from the Bundaberg region and able to move freely across the salinity gradient between the sites shown on Figure 3.2. Using this information an abstracted migrating RSD susceptible fish community food-Web, (Figure 3.11), was constructed.

The flora from different environmental materials and for a total of 391 presumptive *Vibrionaceae,* (Table 3.8), were after numerical analysis,

(3.8.3-6), classified into 31 taxa and four single strains, (Table 3.28.1). Most strains from Table 3.8 were from diseased and control river fish; and diseased Moreton Bay fish (353 & 136; and 99 respectively (3.7.3)). The symptoms of these fish were described in Table 3.6. At least 10 presumptive isolates were obtained from each of the residual isolation materials. Amongst presumptive *Vibrionaceae* phenetically classified, less than 10 OTU's were sampled from Site [1.] water (6), and Site [2.] sediment (7); Site [3.] plankton (5); Site [2.] crab *[Varuna littorata]* (4); Site [2.] algae *[Gracilaria verrucosa]* (7); polychaete *[F.nereidae]* (3); Site [2.] prawn *[Penaeus merguiensis]* (2); Site [2.] Bivalves *[Modiolis* sp.] (6); internal isolates of unfed pseudolanid isopods (3, shown in Table 3.28.1 as a pooled sample), and external isolates from control-fish (2). Except for the pooled isopod sample described separately later, data for these samples are *italicized* in Table 3.28. Data from river fish was tabulated to indicate the distribution of taxa over time (Table 3.28.2), and from early and developed, (necrotic), lesions (Table 3.28.3). Data, (Table 3.28.1), was further tabulated in binary notation to indicate presence or absence of taxa from each ecological niche and Jaccard matrices (3.29.1-2) were assembled (A11.4). The first (untransposed) representing the 13 most common taxa represented by nine or more strains over 23 sites, and the second, (transposed, A11.1), for 23 sites as measured by 35 taxa. Distance matrices were subjected to UPGMA *(Microcluster)* analyses first to indicate taxa by using sites as variables (Figure 3.12.1) and then by sorting niches with taxa as variables (Figure 3.12.2). Further evaluation of the data was on the basis of floral distributions; including the comparative *Vibrionaceae* floras of control and diseased fish; and the ecological distance between trophic strata.

Figure 3.11: Abstracted Migrating RSD Susceptible Fish Community Food-Web.



Key: Sites are indicated by (1,2,3 & MB (Moreton Bay)), and Cross-Trophic Status by (Pa,C,D,H,P), for Parasites, Carnivores, Detritivores, Herbivores & Plants.

3.10.2.2 Results.

3.10.2.2.1 Description of Niche Flora.

Ecological similarities amongst the 13 most commonly encountered taxa, as $(S_{j.})$, from Table 3.29.1 ranged between 0 and 75 %. The highest, measured by mutual occurrence in different ecological niches, were between *Photobacterium*-like and *A.hydrophila*, (75 %); *V.aestuarianus* and *V.carchariae/-V.harveyi*, (71 %); *V.fluvialis/-V.furnissii*, and *S.putrefaciens* (60 %); and between *V.aestuarianus* and *V.orientalis* (57 %). Strains of *V.zobellii* were recovered exclusively from calanoid copepods and so were not measured as ecologically similar to any other taxon.

Similarities $(S_{j.})$, of taxa between ecological sites [Table 3.29.2] ranged between 0, (Site [1.] copepods), and 100 %, (Site [2.], algae and polychaete); however the overlap of taxa between sites mostly ranged between 0 and 50 %. Comparisons of fish samples indicated a greater similarity between internal and external taxa from diseased Moreton Bay Fish than between diseased and control river system fish, respectively 45 % cf. 24 and 14 %, (Control, internal - sample size 2).

More different taxa, (24 %), including some such *V.anguillarum*, only obtained from a single niche, were identified from repeated samplings of diseased river fish, than from any other sampled material. The lowest proportion i.e. lowest species diversity from isolation material was from repeated sub-samplings of calanoid copepod material. Low proportional recoveries also came from Site [1.] crabs, (12 %) Site [2.] algae, (15 %) internal isolates from diseased Moreton Bay fish.

Single taxon floral communities were found in three ecological niches. From the first of these marine copepods; *V.zobellii* only sampled from single copepods; and three taxa, none like *V.zobellii* were found from 13 strains isolated from the same marine plankton sample, but after different (3.7.2.3.2,3) treatments. One taxon *V.fluvialis/-V.furnissii*, was found in

Table 3.28.1: Comparative Environmental Origins and Relative Abundance of 391 Provincial Isolates.

Environmental Origins.	Water. Site.		Sedim-ent. Site.		Plankton, Cope-pod, Site.			Crabs. Site.		Algae. Site.		Polyc-haete. Site.		Prawn. Site.		Bival-ves. Site.		Isopods. C. I.	P. I.	P. E.	River Fish. Control. I.	E.	Test. I.	E.	Bay Fish. Test. I.	E.	Total for Taxon.
	1.	2.	1.	2.	1.	1.	3.	1.	2.	1.	2.	1.	2.	1.	2.	1.	2.										
Taxon:																											
V.alginolyticus	*2*	4	8	*3*	0	9	*0*	24	*1*	N	*0*	N	*0*	N	*0*	13	*0*	2	0	*3*	4	*2*	0	12	0	0	87
V.carchariae/V.harveyi	*0*	0	1	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*1*	4	2	*0*	0	*0*	0	6	21	16	51
V.fluvialis/V.furnissii	*3*	6	0	*0*	0	2	*1*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	4	*3*	5	*0*	12	9	0	0	45
V.parahaemolyticus	*0*	0	1	*0*	0	0	*0*	0	*2*	N	*7*	N	*3*	N	*0*	0	*1*	0	0	*1*	0	*0*	0	10	0	0	25
A.hydrophila	*0*	0	0	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	0	*0*	2	*0*	7	12	0	0	21
V.aestuarianus I & II	*0*	0	0	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	2	5	*0*	0	*0*	0	1	8	2	18
S.putrefaciens	*0*	0	0	*0*	0	0	*1*	0	*0*	N	*0*	N	*0*	N	*1*	0	*0*	0	1	*1*	9	*0*	2	2	0	0	17
V.carchariae	*0*	0	0	*0*	0	0	*2*	0	*0*	N	*0*	N	*0*	N	*0*	4	*0*	2	0	*0*	0	*0*	1	0	6	0	15
V.orientalis	*0*	1	0	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	1	*0*	2	0	*0*	0	*0*	0	5	4	1	14
Photobacterium-like	*0*	0	0	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	0	*0*	1	*0*	1	8	0	1	11
V.fluvialis/furnissii-like	*0*	0	0	*3*	0	2	*0*	0	*1*	N	*0*	N	*0*	N	*1*	0	*0*	0	0	*0*	0	*0*	2	1	0	0	10
V.zobellii	*0*	0	0	*0*	10	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	0	*0*	0	*0*	0	0	0	0	10
V.vulnificus	*0*	0	0	*0*	0	0	*1*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	1	*1*	0	*0*	1	0	3	2	9
V.carc/V.harv/V.para.	*0*	0	2	*1*	0	0	*2*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	0	*0*	0	*0*	1	0	1	0	7
V.natriegens like	*0*	0	0	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*4*	0	0	*0*	0	*0*	1	0	0	0	5
V.anguillarum	*0*	0	0	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	0	*0*	0	*0*	0	5	0	0	5
V.diazotrophicus	*0*	0	0	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	0	*0*	2	*0*	0	3	0	0	5
V.natriegens	*0*	0	1	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	1	*0*	0	0	*0*	0	*0*	0	2	0	0	4
V.costicola-like	*0*	0	0	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	0	*0*	0	*0*	0	0	3	0	3
V.diazotrophicus-like	*0*	0	0	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	0	*0*	0	*0*	2	1	0	0	3
V.mediterranei	*0*	0	0	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	1	*0*	0	0	*0*	0	*0*	0	0	2	0	3
Phenon AC [Batches 1,3,7,& 8]	*1*	0	2	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	0	*0*	0	*0*	0	0	0	0	3
V.campbellii	*0*	0	0	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	0	*0*	0	*0*	0	0	1	1	2
V.gazogenes	*0*	0	2	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	0	*0*	0	*0*	0	0	0	0	2
V.ordalii	*0*	0	0	*0*	0	0	*0*	1	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	0	*0*	0	*0*	0	1	0	0	2
V.pelagius	*0*	0	0	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	0	*0*	1	*0*	0	1	0	0	2
V.splendidus	*0*	0	0	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	0	*0*	0	*0*	0	2	0	0	2
P.shigelloides	*0*	0	0	*0*	0	0	*0*	1	*0*	N	*0*	N	*0*	N	*0*	1	*0*	0	0	*0*	0	*0*	0	0	0	0	2
V.hollisae	*0*	0	1	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	0	*0*	0	*0*	0	0	0	0	1
V.anguillarum-like	*0*	0	0	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	0	*0*	0	*0*	0	1	0	0	1
Serratia-like	*0*	0	0	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	0	*0*	0	*0*	1	0	0	0	1
V.algi/V.carc/V.harv.	*0*	0	0	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	0	*0*	0	*0*	0	0	1	0	1
Vibrio spp. Stn 390 & 421.	*0*	0	0	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	2	*0*	0	0	*0*	0	*0*	0	0	0	0	1
Vibrio spp. Stn 7680 & 7790.	*0*	0	0	*0*	0	0	*0*	0	*0*	N	*0*	N	*0*	N	*0*	0	*0*	0	0	*0*	0	*0*	0	2	0	0	1
Total *Vibrionaceae* Tested. :	6	11	18	7	10	13	7	26	4	N	7	N	3	N	2	23	6	12	13	9	24	2	31	84	50	23	391
Total of Taxa. :	3	3	8	3	1	3	5	3	3	N	1	N	1	N	2	8	3	5	5	5	7	1	11	20	10	6	
Taxa, Proportion (%), Total. :	50	33	44	42	10	23	71	12	75	N	15	N	33	N	100	35	50	42	38	55	29	50	35	24	20	26	

Key: Sites 1,2,3 respectively Winfield, Wartburg & Oyster Ck.; Copepod, *O.Calanoidea,* Crabs (Site) 1 & 2 respectively *Hictyris longicarpus,* & *Varuna littorata;* Algae, *Gracilaria verrucosa;* Polychaete, *F.Nereidae;* Prawn, *Penaeus merguiensis;* Bivalves (Site) 1 & 2; respectively *Crassostrea commercialis,* & *Modiolis* sp.; Isopods C & P, respectively *Cymothoa indica* & *Pseudolana dactylosa;* River Fish - *Mugil cephalus, Nematalosa come,* & *Polydactylus sheridani;* Bay Fish, *Sillago ciliata;* I-Internal, E-External; Control, Disease Lesions Absent; Test, Disease Lesions Present; In data; N- Not Determined; *Italics* indicate < 10 Strains; and Bold the most common Taxon.

Table 3.28.2: Sampled Distribution of River System *Vibrionaceae* Over Time.

Sampling Date (dd.mm.yy) :	25.02.85		13.03.85		19.03.85		29.05.85		02.07.85		--08.85		--02,03.86	
Diseased, D., Control, C.:	D. :	C.	D. :	C.	D. :	C.	D. :	C.	D. :	C.	D. :	C.	D. :	C.
Number of Fish. :	6.	5.	2.	0	3.	0.	3.	0.	3.	0	7	0	0	1
Taxon:														
V.alginolyticus	3E	2I	E	-	0	-	8E	-	0	-	0	-	0	2E
V.carchariae/-V.harveyi	5E	0	E	-	0	-	0	-	0	-	0	-	0	0
V.fluvialis/-V.furnissii	E,12I	5I	0	-	8E	-	0	-	0	-	0	-	0	0
V.parahaemolyticus	2E	0	8E	-	0	-	0	-	0	-	0	-	0	0
A.hydrophila	E,7I	2I	0	-	7E	-	0	-	4E	-	0	-	0	0
V.aestuarianus I & II	0	0	0	-	0	-	0	-	0	-	E	-	0	0
S.putrefaciens	2I	9I	0	-	E	-	0	-	0	-	0	-	E	0
V.carchariae	I	0	0	-	0	-	0	-	0	-	0	-	0	0
V.orientalis	0	0	0	-	0	-	0	-	3E	-	E	-	0	0
Photobacterium-like	2E,I	I	0	-	0	-	0	-	4E	-	2E	-	0	0
V.fluvialis/-furnissii-like	E,2I	0	0	-	0	-	0	-	0	-	0	-	0	0
V.vulnificus	I	0	0	-	0	-	0	-	0	-	0	-	0	0
V.carc/-V.harv/-V.para.	I	0	0	-	0	-	0	-	0	-	0	-	0	0
V.natriegens-like	I	0	0	-	0	-	0	-	0	-	0	-	0	0
V.anguillarum	0	0	0	-	0	-	0	-	0	-	5	-	0	0
V.diazotrophicus	0	2I	0	-	0	-	0	-	1E	-	2E	-	0	0
V.natriegens	0	0	E	-	0	-	0	-	I	-	0	-	0	0
V.diazotrophicus-like	2I	0	0	-	0	-	0	-	0	-	0	-	0	E
V.ordalii	0	0	0	-	0	-	0	-	0	-	0	-	I	0
V.pelagius	0	I	0	-	E	-	0	-	0	-	0	-	0	0
V.splendidus	0	0	0	-	0	-	0	-	2	-	0	-	0	0
V.anguillarum-like	E	0	0	-	0	-	0	-	0	-	0	-	0	0
Serratia-like	I	0	0	-	0	-	0	-	0	-	0	-	0	0
Vibrio spp. Stn 7680 & 7790.	0	0	0	-	E,E	-	0	-	0	-	0	-	0	0

I. Internal, E. External; ND.

Table 3.28.3: Distribution of *Vibrionaceae* Taxa from Diseased River Fish According to Lesion Development.

Lesion Development	**: Control.**	**Early.**	**Necrotic.**
Fish Numbers.	**:** 3,4,7,9,10 11,37,38.	1,2,5,6,8, 14,15,19-25.	6,13,25-27,30 30,32-35,38.
Taxon:			
V.alginolyticus	4	12	0
V.carchariae/-V.harveyi	0	6	0
V.fluvialis/-V.furnissii	5	21	0
V.parahaemolyticus	0	10	0
A.hydrophila	2	15	4
V.aestuarianus I & II	0	0	1
S.putrefaciens	9	3	1
V.carchariae, V.vulnificus	0	1,1	0
V.orientalis	0	3	2
Photobacterium-like	1	2	7
V.fluvialis/-furnissii-like	0	3	0
V.carc./-V.harv/-V.para.	0	1	0
V.natriegens-like	0	1	0
V.anguillarum, V.anguillarum-like	0	0,1	5,0
V.natriegens	0	1	1
V.ordalii, V.splendidus	0	0	1,2
V.pelagius, V.diazotrophicus	1,2	1,1	0,3
Serratia-like	0	1	0
Vibrio spp. Stn 7680 & 7790.	0	1,1	0

Table 3.29.1: Jaccard Similarity (%), as Measure for Ecological Relatedness of the 13 Most Common, (9 or more strains), Taxa from this Work and the Number of Originating Niches.

Most Common Taxa:

A *V.alginolyticus*	:	100											13 Sites.
B *V.carchariae/-V.harveyi*	:	18	100										7 Sites.
C *V.fluvialis/-V.furnissii*	:	38	14	100									9 Sites.
D *V.parahaemolyticus*	:	25	27	14	100								7 Sites.
E *A.hydrophila*	:	14	11	33	11	100							3 Sites.
F *V.aestuarianus* I & II	:	13	71	17	9	14	100						5 Sites.
G *S.putrefaciens*	:	18	17	60	17	43	20	100					7 Sites.
H *V.carchariae*	:	13	20	17	0	14	25	20	100				5 Sites.
I *V.orientalis*	:	27	44	15	8	13	57	8	38	100			6 Sites.
J *Photobacterium*-like	:	13	22	30	10	75	29	38	13	25	100		3 Sites.
K *V.fluvialis/furnissii*-like	:	27	8	25	18	29	10	30	10	9	25	100	6 Sites.
L *V.zobellii*	:	0	0	0	0	0	0	0	0	0	0	0	100 1 Sites.
M *V.vulnificus*	:	6	30	36	8	13	38	44	38	20	25	9	0 100 6 Sites.

Taxon:	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**

Table 3.29.2: Jaccard Similarity (%), between 23 Sites Measured by comparison with 34 Taxa. (Niche Abbreviations are Coded in Table 3.28.1; A - W indicate Origins.

Sample Origin: Site:

A Water,	(1):	100																				
B Water,	(2):	50	100																			
C Sediment,	(1):	22	10	100																		
D Sediment,	(2):	20	20	22	100																	
E Copepods,	(1):	0	0	0	0	100																
F Plankton,	(1):	50	50	10	50	0	100															
G Plankton,	(3):	14	14	8	14	0	14	100														
H Crab,	(2):	20	20	10	20	0	20	0	100													
I Crab,	(1):	20	20	22	50	0	50	0	20	100												
J Algae,	(2):	0	0	13	0	0	0	0	0	33	100											
K Polychaete,	(2):	0	0	13	0	0	0	0	0	33	100	100										
L Prawn,	(2):	0	0	0	25	0	25	17	0	25	0	0	100									
M Bivalves,	(1):	10	22	14	10	0	10	8	22	10	0	0	0	100								
N Bivalves,	(2):	0	0	22	0	0	0	0	0	20	33	33	0	0	100							
O *C.indica,*	(I):	14	33	18	14	0	14	11	14	14	0	0	0	30	14	100						
P *P.dactylosa,*	(I):	14	14	8	0	0	14	43	0	0	0	0	17	0	14	25	100					
Q *P.dactylosa,*	(E):	33	33	18	14	0	33	43	14	33	20	20	17	8	14	11	43	100				
R River Fish (C.),(I):		25	25	7	11	0	25	20	11	11	0	0	13	7	0	9	20	33	100			
S River Fish (C.),(E):		33	33	13	33	0	33	0	33	33	0	0	0	13	0	20	0	20	14	100		
T River Fish (D.),(I):		8	8	6	17	0	17	45	0	8	0	0	18	6	8	7	23	23	29	0	100	
U River Fish (D.),(E):		10	15	17	10	0	15	9	10	15	5	5	10	12	10	19	19	19	35	5	24	100
V Bay Fish, (D.),(I):		0	8	13	8	0	0	25	0	0	0	0	0	20	8	36	25	7	0	0	17	11 100
W Bay Fish, (D.),(E):		0	13	8	0	0	0	10	0	0	0	0	0	8	13	38	38	10	8	0	13	18 45

Origin:	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**P**	**Q**	**R**	**S**	**T**	**U**	**V**

marine and freshwater plankton samples. The remaining two single taxon sites were algal and polychaete niches, of *V.parahaemolyticus.* Unlike *V.zobellii, V.parahaemolyticus* was also co-dominant amongst sampled flora from a third niche, Site [2.] crabs; and most strains of this taxon (10) were obtained from external lesions of diseased fish.

The other common taxon with a restricted distribution was *V.aestuarianus* I & II which was only sampled from fish and isopods. This taxon was recovered principally from internal and external sites on diseased Moreton Bay Fish and from external surfaces of control fish, and also non-exclusively from gut samples of associated cymothoid and fed pseudolanid isopods (3, unfed pseudolanid isopods were represented in the taxa *V.fluvialis/-V.furnissii,* (2), and *V.vulnificus* (1)).

V.alginolyticus, the most common taxon, was dominant or co-dominant amongst sampled flora from seven brackish- and marine- niches as follow: Sites [1.] & [2.] sediment, Site [1.] plankton, crabs, and bivalves (oysters); external isolates from pseudolanid isopods and from external lesions of diseased river fish, (co-dominant with *A.hydrophila). V.alginolyticus* was not recovered from relatively sedentary brackish-water biota such as crabs and bivalves, in contrast to the large numbers of this taxon obtained from correspondent marine niches.

V.carchariae was co-dominant with *V.carchariae/-V.harveyi/-V.parahaemolyticus* amongst sampled flora from freshwater plankton, [Site 2.]. *V.carchariae/-V.harveyi* dominated in samples from three niches, cymothoid internae; and internal and external surfaces of diseased Moreton Bay fish. *S.putrefaciens* was respectively dominant and co-dominant with *V.fluvialis/-V.furnissii* amongst flora from diseased river-fish and the Site [2.] prawn. *V.fluvialis/-V.furnissii* dominated flora water samples from Sites [1. & 2.], external surfaces of pseudolanid isopods and diseased river fish.

From 17 water isolated presumptive *Vibrionaceae* three taxa were found from marine and brackish-water sites, two of these *V.alginolyticus,* and

V.fluvialis/-V.furnissii, were shared, and two *V.orientalis* [Site 2.], and Phenon AC, [Site 1.], were localized. A lesser correspondance occurred from the two sediment samples; from Site [1.] 18 strains were classified into eight taxa, and from Site [2.], seven strains into three taxa. *V.alginolyticus* and *V.carchariae/-V.harveyi/-V.parahaemolyticus* were found in both samples. Apart from *V.alginolyticus* which was present in all abiotic samples the only other taxon found in water and sediments [Site 1.] was phenon AC.

Diseased river fish and diseased Moreton Bay fish shared 9 or possibly 10 taxa: *V.carchariae/-V.harveyi, V.fluvialis/-V.furnissii, V.aestuarianus* I & II, *V.carchariae, V.orientalis, Photobacterium*-like, *V.vulnificus, V.carchariae/-V.harveyi/-V.parahaemolyticus, V.costicola*-like, and possibly *V.alginolyticus.* Thirteen taxa: *V.alginolyticus, V.parahaemolyticus, A.hydrophila, S.putrefaciens, V.fluvialis/-V.furnissii*-like, *V.natriegens*-like, *V.anguillarum, V.diazotrophicus, V.natriegens, V.ordalii, V.pelagius, V.splendidus, Serratia*-like; isolated from diseased river fish were not found on diseased Moreton Bay fish. Diseased river fish bore several co-dominant taxa while most strains from diseased Moreton Bay fish came from *V.carchariae/V.harveyi.*

Amongst internal and external samples from control and diseased river system flora seven taxa, *V.alginolyticus, V.fluvialis/-V.furnissii, V.pelagius S.putrefaciens, V.diazotrophicus, Photobacterium*-like, and *A.hydrophila* were shared; and 15 taxa, *V.carchariae/-V.harveyi, V.parahaemolyticus, V.aestuarianus I & II, V.orientalis, V.fluvialis/-V.furnissii*-like, *V.vulnificus, V.carchariae/-V.harveyi/-V.parahaemolyticus, V.natriegens*-like, *V.anguillarum, V.diazotrophicus*-like, *V.natriegens, V.ordalii, V.splendidus, V.anguillarum*-like and *Serratia*-like; were only found on diseased river fish. However it is indicated, (Table 3.28.2), that amongst flora exclusive to diseased fish no single taxon was consistantly isolated from all diseased fish. Table 3.28.3 further indicates that the taxa

V.aestuarianus, V.anguillarum, Photobacterium-like *V.diazotrophicus* and *V.splendidus* were exclusively or most often isolated from advanced RSD lesions.

3.10.2.2.2 **Review of Numerical Analyses.**

The dendrogram (Figure 3.12.1) formed the taxa into four dissimilar Ecotypes (1-4.); the first represented *V.zobellii,* which associated only with a detritivorous or herbivorous calanoid copepod host, and so had no measured similarity with any other ecotype. The second, $S_{j.}$ *ca* 20 %, *V.alginolyticus, V.parahaemolyticus,* and *V.fluvialis/-furnissii*-like and depicted a diversity of ecological niches and trophic levels ranging between plants, herbivores, detritivores, parasites, carnivores. Ecotype (3.), $S_{j.}$ *ca* 30 %, comprised the taxa *A.hydrophila, Photobacterium*-like, *V.fluvialis/-V.furnissii, S.putrefaciens,* and *V.vulnificus;* these species could be defined as representing two overlapping groups, fish and their crustacean parasites, and miscellaneous crustaceans including prawns and unspecified planktonic crustaceans. Ecotype (4.), ($S_{j.}$ of *ca* 30 %), *V.carchariae, V.carchariae/-V.harveyi, V.aestuarianus* I & II, and *V.orientalis* was representative of niches with broadly differing trophic status i.e. water, sediments, filter feeding detritivores, and carnivorous, detritivorous and herbivorous fish.

The second dendrogram, (Figure 3.12.2), ($S_{j.}$ *ca* 30 %), depicting similarities in ecological niches by comparison of taxa, produced 5 major niche-groups (a-e.) Ecotype (1.), *V.zobellii,* from the first analysis corresponded here to niche-group (a.). Niche-group (b.) fused the other monospecific algal and polychaete niches, first to another niche holding *V.parahaemolyticus* and then to marine sediment which held a completely different flora from the initial niches. Niche-group [c,d.] represented sites which held more taxa than the other niche-groups. Niche-group (c.) was composed by the niches from marine bivalves, internal and external samplings from diseased Moreton Bay fish, and the associated cymothoid

Figure 3.12.1: UPGMA Dendrogram, (UPGMA), of the Jaccard matrix from the 13 most common Taxa Measured over 23 Sites.

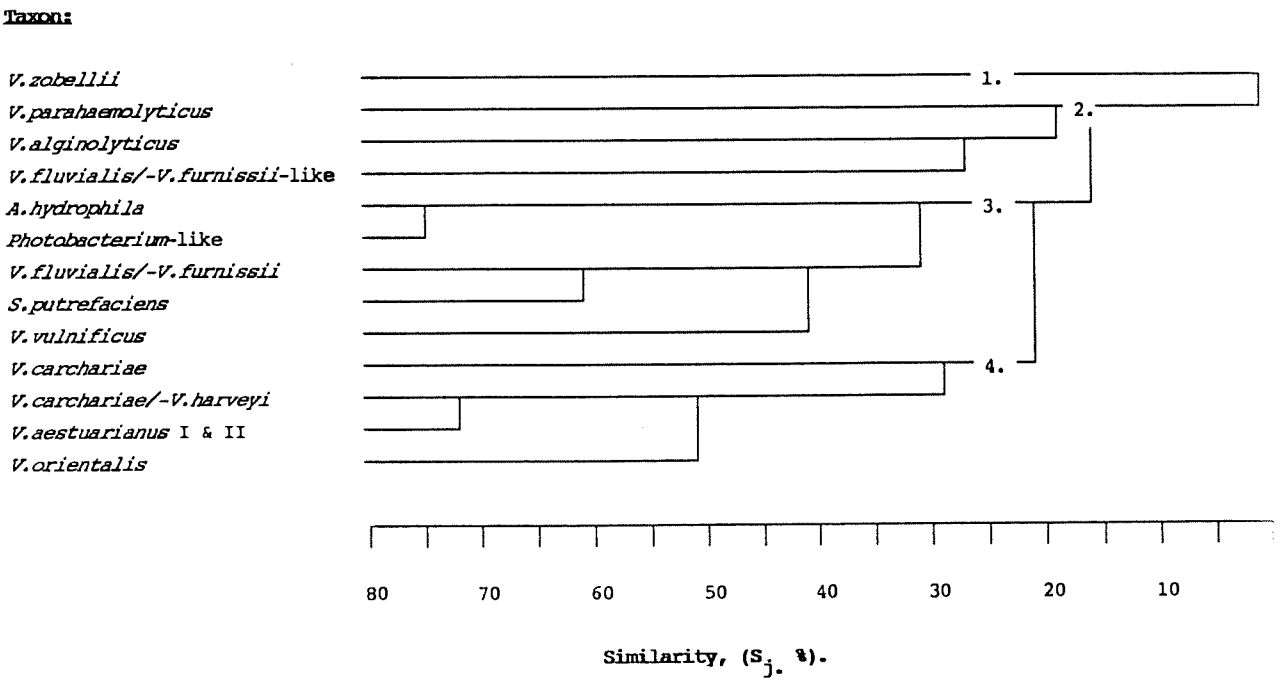
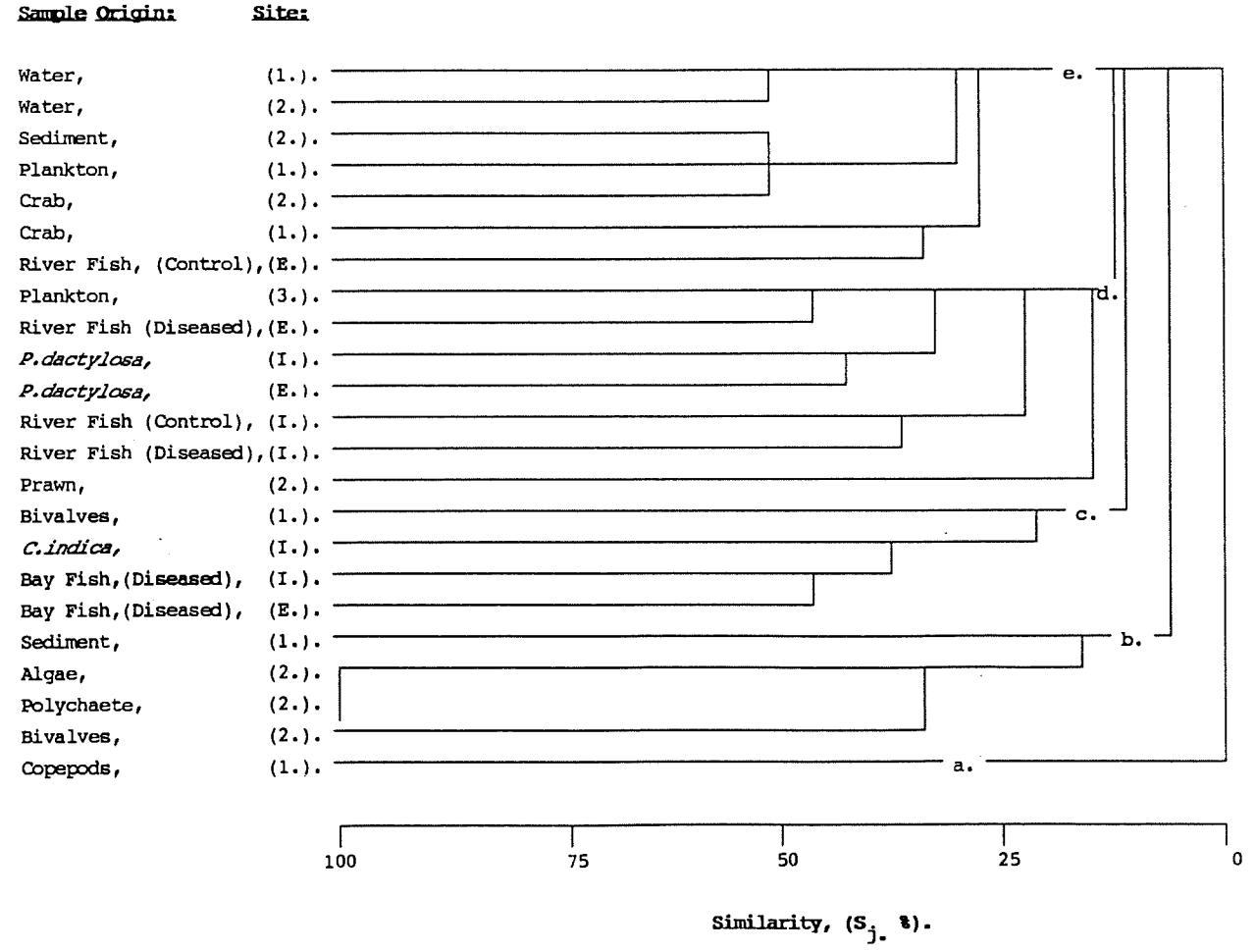


Figure 3.12.2 : UPGMA Dendrogram, (UPGMA), of the Jaccard matrix from the 23 Ecological Niches for 34 taxa.



parasite. Niche-group (d.) held flora from internal and external samplings from pseudolanid isopods with that from external and internal samples from diseased fish and internal samples from control fish. Niche-group (e.) fused the control river fish niche with niches from freshwater plankton, marine and brackish-water crabs, marine and brackish water, marine plankton and brackish-water sediments.

3.10.2.3 Discussion.

The presence of one or two taxa [*V.carchariae*] or [*V.carchariae/-V.harveyi*], as the dominant taxa from Moreton Bay fish and as co-dominant taxa from river system fish affected by RSD is a paradox indicating either the bacteria or the fish, initially assumed as similar, were different i.e. by ecological experience, instantaneous physiology or genotype.

The acute condition of moribund Moreton Bay fish (Table 3.6) with shallow lesions may have been affected by acid stress (See 2.3.3.1.2). This is supported by description of RSD-affected fish as in good condition (Table 3.6), despite in some cases deep and extensive (chronic) lesions.

The similarities of internal floral composition of control fish, (attributed [3.7.4] to mechanical vectoring during migration from the gut to the mesenteries by encysting parasites), and of RS diseased fish might indicate that subject to operation of some stress initiated factor immunological stasis of these deep-tissue bacterial taxa is disrupted and disease starts from within; but is manifested most prominently by external lesions.

Alternatively it may be that internal flora as measured is intrinsically non-pathogenic i.e. if it were pathogenic access to the internal tissues would soon lead to the host fish's death and consequent removal of pathogenic strains from normally sampled flora. If internal and external flora of control fish is non-pathogenic then the taxa additional to *V.anguillarum* which are associated with RSD and which might also be active in RSD, include in order of abundance: *V.parahaemolyticus, V.carchariae/-*

V.harveyi, V.orientalis, V.fluvialis/-V.furnissii-like, *V.carchariae, V.vulnificus, V.carchariae/-V.harveyi/-V.parahaemolyticus, V.natriegens*-like, *V.diazotrophicus*-like, *V.natriegens, V.ordalii, V.splendidus, V.anguillarum*-like and *Serratia*-like. If flora of control fish is pathogenic, but requires an additional stimulus for pathogenesis then some or all of the taxa, *V.fluvialis/-V.furnissii, V.alginolyticus, V.pelagius, V.diazotrophicus, S.putrefaciens,* and *A.hydrophila* may also be involved.

The large number of different associated taxa from both diseased and control fish could also indicate that the primary cause of disease is not the *Vibrionaceae* but rather that these are present incidentally as opportunistic pathogens but as concluded in (2.6.5) their repeated involvement in other ulcerative fish disease makes this appear unlikely. Involvement could be synergistic in a pathogenic gestalt, or different taxa of *Vibrionaceae* might affect fish in series of epizootic waves. In which case it would also seem, except for the sampling of fish in good condition and from an unpolluted habitat, that the health of susceptible fish was somehow impaired. This could not be sufficiently tested by correlation of physical chemical or definition of other parameters because of the qualitative nature of the remote sampling regimen. An explanation for presentation of RSD as a chronic rather than acute condition is that affected fish, by their behavior disrupt disease progression i.e. by swimming upstream into lower salinities or osmolarities to displace invading halophilic *Vibrionaceae* and allow non-invasive saprophytic colonization of dead tissue e.g. by normal skin flora. This differs from interpretation by Burke and Rodgers, (1981) by consideration of secondary colonization of lesions as non-invasive and initial invasion by many species of *Vibrionaceae,* apart form *V.anguillarum.*

Generally sufficient environmental material was sampled to determine individual niche floral profiles for only a few niches. However direct and exclusive isolation of *V.zobellii* from individual calanoid copepods but not from the aggregated originating plankton sample strongly suggests this species is a site specific symbiont. The exclusive occurrence of

V.parahaemolyticus on algae and isolation from diseased rather than control river fish which feed on such food is a potential but unproven route to pathogenesis. It seemed from the larger samplings of diseased and control fish and their sedentary, (cymothoid), and transitory, (pseudolanid), isopod parasites that components of their flora were common or shared. Despite experimental evidence which indicated that one shared taxon [*V.aestuarianus* I and II] might have an ecologically restricted distribution the description (Tison and Seidler, 1983) of biogroup I, was originally from water and shellfish so here its restricted distribution may have been a sampling artifact. *V.carchariae* also was found on both fish and isopods; however G + C determination of UQM 2849 (Appendix 9.; Table 3.5) isolated from marine bivalves, and phenetically resembling *V.carchariae/-V.harveyi* indicated this strain as a *V.carchariae.* Consequently on the basis of this non-specific association it would appear that if transmission of bacteria by isopods between fish occurs, as seems possible because of the transitory pseudolanid associations, their action would be as mechanical rather than as biological vectors.

Similarities in the specific component flora of parasitic and free living crustaceans such as crabs, prawns, and amongst plankton suggests the possibility that bacterial flora is dictated by two parameters; the first defining physical and chemical characteristics of surfaces, is phylogenetically determined and the second governed by host ecology i.e. parasitic or free-living.

The absence of taxa such as *V.cholerae,* from the relatively large sample of provincial strains and despite occurrence in other Queensland or New South Wales regional surveys (See Table 3.5), reflects the relative taxonomic intensity of this work i.e. by reclassification of non-01 strains such as UQM 2729,2730 from sucrose negative *V.cholerae* to *V.vulnificus* and *V.cholerae* DC 276 (UQM 3061) to *V.campbellii,* and probably also the seasonal distribution of individual taxa, (See 2.4.1) and perhaps absence of specific floral components from single river systems.

The application of cluster analysis to objectively classify different taxa and niches is apparent, and here by complimentary interpretation dendrograms artifacts could be separated from stable groupings. Apart from their composition by different classificatory units, differences between the two cluster analyses can be also be attributed to the properties of the measure used to assemble similarity matrices. The binary data set composed and transposed here only partially offset the effects of unbalanced sampling. The application of Jaccard rather than the euclidean coefficient is supported because negative matches are excluded from consideration, and UPGMA clustering of the Jaccard coefficients was concluded (3.8.7) to be the most suitable for systematics. The Jaccard coefficient here recorded presence in one site and absence in another as an indication of dissimilarity. This may have been valid in larger samples but is difficult to justify with smaller samples. A correction for this false premise could be to code all data where taxon absences occurred or occurred in samples of less than, e.g. 10, as missing and again measure similarity. This would cause the Jaccard coefficient in some cases locally to approximate the pattern coefficient of Sneath (1968).

3.10.2.4. Conclusions.

Specific differences existed between the flora and symptoms of diseased Moreton Bay fish and river system fish with RSD. The condition in Moreton Bay fish was acute and principally *V.carchariae/-V.harveyi* and *V.carchariae* were found. Among fish susceptible to RSD, control fish carried external and internal *Vibrionaceae* flora which was attributed (3.7.4) to mechanical vectoring of gut bacteria by invasive mesenteric encystation of cestode parasites. In diseased river fish with RSD many *Vibrionaceae* were isolated including some previously reported as fish pathogens and others including some described through sub-divisions (3.8.6.2.3.3) and further distinct taxa. These results may indicate that *Vibrionaceae* associated with RSD are opportunistic secondary colonizers or that the condition occurs in random waves of epizootics initiated by different taxa perhaps induced by weather

changes as reported by Burke and Rodgers (1981) and Rodgers and Burke (1981) and not able to be evaluated here but not as ordered pathogen seres such as initiated by their *(V.anguillarum)*. Here *V.anguillarum* was only isolated from advanced lesions.

It was also suggested here that the chronic rather than acute symptoms expressed in fish with RSD might occur because of fish behavior i.e. affected fish migrate to lower salinities or osmolarities and so displace halophilic pathogens and allow saprophytic recolonization of necrotized tissue by non-invasive bacteria and fungi.

Cluster analysis was seen as a means to objectively classify taxa into ecotypes, and isolation sites into niche-groups, but the analysis procedure for small examination of unbalanced sample sizes needs to be further investigated, and a method for such analysis was proposed.

3.10.3 Evaluation of the Pathogenicity of *Vibrio* spp. using Silver Mullet, *Liza argentea*.

3.10.3.1 Experimental Design.

No suitable fish-model species for RSD was commercially available, so wild cohorts of juvenile silver mullet *Liza argentea* were used in pathogenicity assays. These were undertaken to examine the relative virulence of environmental and fish isolates of *Vibrio* species towards a RS susceptible indigenous fish. Measurement of virulence was based upon determination of lethal median dose (LD_{50}).

Experimental fish were difficult to catch in large numbers, consequently assays were predominantly limited to provincially isolated bacteria from phena previously reported as pathogenic and referenced by comparison with *V.anguillarum* UQM 2771. An initial determination was made to evaluate the stability of the procedure when five rather than ten fish subjects were used at each dose level.

Chemical enhancement to virulence of *V.anguillarum* UQM 2771 by induction of siderophores (2.3.1) was also investigated. Bacteria which shared similar or different ecological niches with RS susceptible fish were examined e.g. from sediments upon which the fish feed, from an ecto-parasitic isopod of fish, and from planktonic copepods with little ecological similarity to fish. As a further consequence of limited fish supplies, effects of background stresses, e.g. temperature, chemicals such as herbicides, or salinity, upon virulence could not be determined. Virulence, (as LD_{50}) was determined by probit analysis and for comparative purposes, results are reported within 95 % confidence intervals. Late log or early stationary phase cultures were used for assays of virulence in acknowledgment of the findings of Watkins *et al.,* 1981 (See 2.3.2). All assays were conducted using washed cells to minimize effects from soluble toxins, i.e. LPS, as reported by Umbreit and Tripp, (1975).

3.10.3.1.1. Maintenance of *Liza argentea*.

Cohorts of juvenile *L.argentea* 50-70 mm long (*ca* 2 g) were beach-seine netted from the North Pine river. All fish were maintained in oxygen saturated sea water diluted with demineralized water to a salinity of 5 ‰, and continuously sparged by air entering sintered bubblers. New fish were held for 2 d prior to experimentation. Fish under test were held in 1 l glass jars, (95mm diameter), containing 750 ml seasoned water aerated (at least 2 d), as above.

3.10.3.1.2 Bacteria.

The following bacteria were used in assays: *V.anguillarum* UQM 2771 (type strain) from a nasal abscess of Cod, *G.morhua* (Traxler and Li, 1972) and UQM 2843, (Σ 1017), from the liver of a mullet, *Mugil cephalus; V.carchariae* UQM 3010, (Stn 6660), from whiting, *S.ciliata* interstitial fluids; *V.gazogenes* UQM 2840, (Stn 180), from an estuarine mud-beach; *V.vulnificus,* a symbiont from the ectoparasitic isopod *Pseudolana dactylosa,* UQM 3032, (Stn 1620), and *V.zobellii* UQM 3029, (Stn 770), a symbiont from free living, planktonic, calanoid copepods.

3.10.3.1.2.1 Inoculum Preparation.

Bacterial strains were removed from storage to active culture as in (3.3.4.1), and inoculated into 30 ml SEBHIB in centrifuge tubes and incubated 15 h to late log phase or early stationary phase, (See Appendix 1.). Bacteria were harvested at 1 500 g (International B 20), 10 minutes. The cell pellet was washed as above two times in 5 ml MPBS (See Appendix 3.) and made to 5 ml. Cells were prepared in a series of $\log_{10}$ dilutions and counted as in (Appendix 3).

3.10.3.1.2.2 Inoculation Procedure.

The fish were held firmly between 2 layers of plastic food wrap while 0.1ml of inoculum was administered ip., with the needle facing posteriorly, using 1ml syringes with 27 gauge needles *(Terumo)*. To evaluate the stability of determinations based upon 5 rather than 10 subjects per dose level

V.anguillarum, UQM 2771 was assayed in duplicate batches of five fish at doses from six decimal dilutions. The fish from each treatment were placed in separate aerated jars as above, and covered with food wrap.

3.10.3.1.2.3 Determination of Median Lethal Dose (LD_{50}).

LD_{50} was determined for the following cases:

3.10.3.1.2.3.1 Evaluation of Chemical Enhancement to Virulence.

The induction of virulence by an iron chelator (See 2.3.1) EDDA was assayed by incubating *V.anguillarum* UQM 2771 in SEBHIB supplemented to contain 1 µM/ml deferrated EDDA (3.4.2.3) and inoculated as above.

3.10.3.1.2.3.2 Evaluation of Provincial Isolates.

The provincial isolates *V.anguillarum* UQM 2843, *V.carchariae* UQM 3010, *V.gazogenes* UQM 2840, *V.vulnificus* UQM 3032, and *V.zobellii* UQM 3029 with origins as specified in 3.10.3.1.2.

3.10.3.1.2.4 Post-inoculation Observation and Handling.

Fish were examined twice daily for 24 h after inoculation. Fish still alive were humanely sacrificed. In some cases autopsies were performed on experimentally infected fish and bacterial isolates were taken. Cultures so derived were checked for purity and phenetically compared with the original culture.

3.10.3.1.2.5 Analysis Procedure.

Probit analysis *(SAS[r] 5.08)* was used to determine the Log_{10} LD_{50} of bacteria towards fish with 95 % confidence limits (A11.6.6). Where, because of insufficient fish, only two points were available for estimation (e.g. 0 and 80 % mortality), and results not analyzable through *SAS,* LD_{50} was estimated without calculation of confidence limits, according to Reed and Muench (1938). Results from determinations are in Table 3.30.

3.10.3.2 Results.

In no case where the fish were injected ip. by bacteria for LD_{50} assays were RSD symptoms expressed. LD_{50} determination from pooled analysis of

V.anguillarum UQM 2771 was within 5 % of the single calculation (Table 3.30). *V.anguillarum* UQM 2771 grown in the presence of 1 μM EDDA had a lower LD_{50} than untreated strains UQM 2771 and UQM 2843; but enhancement to virulence was minimal (statistically beyond the resolution of the sampling procedure i.e. the 95 % confidence intervals were overlapping).

Species.	Category.	Log LD_{50})	95 % Confidence Interval.
V.anguillarum UQM 2771.	Type Culture.	5.8	2.9 - 8.2
V.anguillarum UQM 2771.	Progressive Assay.	6.0	ND
V.anguillarum UQM 2771.	1 μM EDDA.	5.1	> 3.8 - < 5.8
V.anguillarum UQM 2843.	Fish Isolate.	7.7	> 5.0 - < 8.0
V.carchariae UQM 3010.	Fish Isolate.	9.7	ND
V.gazogenes UQM 2840.	Sediment Isolate.	8.3	ND
V.vulnificus UQM 3032.	Isopod Symbiont.	8.4	ND
V.zobellii UQM 3029.	Copepod Symbiont.	8.7	7.7 - 9.6

Table 3.30: Results of Median Lethal Dose (Log_{10}, LD_{50}) Determinations for *Vibrio* species Towards Juvenile Mullet, *Liza argentea*.

Statistically significant differences (no overlap of 95 % confidence intervals), indicated that the copepod symbiont *V.zobellii* was less virulent towards fish than *V.anguillarum* UQM 2771 treated by EDDA, but not less virulent than untreated cells of UQM 2771 or UQM 2843. The calculated LD_{50} (Reed and Muench, 1937) for *V.gazogenes* UQM 2840, isolated from the sediments and *V.vulnificus* UQM 3032 from copepods were less than those *V.carchariae* UQM 3010.

3.10.3.3 Discussion.

The absence of RSD symptoms amongst experimentally inoculated fish seems to indicate that rather than for RSD to be initiated from within as earlier postulated (3.10.2.3) short term ip. assays seemed to indicate mortality or recovery but not chronic infection.

Log_{10} LD_{50} determinations indicated a lower virulence of *Vibrio* species

towards *L.argentea* than previously reported for other fish species (See 2.3.2). Levin *et al*, (1972) found the $\log_{10}$ LD_{50} of *V.anguillarum* towards flounder, *P.americanus*, was 3.7 cf. 6.0 here. Similarly from Grimes *et al.*, (1984) the $\log_{10}$ LD_{50} of *V.carchariae* towards sharks was 6.6 cf. 9.7 for *L.argentea*. When Muroga's (1975) LD_{50} doses of *V.anguillarum* towards eels, *A.japonica*, are converted to Log_{10} for comparison; (8.9 mg cells/ 100 g fish is equivalent to the Log_{10} LD_{50} of (8.9/50 8 X 10^8 is 8.2 cells/ 2 g fish) they were similar to those found here for *V.anguillarum* towards *L.argentea* (Log_{10} LD_{50} 5.8-7.7). Likewise for eels Tison *et al.*, (1982) found Log_{10} LD_{50} of *V.vulnificus* Biogroup II towards *A.japonica*, 8.4 cf. 8.6-8 for *V.vulnificus* UQM 3032 here.

Rasmussen's (1987) (2.3.1) suggestion that capsular (K) antigens of *V.anguillarum* may contribute to its virulence seems initially to be supported by this work, in that washing steps introduced to ensure the absence of soluble toxins (LPS) from broths may also have washed away (K) antigens from *V.anguillarum* and *V.carchariae*. However capsule removal, if it occurred would also have occurred when cells were suspended in normal saline by Watkins *et al.*, (1981), who used three washes, and Grimes *et al.*, (1984) who used one wash. The use of a washing procedure where eel subjects were used was not stated by Muroga (1975) or Tison *et al.*, (1982). Where cell washing was reported for assays upon different fish species, strains of *V.anguillarum* appeared as *more virulent* than found here. This infers that differences might be related to different resistances of different fish species rather than from inoculum preparation method.

Wild fish assayed here may also have received prior sensitizing doses to *Vibrio* immunogen e.g. C2, (2.7.2.5) and could consequently have been resistant to doses lethal to naive fish. Or possibly fish assayed were genetically resistant, e.g. by transferrin genotypes as a result of exposure of progenitors to previous epizootics of RS disease, (See 2.3.2). This last possibility is endorsed by the early historical records of zoonoses in eels (2.1) and an absence of such records for, Flounder, as used by Levin *et al.*,

1972, and Watkins *et al.* 1981; and sharks, used by Grimes *et al.*, 1984.

Consequently it would seem that *L.argentea* rather than suddenly being exposed to a new disease has also adapted to periodic occurrences of RSD such as by adaptation of transferrin genotype as reported (2.3.2) for rainbow trout; i.e. zoonoses of RS disease are likely a normal feature of the ecology of this and other presently affected estuarine species and these too have probably developed resistance to infection.

V.carchariae UQM 3010 was an external isolate from a diseased whiting, *S.ciliata,* but did not appear here to have been very pathogenic towards healthy *L.argentea.* However if as reported by Watkins *et al.* (1981) stress reduced LD_{50} by 3 logs this species is potentially as virulent as *V.anguillarum* to healthy fish i.e. the initial infection to whiting may have been stress related such as from acid pH (from Fish 16-18, Table 3.6). Another, possible explanation for low virulence relates to associative behavioral qualities of bacteria which might serendipitously also induce them to act as *smart* pathogens.

The LD_{50}'s of the species *V.zobellii* and *V.gazogenes* were less than or similar to those of the known pathogens *V.vulnificus* and *V.carchariae.* It would seem axiomatic then that these species should also be regarded as pathogenic. These species are both easily cultivated and biochemically distinct and as such the absence of published reports in literature of these species as pathogens may indicate that they do not have micro-ecologies which overlap sufficiently with fish for infections to result. The corollary to this premise is that *V.vulnificus* and *V.carchariae* may be fish pathogenic species not because of any intrinsically superior currently recognized and quantifiable virulence mechanisms but because of associations with other multicellular species which in turn predispose fish to the possibility of infection.

3.10.3.4 Conclusion.

Acute rather than chronic symptoms were generated as a result of ip. LD_{50} injections of *V.anguillarum* UQM 2771, and other RSD associated bacteria and fish either lived or died. This suggested that RSD has dermal rather than mesenteric origins as postulated earlier (3.10.2.3).

The LD_{50}'s of *Vibrio* species towards the mullet *Liza argentea* were similar to those encountered for eels with a longer recorded history of bacterial epizootics. These results imply that mullet may too have a similar capacity to develop tolerance or have a *natural resistance* to fish pathogenic *Vibrio* species. Whether this resistance was as a result of recent or historical selection is unknown but indicates the possibility for control of future epizootics through selective breeding.

Other experiments indicated that $\log_{10} LD_{50}$ of *Vibrio* species towards *L.argentea* was up to 4.9/g and that strains of *environmental* species assayed here were more virulent than strains from a diseased fish and of a known shark pathogen. This feature may be attributable in part to stress but otherwise seems related to the interactive micro-ecologies of the fish, and environmental species not overlapping sufficiently for infections normally to result.

For laboratory induction of chronic RSD to test if similar symptoms can arise by lowering salinity as proposed in 3.10.2.4 single fish scales might be removed and test fish scraped by marine *Vibrio* inoculum paste and allowed a short period for pathogenesis. A declining osmolarity gradient could then be slowly imposed in the test jar by a peristaltic pump adding unsterile river water.

These assays were useful, reasonably reliable and provide direction for future work; but suffered from the lack of suitable hatchery bred fish i.e. from difficulties in obtaining sufficient numbers of juvenile wild fish and for the unproven assumptions of their naivete to previous immunogen exposure and genetic homogeneity. For confident conclusions about virulence, the

effects of stress, and extrapolation to field ecology, assays must be done using sufficient numbers of such reared fish, preferably *L.argentea* or another species prone to RSD, of known age, history and genetic stock.

3.10.4 Experimental Evaluation of Associations Between *Vibrio* spp. and Larval Parasitic Copepods.

3.10.4.1.0 Experimental Design.

Because the median lethal doses of *Vibrio* spp. from (3.10.3) were higher than could normally be expected to attach to fish by random strikes of single cells, further investigation of the interactive micro-ecologies of *Vibrio* spp. with possible vector crustacean parasites was made.

Planktonic naupliar larvae of parasitic copepods were chosen as experimental subjects; these after several moults attach to and penetrate fish and grow to adults. Presumably these larvae are as prone to colonization by bacteria as free-living copepods. The possible shedding of ectocommensal *Vibrio* species with each moult stage is possibly addressed by recolonization from a residual flora associated with the gut and mouth-parts or faecal pellets of calanoid species as reported earlier (2.4.4.8). If a similar affinity was found between these larval copepods and fish pathogenic *Vibrio* spp. as was found between *V.cholerae* and *V.parahaemolyticus* and planktonic copepods (2.4.4.5) then the targeting and delivery of high, infective, doses to fish may be explained in terms of vectoring. The bacteria selected for comparative evaluation included *E.coli* and *Vibrio* sp. from fish and copepods. These bacterial species had differing flagellation and perhaps also different modes of adherence as shown for *V.cholerae* and *V.parahaemolyticus* by Belas and Colwell, (1982).

To ensure copepod larvae were not subjected to osmotic stress all assays were conducted in filter sterilized seawater. To ensure label uptake was not as a result of copepod feeding, colonization by bacteria was tested *in vitro* by combining bacteria with naupliar larvae for a period, and then applying methyl-^{3}H-thymidine and allowing a further period for the label to be absorbed. Non-adhering labeled bacteria were washed away through a coarse membrane to leave copepod larvae with attached labeled bacteria, which were counted for residual radioactivity. This labeling method is suitable because methyl-^{3}H-thymidine is a compound which is rapidly absorbed from nutrient

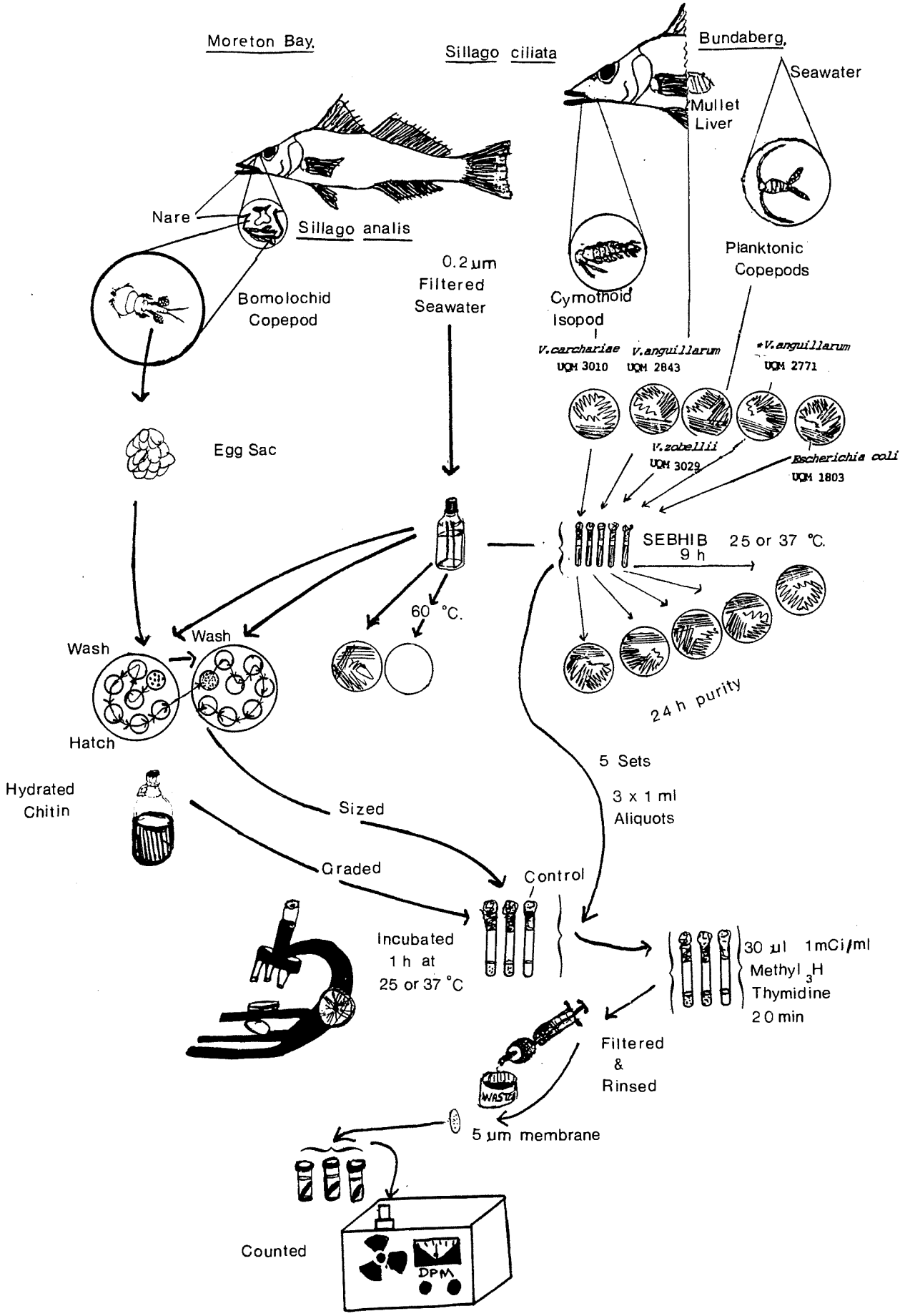
solution and expressed uniformly in the DNA of growing cells (Pollard and Moriaty, 1984). Such label absorbed by separate reproducing pools of attached and unassociated cells would be distributed uniformly and diluted amongst progeny, but still contained within cellular pools. Comparison between species with different generation times can be undertaken by examination of relative effects of different treatments. The experimental procedure is summarized by Figure 3.13.

3.10.4.1.1 Bacteria.

The fish pathogenic species *V.anguillarum*, with only polar flagellation and *V.carchariae* with variable flagellation, (See 2.7.2.5.5) and the polar-flagellate calanoid copepod symbiont, *V.zobellii*, and the peritrichous flagellated human enteric symbiont *E.coli* were used. Cultures of *E.coli* UQM 1803, *V.anguillarum* UQM 2771, *V.anguillarum* UQM 2843, *V.carchariae* UQM 3010 and *V.zobellii* UQM 3029 were removed from storage and pure cultures demonstrated. These were inoculated to 7 ml Brain Heart Infusion Broth (Gibco-*M06800)* prepared with 0.2 μm filter sterilized seawater and incubated at 37°C., *E.coli* and 25°C. for *Vibrio* spp. for 9 h i.e. to log phase (Appendix 1.). Cultures were streaked onto plates of SENA medium for subsequent examination of culture purity. One ml of each culture was placed in each of three polystyrene 13 X 70 mm culture tubes (Kimble).

3.10.4.1.2 Preparation of Copepod and Chitin Material.

Eight egg sacs from bomolochid type poecilostome copepods were identified and extracted from sand whiting *Sillago inaba* by A. West. These were placed in petri dishes containing 0.2 μm filter sterilized seawater. With fresh sterile pasteur pipettes these were washed through eight successive drops of 0.2 μm filtered sea water and allowed to incubate overnight. Naupliar larvae from the four sacs which had hatched by the next morning were used for subsequent experimentation. Nauplii were washed as above in filtered seawater and their size was estimated by microscopy, i.e. (50 μm). Hydrated chitin, (3.4.4.20), was examined under a dissecting microscope (Olympus) and fragments in the range 30-60 μm were aseptically aspirated.


Moreton Bay.
Sillago ciliata
Bundaberg.
Seawater
Mullet
Liver
Nare
Sillago analis
Planktonic
Copepods
0.2 µm
Filtered
Seawater
Bomolochid
Copepod
Cymothoid
Isopod
V.carchariae
UQM 3010
V.anguillarum
UQM 2843
*V.anguillarum
UQM 2771
V.zobellii
UQM 3029
Escherichia coli
UQM 1803
Egg Sac
SEBHIB 25 or 37 °C.
9 h
60 °C.
Wash
Wash
Hatch
24 h purity
5 Sets
3 x 1 ml
Aliquots
Hydrated
Chitin
Sized
Graded
Control
Incubated
1 h at
25 or 37 °C
30 µl 1mCi/ml
Methyl 3H
Thymidine
20 min
Filtered
&
Rinsed
WASTE
5 µm membrane
Counted
DPM

third tubes for UQM 1803, UQM 2771 and UQM 3010 received 20 copepod nauplii and 20 chitin fragments respectively. To make some assessment of relative site saturation, tubes for UQM 2843 received only 8 nauplii and fragments. All tubes were incubated for 1 h at 25°C.

3.10.4.1.4 Labeling of Bacteria.

To each tube 30 µl tritiated thymidine (**Amersham** *TRA.310)* was added from an aqueous 1 mCi/ml stock solution and cultures were incubated for a further 20 min at 25°C. The contents from each tube were poured to separate 2 ml syringes (**Terumo**) and filtered through 5 µm membrane filters (**Millipore** *SMWP 01300)* mounted in filter cartridges (**Millipore** *Swinnex-13).* The membranes were washed slowly over *ca* 20 s with 1.5 ml 0.2 µm filtered seawater. The membrane from the preparation of *V.carchariae* with chitin was improperly sealed and was filtered and washed again over a fresh membrane and both membranes were pooled into one sample.

3.10.4.1.5 Immersion of Membranes and Scintillation Counting.

Washed membranes were transferred from cartridges to scintillation vials (**Canberra Packard** *6000186)* containing 4 ml scintillant (**Canberra Packard** *Hionic-Fluor).* Additionally a scintillation blank containing only a membrane and scintillant was prepared. The tubes were examined in duplicate for scintillation counts in an (**LKB** *1219 Rack Beta)* within 3 h. Counts were transformed to decompositions per minute (DPM) according to a tritium quench curve developed by T.Harriot-Smith and these then adjusted for the blank. Results from determinations are in Table 3.31.

Figure 3.13: Methyl-^{3}H-Thymidine Labeling and Bacterial Association with Larval Copepods.

3.10.4.2 Results and Discussion.

Purity plates were checked after 24 h, and it was found that all cultures except UQM 1803 and UQM 3010 showed small numbers of contaminants, as a result of the presence in filtered seawater of bacteria with a cell diameter of less than 0.2 μm; (evaluated by refiltering seawater sample and observing growth after incubation with sterile BHIB base. These cells were capable of growth in full strength nutrient broth unlike those reported by MacDonell and Hood (2.4.2). Filtered (0.2 μm) seawater treated at 60°C. for 1 h did not show growth after incubation with sterile BHIB base). Copepod larvae although washed were not sterile, and upon consideration of prior difficulties in obtaining gravid copepod material at the right stage of egg development results were further evaluated.

Because seawater used to prepare media was contaminated it was reasoned from purity plates, that an absence of contamination was probably correct for *E.coli* 1803, (incubated at 37°C.), because of the contaminants susceptibility to heat, but less likely for *V.carchariae,* where confluent growth made interpretation of plates more difficult.

Comparative evaluation between species was on the basis that the contaminant was initially uniformly present amongst all preparations except *E.coli* and by inference also present in this preparation after seawater washes.

Control counts, Table 3.31, ranged between *ca* 30 000 and 70 000 decompositions per minute (DPM) and test counts up to *ca* 110 000 DPM. In all sets except, *V.zobellii* UQM 3029 and *V.carchariae* UQM 3010, counts were less in control than test (chitin and nauplii) tubes. The DPM for *V.zobellii* in the presence of nauplii was only 4 % lower than the control i.e. sufficiently similar to indicate that no associations were formed between *V.zobellii,* and the nauplii of this species of parasitic copepod. The DPM for *V.carchariae* UQM 3010 exposed to nauplii was so much lower than the control that the result appeared to be the result of some other factor. Two possibilities are apparent, all available sites on nauplii may already have

been occupied by volunteer bacteria from washed but unsterile nauplii or the filter membrane may have been damaged. Because the DPM for this sample was low by comparison with all counts of from other batches of treated nauplii it would seem most likely this sample was filtered through a damaged membrane.

For *E.coli* and *V.zobellii* there were more DPMS for samples exposed to chitin than copepod nauplii. Associations with chitin fragments by *V.carchariae* and *V.anguillarum* UQM 2843 showed similar relative increases in the number of DPM but for reasons mentioned above no valid comparison was possible for comparative associations of these species with naupliar larvae. It would seem that because of the proximity of proportional increases of DPM for *V.anguillarum* UQM 2843 and *V.carchariae* that production of lateral flagella by this species either did not occur, or was not a significant factor in determining associations. This interpretation is further supported by the lesser number of chitin fragments to which *V.anguillarum* was exposed compared to *V.carchariae.* The possibility that overwhelming numbers of bacteria were present in both cases is at least partly addressed by this difference of in substrate quantity i.e. *V.carchariae* had the potential for at least *ca* 110 000 DPM but a maximum of only 85 000 was recorded.

Species.	*E.coli*	*V.anguillarum*		*V.zobellii*	*V.carchariae*
Accession.	UQM 1803	UQM 2771	UQM 2843	UQM 3029	UQM 3010
Count.(% Increase.)	DPM (%)	DPM (%)	DPM (%)	DPM (%)	DPM (%)
Control.	46 359 (0)	30 945 (0)	33 951 (0)	71 073 (0)	45 143 (0)
Chitin.	55 756 (20)	55 729 (80)	64 763 (90)	77 109 (8)	85 223 (89)
Nauplii.	51 522 (11)	59 109 (90)	112 978(332)	68 443 (-4)	25 852 ND

Table 3.31: Blank Adjusted Decompositions per minute (DPM) and (Percentage Increase) Relative to Control for Bacteria Exposed to Chitin and Copepod Naupliar Larvae and then Labeled with Methyl-^{3} H-Thymidine.

Both strains of *V.anguillarum* had proportionally higher associations with copepod nauplii than with chitin particles. The provincial isolate UQM 2843

in spite of there being fewer particles for adherence, showed in both chitin and copepod nauplii proportionally greater associations than UQM 2771.

3.10.4.3 Conclusions.

3.10.4.3.1 Review of Methods.

Association experiments were undertaken in the expectation that 0.2 μm filterable bacteria were dormant forms induced in stored seawater or confined to oligotrophic pelagic rather than more organically rich coastal seawaters. However almost concurrently, Hood *et al.,* (1986) reported small starved bacteria were present in fresh coastal seawater. In future to ensure exclusion of such cells and sterility of 0.2 μm filtered seawater, it is also necessary to use supplementary procedures e.g. heat treatment at 60°C. 1 h.

Apart from further problems associated with damaged 5 μm membranes the methodology seemed appropriate for these association experiments. However the small number of eggs available from each gravid copepod makes procedure difficult. Bopyrid isopods such as are found on Queensland penaeid prawns (Nearhos and Lester, 1984) contain many thousands of synchronously developed eggs or larvae and so as might provide a more suitable vehicle for such association experiments.

Contaminants were assumed to have been uniformly present in all bacterial cultures except *E.coli*, and to also have been present in seawater used to wash copepod nauplii. As such these contaminants would have been a constant component of background noise consequently allowing comparative evaluation of associations formed by *Vibrio* spp.

3.10.4.3.2 Review of Lateral Flagella Function.

The results indicated that more cells of polar flagellated bacteria were able to colonize surfaces than those with lateral flagella. This is indirectly consistent with classification of species in planktonic copepod flora (Thompson *et al.,* 1976). The possibility that this conclusion was the inevitable result of a surplus of bacteria was addressed by the occurrence

of proportionately similar increases in DPM after exposure to fewer chitin particles shown by *V.anguillarum* UQM 2843 cf. *V.carchariae* UQM 3010.

Relative adherence success by polar and lateral flagellation is best determined by dual labeled competitive assays such as by Belas and Colwell (1982).

3.10.4.3.2 Review of Association of Bacteria to Copepod Nauplii.

Both strains of *V.anguillarum* tested showed a higher affinity towards parasitic copepod nauplii than towards chitin particles alone. This was contrary to findings for *E.coli* and *V.zobellii* perhaps indicating for *V.zobellii* that specific associations only occur with calanoid species or one of the calanoid species listed (3.7.2.3.3). Because of the differences in proportions of cells attached to copepod nauplii it would seem that the association is by bacteria with copepods rather than of copepods collecting bacteria e.g. in feeding mucus (2.4.4.4).

3.10.4.3.3 Ecological Significance.

The results indicate an association might exist between parasitic copepods and fish pathogens such as *V.anguillarum,* and consequently further indicates the feasibility for commensal pathogenic *Vibrio* species to perhaps opportunistically use copepods as biological vectors to seek out fish species and cause the primary lesions involved in subsequent bacterial pathogenesis. The difference in degree of association between strains of *V.anguillarum* may be as a result of culture attenuation from storage of *V.anguillarum* UQM 2771 or perhaps as a result of sub-specific compatibility between strains and parasites. The failure of *V.zobellii* to associate with parasitic copepod nauplii is consistent with this later premise and supports the possibility that the associations formed by *Vibrio* species may be specific rather than opportunistic.

3.10.5 Review of Ecological Analyses.

The previously reported principal pathogen of RSD (Burke and Rodgers, 1981), *V.anguillarum,* was exclusively isolated from fully developed rather than early RSD lesions. Amongst internal and external samples of flora from control and diseased river system fish, seven taxa, *V.alginolyticus, V.fluvialis/-V.furnissii, V.pelagius S.putrefaciens, V.diazotrophicus, Photobacterium*-like, and *A.hydrophila* were shared; and 15 taxa, *V.carchariae/-V.harveyi, V.parahaemolyticus, V.aestuarianus I & II, V.orientalis, V.fluvialis/-V.furnissii*-like, *V.vulnificus, V.carchariae/-V.harveyi/-V.parahaemolyticus, V.natriegens*-like, *V.anguillarum, V.diazotrophicus*-like, *V.natriegens, V.ordalii, V.splendidus, V.anguillarum*-like and *Serratia*-like were only found on diseased fish. It was concluded that different RSD epizootics resulted from not necessarily the same pathogen or pathogenic gestalt.

Diseased marine fish were concluded to have suffered from an acute infection apparently caused by *V.carchariae,* and *V.carchariae/-V.harveyi* and that this was probably acid stress related. The same pathogenic *Vibrionaceae* taxa were non-exclusively present amongst river system fish with chronic RSD. The differences in pathogenesis of these two conditions were attributed potentially to behavioral qualities of RSD affected fish which by migration in the rivers osmolarity or salinity gradients might disrupt pathogenesis of halophilic marine bacteria and allow displacing colonization of dead tissues by non-invasive, (affected fish were in good condition), saprophytes such as *S.putrefaciens* and *A.hydrophila* and other species found on control fish.

Pathogenesis experimentation upon juvenile *Liza argentea* indicated both a high resistance to previously reported fish pathogenic *Vibrionaceae* such as *V.anguillarum, V.vulnificus,* and *V.carchariae* which was suggested as indicating a complimentary co-evolutionary history between fish and their bacterial pathogens. Because the LD_{50}'s of the environmental species *V.zobellii* and *V.gazogenes* were less than or similar to those of the known pathogens *V.vulnificus* and *V.carchariae* it was suggested that these

environmental species are not pathogenic because their micro-ecologies do not overlap sufficiently with fish for infections to occur.

These species *[V.vulnificus* and *V.carchariae]* and other taxa including *V.aestuarianus* I and II, from diseased fish were also recovered from their transitory and/or sedentary isopod parasites and so indicating their ecological potential to act as mechanical vectors of low virulence pathogens. Insufficient repetitive evidence of floral compositions of most biota prevented determination of distinct micro-ecological associations between different groups except between *V.zobellii* and single probably calanoid copepods.

It appeared from labeled bacterial association experimentation, (3.10.4) that failure of large numbers of bacteria to associate more with copepods than with hydrated chitin fragments, and that perhaps some species might form specific associations with copepod hosts. This is obviously both speculative and highly significant and before publication needs to be repeated using sterile filtered seawater and both with different species of bacteria and larval crustaceans e.g. from isopods which have a greater fecundity than the inaccessible and difficult bomolochid species used here.

4.0 CONCLUSION.

Various procedures were used to perform numerical analyses upon hybrid datasets (3.8.7.2) and the best procedure was objectively selected and tested by different (multivariate) procedure. This method was used in the phenetic classification of 391 presumptively identified *Vibrionaceae* from diseased and control fish and other aquatic reservoirs isolated under differing enrichment protocols. In some cases DNA of strains was also examined by thermal methods (Appendix 9.).

Thirty-one taxa of *Vibrionaceae* were resolved (3.8.7.1), most corresponding to single species but others were represented by groups of closely related species as reported in the literature by DNA homology or by phenetic (ecologic) similarity. The first substantive Australian records for several *Vibrionaceae* were indicated. It was suggested that two validly described xanthinolytic species *V.orientalis,* and *V.tubiashii* should be synonymized and that the former taxon has taxonomic priority. Some further phenetically distinct groups and biogroups of strains were described, one from planktonic copepods was also distinct by DNA based procedure and provisionally classified as the species *V.zobellii sp. nov.* Another strain which fell outside previous presumptive *Vibrionaceae* criteria and which phenetically resembled *Serratia* spp. but which had mixed flagellation was provisionally classified as a *Serratia*-like *Vibrionaceae.*

Two cross-batch damped probability matrices were developed for the comparatively rapid identification of *Vibrionaceae.* The second and most rigorous of these differed from a previous comprehensive probability matrix (Bryant *et al.,* 1986b) by a substantial number of characters and this was concluded to indicate support for Sneath's (1957) concept of stability of relative interrelationships. Some features from these probability matrices were also present in commercial identification schemes (Appendix 8.) however if reliable identifications were required these schemes were based upon to few tests and had inadequate profile libraries.

Diseased fish from which bacteriological samples were taken had both acute and chronic symptoms. Fish from Moreton Bay, with acute symptoms were found to have one or two *Vibrionaceae* taxa predominant. Those from River systems, had a number of co-dominant taxa present including those from diseased Moreton Bay Fish. It was reasoned (3.10.2.4), that the difference between the acute and chronic symptoms may be related to fish behavior rather than low virulence pathogens i.e. affected fish might move into lower salinities to displace invading halophilic pathogens with non-invasive normal skin flora.

Control fish were found to carry both an external and an internal bacterial flora, these latter bacteria were suggested as mechanically vectored artifacts of invasive mesenteric encystment by trypanorrhynch cestode larvae (3.7). Generally sufficient taxonomic resolution was available to make purposeful conclusions about individual species distributions however time permitted only relatively few samples to be taken from other than fish material. Sedentary and transitory isopod parasites of fish were found to contain flora common to both associated fish and isopods indicating the feasibility of their mechanical vectoring of pathogens (3.10.2.3-4).

The specific occurrence of *V.zobellii* on single calanoid copepods but not from aggregated plankton supports the proposal from 2.4.4.8 about specific associations formed by *Vibrionaceae.* Association studies undertaken with tritium-labeled bacteria (3.10.4.2-3) tentatively seem, by the failure of cells of *V.zobellii* to associate more with bomolochid larvae than with chitin particles, to be consistent with this possibility.

Pathogenicity experimentation (3.10.3) led to the conclusion that RSD is not a result of a low virulence pathogen as reasoned by Burke and Rodgers, (1977-unpublished) but rather an indication that an adapted fish-genotype perhaps of lactoferrin or transferrin, and which confers disease resistance, has come to prevail in these fish. Specific *in situ* disease-management measures were suggested (Appendix 15.) on the basis of this pathogenesis and other (Appendix 5.) experimentation.

Examination for isolation media redundancy indicated (3.9.3) that in future work the same bacterial taxa as found can be recovered using a reduced number of isolation protocols i.e. Simidu, TCBS and ATW24-TCBS, to permit more material to be examined. Rather than to use phenetic classification of isolates in future, it may be faster and as efficient to use a reduced set of isolation protocols and then to monitor the resultant isolates for their base sequences from a small *ca* 50 base sub-unit of the 16S or 23S rRNA molecule. Such analysis would also provide useful information about the *Vibrionaceae* phylogeny particularly with respect to atypical strains such the red-pigmented UQM 3256.

5.0 REFERENCES.

Note: References preceded with an "*" were in Japanese and work was interpreted only from English text i.e. Abstracts, Figures, and Tables.

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Taxonomy and Ecology of *Vibrionaceae* Associated With *Red-Spot* Disease of Queensland Aestuarine Fish.

Steven P. Nearhos

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APPENDIX 1.

A1.0 *Vibrionaceae* Growth Characteristics in Different Nutrient Broths.

A1.1 Experimental Design.

Because in this study both sodium requiring, (SR), and non-sodium requiring isolates were likely to be encountered it was necessary to find a basal growth medium suitable for general use and for phenotypic tests. The medium advocated by Heinis, Beuchat and Jones (1977) as most complimentary to the nutrient requirements of *V.parahaemolyticus* was tryptic soy broth with 3% sodium chloride. This medium with only 2% sodium chloride (SETSB, 3.4.1.07) was compared with non-acid forming, (glucose free), sodium-enriched (2 % NaCl) nutrient broth SENB.

Twenty-five strains of *Vibrionaceae,* from Tables 3.4-5 with UQM accession codes as follow : 211, 2732, 2745, 2768-76, 2778-86, 2838-9, 2843, 2855 were taken from ampoules and inoculated to 2ml SEN broth in bijoux and incubated 36h. For each strain, 50 μl was transferred to each of two sterile borosilicate 13 X 150mm tubes containing 7ml SENB (3.4.1.01), or SETSB, and incubated. At intervals of 1 h the tubes were lightly vortexed (Labline Instruments Inc.) and their absorbance measured at 540 nm (Bausch and Lomb, *Spectronic 20).*

Growth curves, (Figure A1.1, (A) (SENB), (B) (SETSB) were constructed and culture phase periods estimated. Generation times were estimated from the doubling period for absorbances.

A1.2 Results and Discussion.

Except for reference cultures e.g. *V.metschnikovii* UQM 211, *A.hydrophila* UQM 2768, and *V.cholerae* UQM 2773; all the strains grew well in media with 2% salt with lag-phases persisting for up to 5 h. Final absorbances, (most strains were 0.4-0.6), were usually higher in SETSB than SENB probably because of glucose in SETSB. The maximum absorbance found was *ca* 0.7 for *V.harveyi* UQM 2839 in SETSB and the lowest, (*ca* 0.2), was from *A.hydrophila* UQM 2769 in SENB and SETSB and *V.cholerae* UQM 2773 in SENB. Generation times

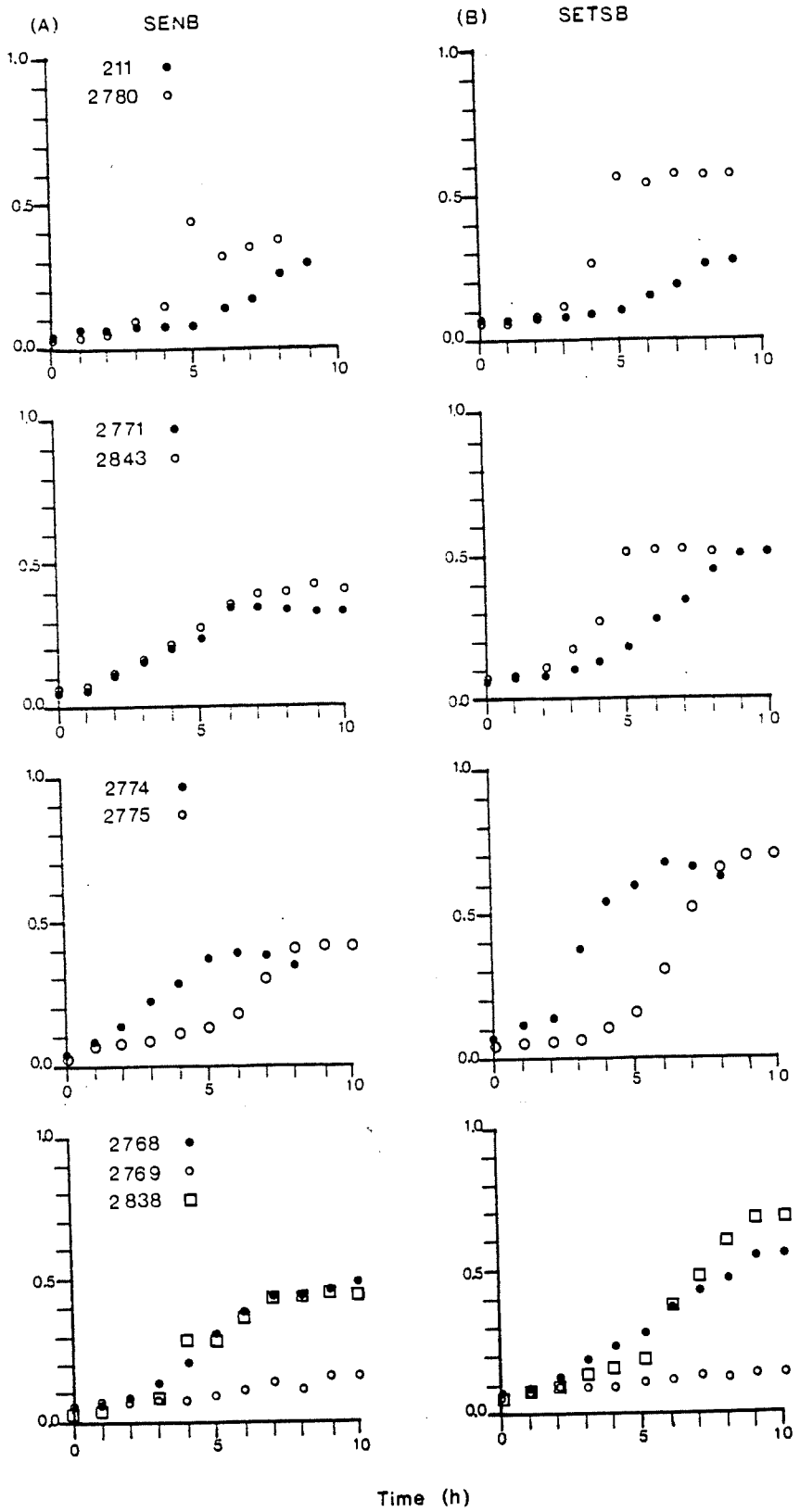


Figure A1.1: Growth Curves For *Vibrionaceae* in; **(A) SENB, (B) SETSB:-** UQM 211 *Vibrio metschnikovii;* UQM 2780 *V.diazotrophicus;* UQM 2771, UQM 2843 *V.anguillarum;* UQM 2774 *V.fluvialis;* UQM 2775 *V.furnissii;* UQM 2768, UQM 2769, UQM 2838 *A.hydrophila.*

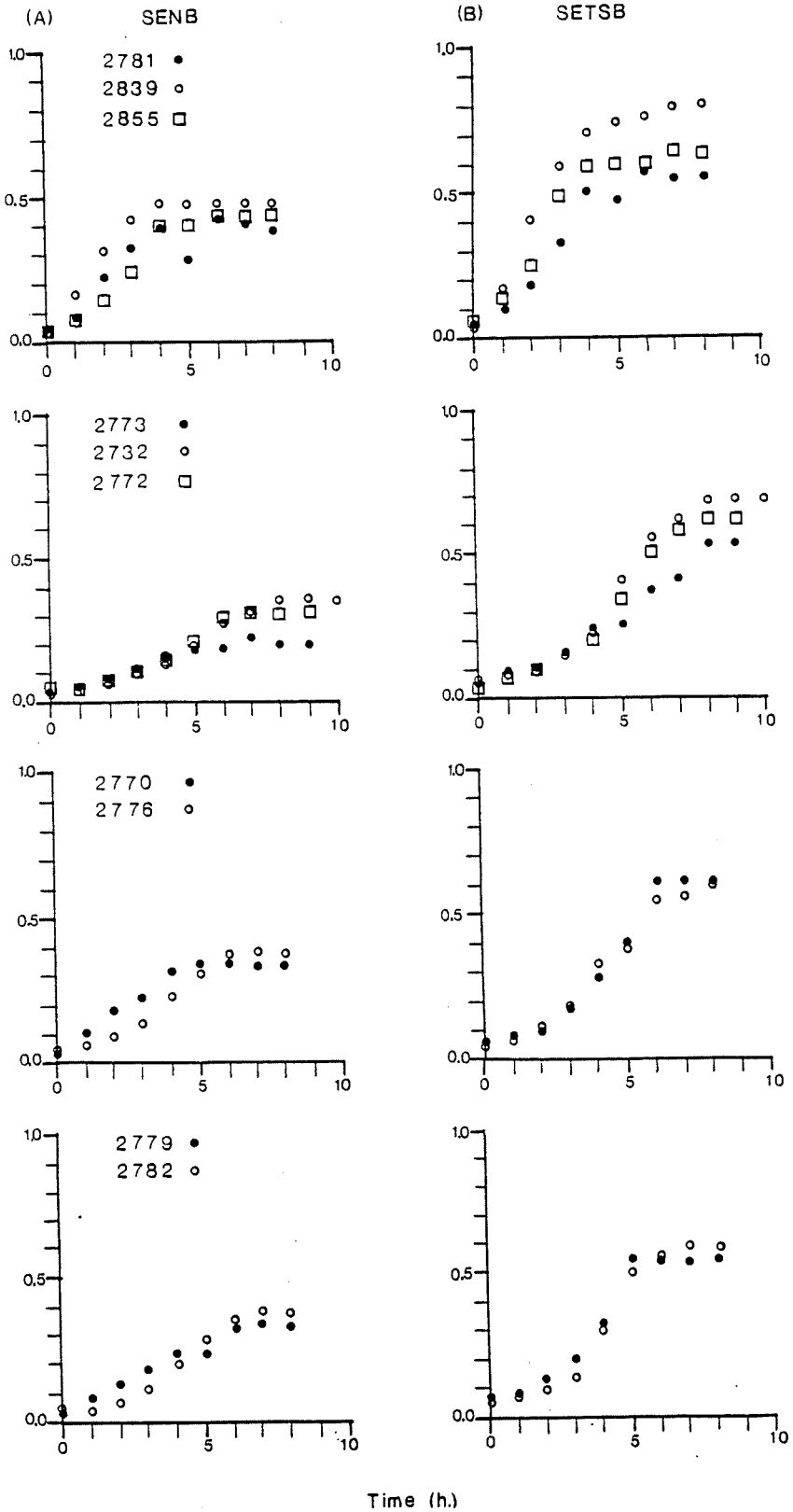


Figure A1.1 (Cont.): Growth Curves For *Vibrionaceae* in; **(A) SENB, (B) SETSB:-** UQM 2781, UQM 2839, UQM 2855, *Vibrio harveyi;* UQM 2773, UQM 2772, UQM 2732 *V.cholerae*; UQM 2770, *V.alginolyticus* UQM 2776, *V.parahaemolyticus,* UQM 2779 *V.campbellii,* UQM 2782 *V.natriegens.*

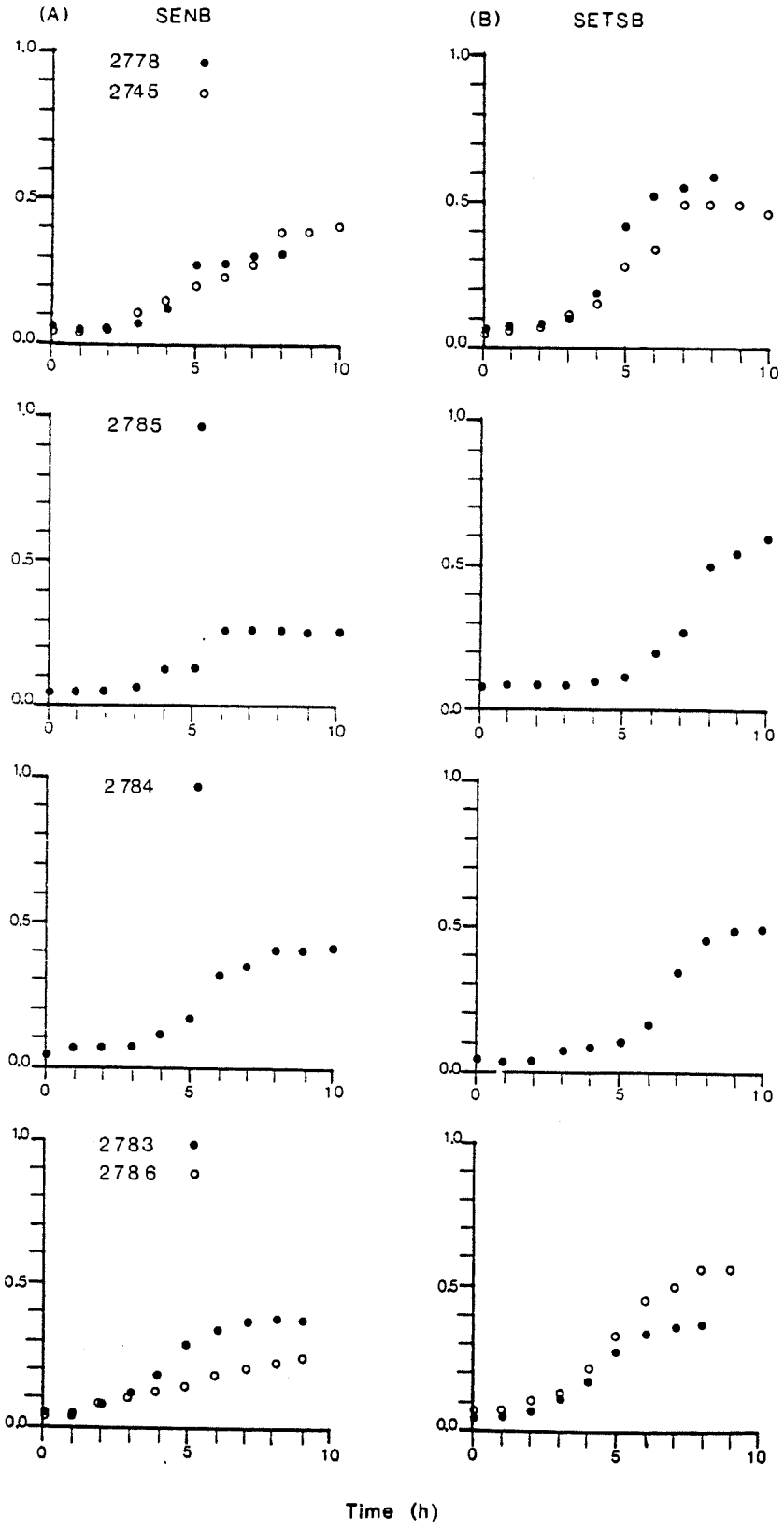


Figure A1.1 (Cont.): Growth Curves For *Vibrionaceae* in; (A) SENB, (B) SETSB:- UQM 2778, UQM 2745 *Vibrio vulnificus;* UQM 2785 *V.pelagius;* UQM 2784, *V.nigripulchritudo;* UQM 2783 *V.nereis;* UQM 2786 *V.splendidus.*

were less than 1 h, (the assay interval), except for the following strains *V.metschnikovii* UQM 211, *V.vulnificus* UQM 2745, *A.hydrophila* UQM 2769 and *V.cholerae* UQM 2773, and *V.splendidus* UQM 2786 which all showed protracted arithmetic growth. Cultures, in both media had similar log phase periods and reached late log or stationary phase after 6 to 10 h.

A1.3 Conclusions.

Media with 2% salt supported both SR and non-SR species and did not entirely suppress non-sodium requiring species. Comparable lag-phase periods were seen in both media, and most strains reached stationary phase in less than 10 h. SENB medium produced less growth than SETSB, but because it lacks fermentable carbohydrate it is a suitable nutrient base for phenotypic tests.

On the basis of these trials *Vibrionaceae* cultures in experimentation were regarded as in stationary phase in the period between 10 and 24 h post-inoculation.

APPENDIX 2.

A2.0 Evaluation of Survival by *Vibrionaceae* on Amies Transport Medium.

A2.1 Experimental Design.

The convenient transport of specimens and mass storage of bacteria are intrinsic requirements of any ecological and taxonomic analysis. In the present work it was required that isolate's specific growth needs (e.g. sodium) were provided. A commercially available transport medium was consequently evaluated for its fulfillment of this criterion over a maximum 7d short term period.

To evaluate the transport medium for retention of long-term viability in a representative distribution of species, (i.e. more common species than rare), the viability of cultures on inoculated swabs was checked repeatedly for almost a year.

The following 27 strains representing 17 species from Tables 3.4-5 with UQM or other accession codes as follow: 211, 2732, 2740, 2743, 2769, 2771-2, 2774-76, 2778-84, 2838-9, 2843, 2849, 2855, and isolates B 55, ϕ 1 and ϕ 29 were incubated for 24 h in SENB and diluted with 2 % saline to a MacFarland turbidity equivalent of one (Finegold and Barron, 1986). Alginate swabs (Medical Wire and Equipment Co.) were immersed into the culture suspensions, patched to a single plate of SENA medium and stored in a vial of Amies transport medium (Medical Wire and Equipment Co.). Duplicate swabs were prepared for *V.cholerae* UQM 2772 and *V.harveyi* UQM 2839. The swabs and the patch-plates were incubated at 25°C. All swabs were used to inoculate subsequent patch plates after 1,2,4,7,14,21,62,127, and 350 days. These plates were examined for growth after 24 and 48 h incubation. Results were expressed graphically as percent recoverable strains (Figure A2.1) and the percent species recovered (Figure A2.2) over 350 d both against a $((\log_{10}(n+1))-1)$ transformed time scale.

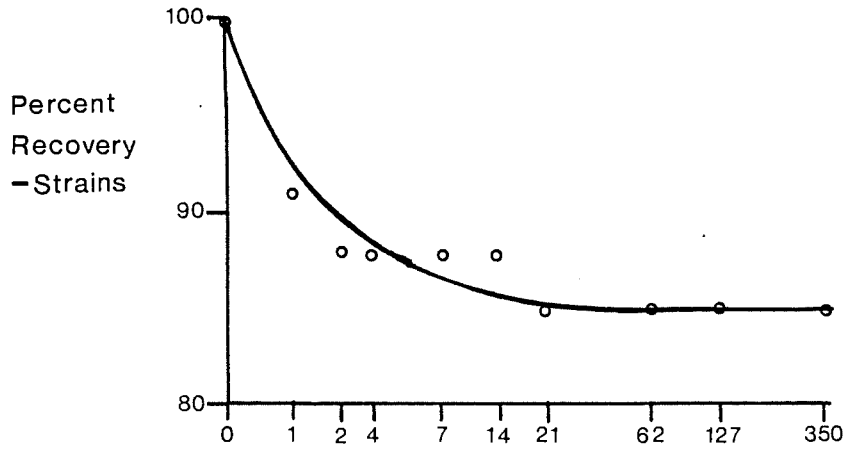


Figure A2.1: Percentage of Strains Recovered after 350 d.

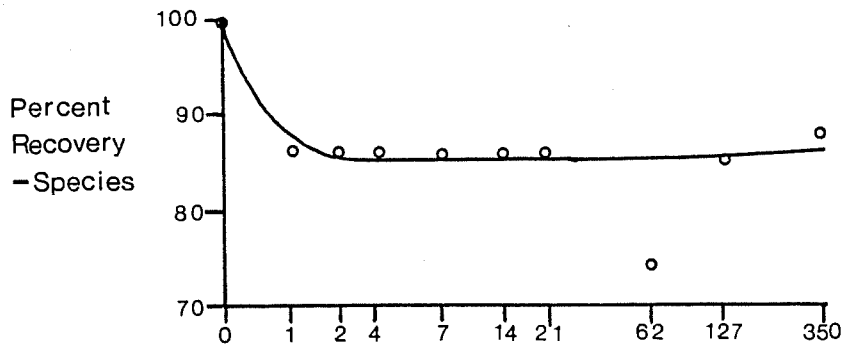


Figure A2.2: Percentage of Species Recovered after 350 d.

A2.2 Results and Discussion.

Duplicate cultures of *V.cholerae* and *V.harveyi* were recovered consistently at every sampling. Variable recoveries for *V.orientalis* B55, *V.campbellii* UQM 2779, *V.natriegens* UQM 2782, *V.nigripulchritudo* UQM 2784 may have been attributable to uneven sampling or survival on the swab surfaces. Figures A2.1-2 show that percentages of recovered strains and species after 7 d and 350 d for both assay procedures were approximately equal at *ca* 85 %..

A2.3 Conclusion.

Commercially prepared tubes of Amies transport medium provide a cost effective and non-selective means for dispatching material from remote sites. This medium is also a convenient method for mass culture storage possibly for periods greater than 1 year.

APPENDIX 3.

A3.0 Evaluation of *Vibrionaceae* Survival in Different Dilution Buffers.

A3.1 Experimental Design.

The use of an inoculation buffer which is both inert to suspended bacteria and the inoculated host is a fundamental requirement for determination of median lethal dose. Beuchat (1977) found that MPBS was the most suitable diluent for the survival of temperature stressed *V.parahaemolyticus*. MacLeod (1985) additionally reported the leaching of magnesium from cell walls of "washed" marine bacteria.

A3.2 Efficacies of Dilution Buffers for Recovery of Sodium-Requiring and non-Requiring *Vibrionaceae*.

A3.2.1 Experimental Design.

Dilution buffers used for maintenance of terrestrial bacteria i.e. normal saline and phosphate buffered saline, were comparatively evaluated with media supplemented to contain 2% saline, and 0.1% magnesium chloride, to evaluate their effectiveness for maintenance of viability of washed cells of *Vibrionaceae* with varying sodium requirements.

The bacteria assayed had average sodium requirements, (as % NaCl), which ranged from less than 0.5 % for *A.hydrophila* and *V.cholerae*, to 1.6 % for *V.gazogenes* (recalculated from Baumann *et al.*, 1980).

Four strains, *Aeromonas hydrophila* UQM 2769; *Vibrio cholerae* UQM 2772; *Vibrio anguillarum* UQM 2771 and *Vibrio gazogenes* UQM 2840 were removed from ampoules and inoculated into 50 ml brain heart infusion broth supplemented to contain 2.0% sodium chloride and incubated for 24h.

Cultures were gently mixed and 10 ml portions and transferred to five 10 ml centrifuge tubes labeled a-e. These were centrifuged, at 1 500 g for 10 min and each suspension washed three times in 10 ml of one of the following sterile diluents:

(a) 0.9% Sodium Chloride.

(b) Phosphate Buffered Saline, (3.4.3.1 in 0.5 % NaCl).

(c) 2.0% Sodium Chloride.

(d) 2.0% Sodium Chloride with 0.1% Magnesium Chloride.

(e) Marine Phosphate Buffered Saline, (3.4.3.1).

Decimal dilutions were prepared in MPBS and after 30 minutes, and 5 h (300 min), 20 µl of mixed suspension was spotted onto duplicate plates of SENA. Counts for both retention times were made after 24 h incubation. These were standardized by first averaging counts for diluents and then determining the relative percentages of the highest counts (assumed best recovery). Standard proportions were analysed by two-way analysis of variance and the mean percentage recoveries and standard deviations for each diluent were calculated. Results are given in Tables A3.1, A3.2 and A3.3.

A3.2.2 Results.

Species recoveries (Table A3.1) showed considerable variation across diluents. Counts for *A.hydrophila*, *V.cholerae*, *V.gazogenes* and *V.anguillarum* respectively ranged over 1,2,3, and 4 logs. Maximum counts were in 2 % saline for *A.hydrophila* and *V.cholerae*, and in MPBS for *V.anguillarum* and *V.gazogenes*. Highest recoveries across species were found with MPBS, this buffer also had the smallest standard deviation across diluents and species.

The two-way analysis of variance, (Table A3.2), did not indicate differences between sampling times but did resolve significant differences between species and dilution buffers. The buffer which produced recoveries closest to the maximum for both SR and non-SR species, and which also had the lowest standard deviation was MPBS (Table A3.3).

Table A3.1: Comparitive Viability of Vibrionaceae Retained in Five Dilution Buffers.

	Normal Saline	Phosphate Buffered Saline	2 % Saline	2 % Saline / 0.1 % Magnesium Chloride	Marine Phosphate Buffered Saline
Time (Minutes)	**30 ,300**	**30 ,300**	**30 , 300**	**30 , 300**	**30 , 300**
A.hydrophila UQM 2769	$6.0x10^5$,$3.6x10^5$	$2.1x10^6$,$1.5x10^6$	$2.5x10^6$,$1.8x10^6$	$2.4x10^6$,$1.8x10^6$	$2.2x10^6$,$1.8x10^6$
(Average %)	(19.2)	(72)	(86)	(84)	(80)
V.cholerae UQM 2772	$1.0x10^8$,$4.4x10^7$	$9.0x10^7$,$1.2x10^8$	$6.2x10^7$,$4.4x10^6$	$7.3x10^6$,$4.7x10^6$	$3.2x10^7$,$2.8x10^7$
(Average %)	(60)	(87.5)	(28)	(5)	(25)
V.anguillarum UQM 2771	$2.2x10^6$,$2.0x10^5$	$1.1x10^7$,$2.0x10^7$	$8.8x10^6$,$6.6x10^6$	$5.2x10^6$,$3.5x10^6$	$2.5x10^8$,$3.0x10^8$
(Average %)	(0.4)	(5.17)	(2.6)	(1.45)	(91.7)
V.gazogenes UQM 2840	$4.0x10^7$,$1.5x10^6$	$2.1x10^6$,$4.2x10^5$	$1.2x10^5$,$3.7x10^5$	$2.3x10^6$,$1.2x10^6$	$1.9x10^7$,$2.4x10^7$
(Average %)	(51.9)	(3.2)	(0.6)	(4.4)	(53.8)

Table A3.2: Results of Two-Way Analysis of Variance.

(1) Difference Between Times: $F_{calc.} = 5.1 < F\ tab._{(0.05)}^{12,4} = 5.91$

(2) Difference Between Species: $F_{calc.} = 4.3^{*} > F\ tab._{(0.05)}^{4,12} = 3.26$

(3) Difference Between Diluents: $F_{calc.} = 11.5^{**} > F\ tab._{(0.01)}^{3,12} = 5.95$

Table A3.3: Mean Percentage of Maximum Counts and Standard Deviation for Five Dilution Buffers.

Dilution Buffer	Mean. %	Standard Deviation. %
(a) Normal Saline	32	39
(b) PBS	41	42
(c) 2 % Saline	29	40
(d) 2 % Saline, 0.1 % Magnesium Chloride	23	38
(e) MPBS	62	28

A3.2.3 Discussion.

The results demonstrated susceptibility of SR bacteria *[V.anguillarum* and *V.gazogenes]* to inappropriate support buffers and the consequent importance of using an appropriate dilution buffer for quantitative assays.

Higher counts of the non-SR species *[A.hydrophila* and *V.cholerae]* in 2 %

saline than in normal saline and PBS may have been attributable to the equal osmolarity of this solution with the original growth media and washing solutions.

There was no evidence that indicated an enhancement to recovery of washed cells buffered in magnesium ions.

A3.3.0 Comparative Toxicity of Inoculation Buffers Towards Fish.

A3.3.1 Experimental Design.

Each of the dilution buffers from A3.2 above was injected ip. in 0.1 ml portions to 5 mullet, *L.argentea*. Five control fish were not injected. All fish were observed over 24 h for mortality or other deterioration of condition. Results were evaluated by the Chi square, (χ^2), test.

A3.3.2 Results.

After 24 h one fish from the normal saline, 2 % Saline, and 2 % saline in 0.1% magnesium chloride treated groups had died. No fish died in the control, PBS or MPBS classes. The data were pooled for the groups containing saline and no pH buffer and the control group and those containing phosphate buffer and the χ^2 calculated. This value, (3.33), was greater than the 10 % tabulated value of χ^2 for 1 degree of freedom (2.71).

A3.3.3 Discussion.

Dilution buffers containing potassium or phosphate seemed to have been marginally less injurious to fish than those containing only saline or magnesium saline. These results may indicate the non-injurious effects of pH control to the operation of cellular sodium pumps of fish.

A3.4 Conclusion.

The above two experiments indicate that MPBS, the most suitable dilution buffer for *Vibrionaceae* is also a suitable inoculation buffer for fish.

APPENDIX 4.

A4.0 Evaluation of Chemical Swarming Inhibitors.

A4.1.0 Experimental Design.

Phenotypic analysis of *Vibrionaceae* using conventional multiple inoculation is frustrated particularly in prolonged incubations by swarming strains. This problem is further compounded by chemical induction of non-swarming mutants by other strains (Ulitzur 1975b). Lee *et al.*, (1978) examined swarming strains by cutting gutters into agar plates and reducing the number of strains examined per plate. This is both labour and media expensive. Initially hot wax rings were set around inoculum loci to contain swarming strains, and consequently media costs; however manual application of 21 such rings per plate for more than 100 plates as would be required in numerical analysis was unfeasible.

Apart from increased agar concentrations and provision of use of media such as CLED various chemical measures have been employed for the prevention of swarming by *Proteus* spp.; boric acid (Sykes and Reed, 1949), sodium tetradecylsulphate, β-phenylethyl alcohol, and *p*-nitrophenylglycerol, (Kopp, Müller and Lemme, 1966), and tannic acid (Smith 1975). De Boer, Golten, and Scheffers (1975) found that greater than 4% saline and surfactants, particularly sodium dodecylsulphate and teepol inhibited swarming by *V.alginolyticus*. Ulitzur (1975b) reported that 0.3% glycerol in minimal medium and filter paper disks impregnated with 5M alkali attached to petri dish lids also prevented swarming in strains of *V.alginolyticus*. This last approach was discarded for the necessity to keep these disks wet. Instead sodium dodecylsulphate (SDS), β-phenylethyl alcohol (PEA), *p*-nitrophenylglycerol (PNPG), glycerol (Gly), structurally similar ethylene glycol (EtGl), and neutral solutions of boric (Bor) and tannic acid (Tan) at concentrations which bracketed the active range of inhibition for *Proteus* were comparatively assessed against reference and type cultures of *V.alginolyticus*, *V.harveyi* and *V.anguillarum* according to their inhibition of growth and sustained prevention of swarming on different media.

A4.2.0 Methods.

A4.2.1 Preparation of Support Media.

One litre portions of salt enriched tryptone broth (SETB) (3.4.1.09) and SENB were prepared, and the pH adjusted to 7.0. Broths were dispensed to 500ml (Schott) bottles designated A and B, (SET broth) and C and D, (SENB). To bottles A and C, 6.6 g, (1.3%) agar (Oxoid L13) was added, and 13.2 g (2.6%) agar was added to bottles B and D. The media were melted in an autoclave at 108°C. 15 min and dispensed in 20 ml portions to series with 22 labeled, 25ml screw capped bottles and sterilized.

A4.2.2 Preparation of Inhibitor Stock Solutions.

A4.2.2.1 Sodium Dodecylsulphate, 0.05 M, (SDS).

Sodium dodecylsulphate, 0.7210 g, (Ajax Chemicals) was dissolved in 50 ml distilled water to produce a 0.05M solution and sterilized by autoclaving.

A4.2.2.2 Phenylethyl Alcohol, 0.2 M, (PEA).

Phenylethyl alcohol, 1.22 g, (BDH) was aseptically added to 50ml sterile distilled water.

A4.2.2.3 α-p-Nitrophenylglycerine, 0.047 M, (PNPG).

α-*p*-Nitrophenylglycerine, 10 mg, (Sigma) was added to 1 ml distilled water in a bijou bottle and sterilized by autoclaving.

A4.2.2.4 Glycerol, 0.5 M, (Gly).

Glycerol, 2.30 g, (Ajax Chemicals) was made up to 50 ml with distilled water, and sterilized by autoclaving.

A4.2.2.5 Ethylene Glycol, 0.5 M, (EtGl).

Ethylene Glycol, 1.55 g, (Ajax Chemicals) was made up to 50 ml with distilled water, placed in a 50ml screw capped bottle, and sterilized by autoclaving.

A4.2.2.6 Tannic Acid, 0.001 M , (MW *ca* 1700), (Tan).

Tannic acid, 0.58 g, (Ajax Chemicals) was dissolved in 45 ml distilled water and the pH adjusted to 7.0 using 5 N sodium hydroxide. The solution was made up to 50 ml with distilled water, and sterilized by autoclaving.

A4.2.2.7 0.25 M Borate, (Bor).

Boric acid, 0.772 g, (Ajax Chemicals) was dissolved in 45 ml distilled water and the pH adjusted as above to 7.0. The solution was made up to 50 ml with distilled water and sterilized by autoclaving.

A4.2.3 Dispensation of Inhibitors.

Three concentrations of each of the seven inhibitor stock solutions were prepared by dilution into the four media. Final concentrations in Table A4.1 were obtained by introducing with sterile graduated 1 ml pipettes, aliquots of inhibitor and sterile distilled water to make medium volumes of 22 ml. A control to which only 2 ml of sterile distilled water was added was prepared for each medium.

Table A4.1: Stock and Test Concentrations of Swarming Inhibitors.

Inhibitor	[Stock]	[Conc. 1]	[Conc. 2]	[Conc. 3]
SDS	0.05M	2.5 mM	3.5 mM	5 mM
PEA	0.2M	5 mM	10 mM	20 mM
PNPG	0.047M	2.5 μM	5 μM	10 μM
Gly	0.5M	20 mM	30 mM	50 mM
EtGl	0.5M	20 mM	40 mM	60 mM
Tan	1.0mM	20 μM	60 μM	100 μM
Bor	0.25M	5 mM	15 mM	25 mM

Media were mixed, poured into labeled plates and dried 48h.

A4.2.4 Culture Preparation and Inoculation.

Swarming type and reference strains of *Vibrio alginolyticus* respectively UQM 2770, and UQM 2722; swarming, (Σ 182, Σ 200), and non-swarming (UQM 2723) reference strains of *V.harveyi,* and the non-swarming type strains of *V.anguillarum* UQM 2771, (Tables 3.4-5) were taken from storage inoculated onto SENA and incubated 24 h. Growth was harvested and suspended in 3ml of sterile 2% saline, to a turbidity equivalent of 3 on the MacFarland scale. One ml of suspension was aseptically pipetted to each of six sterile 10 mm cupules arranged for equidistant separation on a brass multiple inoculation template. Six fixed 1 mm diameter stainless steel inoculation pins, were arranged on a brass template in a pattern complimentary to the one described

above and mounted the the modified drill press from (3.8.2.1). Lids were removed after petri dishes were located under the inoculation head, then replaced when the inoculum was deposited. Control plates were inoculated first, and inhibitor plates were inoculated at random but with the lowest concentrations of inhibitors inoculated first to reduce possible carry-over problems. Inoculated plates were incubated in sealed plastic bags. The experiment was conducted over 7 days and after which certain characteristic plates were stored for 2 d at 4°C. until they could be photographed.

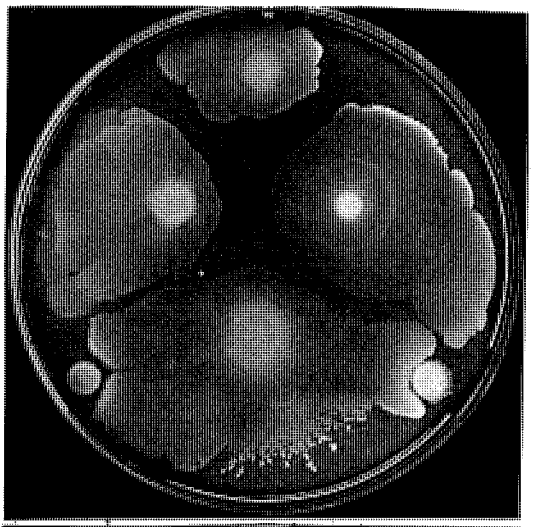
A4.2.5 Scoring and Analysis of Results.

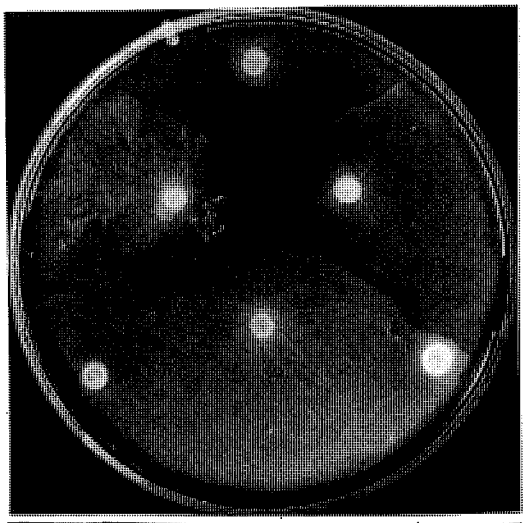
Colony diameters, (mm), were measured on each plate after 2, 4, and 7 days. When growth was confluent and scoring not possible all strains except *V.anguillarum* UQM 2771 and *V.harveyi* UQM 2723 were assumed to have been induced to swarm and assigned to a nominally unacceptable diameter of 40 mm. The non-swarming strains were assumed not to have grown since the last recording. Colony diameters from all times for swarming and non-swarming strains were pooled and averaged. Totals were made for colony diameters on media with *ca* 1 and 2% agar and on SET and SEN media (Table A4.2). Effects of agar concentration and nutrient composition upon swarming were statistically tested by paired 1 tailed *t*- tests. Results were also evaluated for side-effects to colony size or appearence, and and for inhibitor utilization.

A4.3 Results.

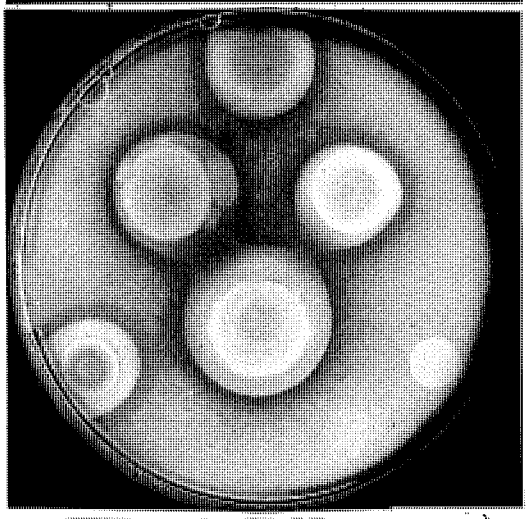
On control plates incubated 48 h all strains grew except *V.harveyi* UQM 2723 and *V.anguillarum* UQM 2771 which did not grow on SET medium with 1 % agar. These strains had grown after 7 d incubation but as can be seen from Plate A4.1 (top row) growth was encroached or surrounded by swarming strains. After 7 d the pooled average colony diameters from control and all inhibitor plates were *highly significantly* greater on media with 2 % agar than 1 % t = 6.6, 6.22 (Swarming, Non-Swarming) and significantly greater for swarming strains on SET than SEN media *(t = 2.12)* but for non swarming strains colony diameters were greater on SEN than SET media (t = 2.04).

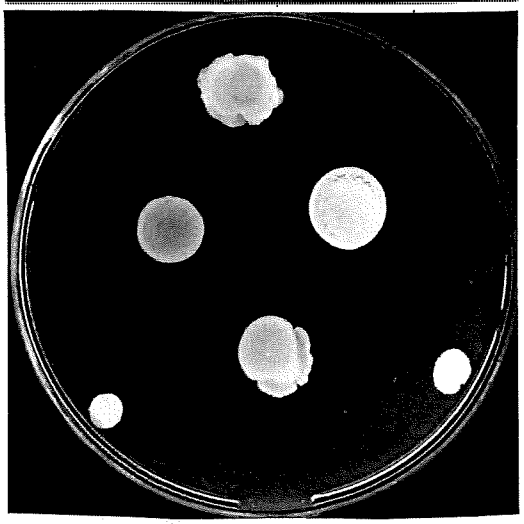
Plate A4.1: Colony Appearences of *Vibrionaceae* Exposed to Different Growth Conditions and Swarming Inhibitors.





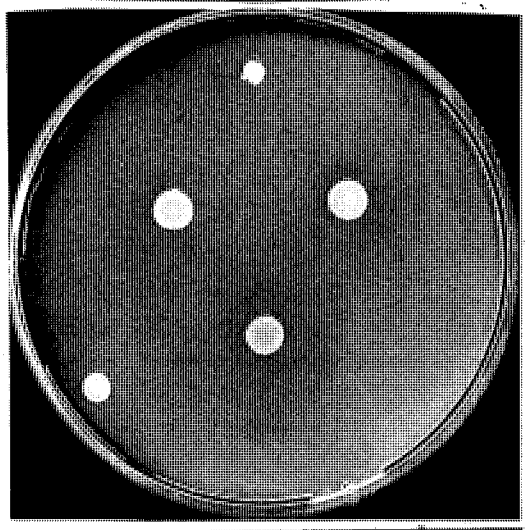
Left,
Control
SEN
1%
Agar.
Right,
Control
SET
1%
Agar.

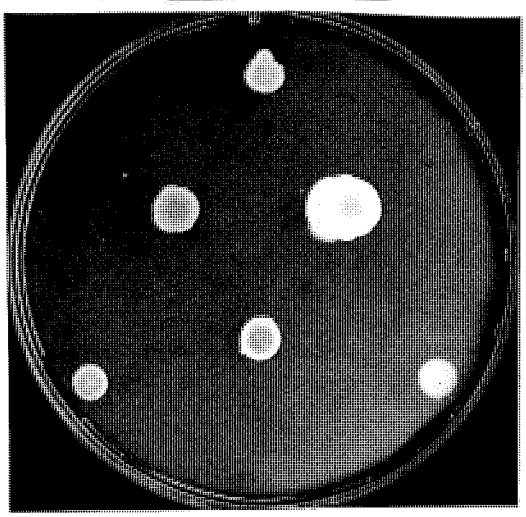




Left,
SDS
5 mM
SEN
1%
Agar.

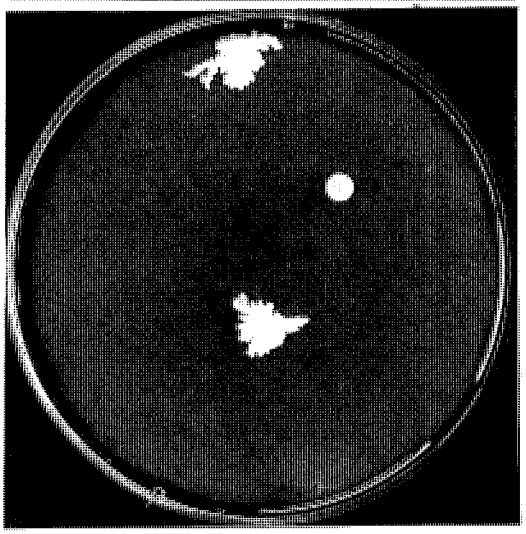
Right,
PNPG
2,5 µM
SET
1%
Agar.





Left,
PEA
20 µm
SEN
1%
Agar.

Right,
Bor
25 mM
SET
1%
Agar.



Left,
Tan
20 µm
SEN
1%
Agar.

Right,
Strain
Loci.

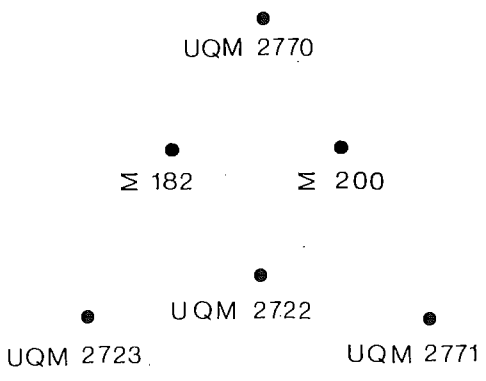


Table A4.2 : Average Colony Diameters (mm) For Swarming And Non-Swarming Strains with Different Inhibitor Treatments.

Inhibitor.	SET MEDIUM.				SEN MEDIUM.				1 % Agar Media.		2 % Agar Media.		SET Media.		SEN Media.	
	1 % Agar.		2 % Agar.		1 % Agar.		2 % Agar.									
	Swa	Nswa	Swa	Nswa	Swa	Nswa	Swa	Nswa	Swa	Nswa	Swa	Nswa	Swa	Nswa	Swa	Nswa
Control.	38	0	40	0	24	5	31	9	62	5	71	9	78	0	55	14
SDS 2.5 mM	10	6	17	14	11	7	14	10	21	20	31	34	27	20	25	17
SDS 3.5 mM	9	7	11	7	14	12	13	11	23	19	24	18	23	14	27	23
SDS 5.0 mM	10	6	15	12	10	7	15	11	20	13	30	23	25	18	25	18
PEA 5 mM	35	4	40	0	20	6	29	9	55	10	69	9	55	4	49	15
PEA 10 mM	35	4	40	0	14	5	29	9	49	9	69	9	75	4	43	14
PEA 20 mM	7	5	10	7	4	0	24	4	11	5	34	11	17	12	28	4
PNPG 2.5μM	27	5	28	7	13	6	21	9	50	11	49	16	49	12	49	15
PNPG 10 μM	9	6	12	9	8	5	8	7	17	11	20	16	21	15	16	12
Gly 20 mM	38	5	38	7	30	6	38	9	68	11	76	16	76	12	68	15
Gly 30 mM	38	5	40	7	33	5	37	7	71	10	77	14	78	12	70	12
Gly 50 mM	38	4	39	7	30	7	36	10	68	11	75	17	76	11	66	17
EtGl 20 mM	37	4	39	4	25	5	33	9	62	10	71	13	62	9	71	14
EtGl 40 mM	36	5	38	5	31	5	33	9	67	12	70	14	67	10	80	14
EtGl 60 mM	31	7	37	5	27	5	33	8	58	10	69	13	58	10	60	13
Tan 20 μM	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Only UQM 2722, Σ182	24	0	40	0	5	0	40	0	29	0	80	0	64	0	45	0
Bor 5 mM	29	5	36	5	32	6	36	5	61	5	72	6	65	10	68	11
Bor 15 mM	33	4	36	5	18	8	31	10	51	10	67	13	69	9	49	14
Bor 25 mM	36	5	38	13	5	7	8	9	41	9	45	20	74	18	13	12

Swa = Swarming, Nswa = non-Swarming.

No dose of SDS was inhibitory to growth, and swarming was initially suppressed by this agent. Strains however excreted SDS esterase and eventually crept out as SDS was utilized (Plate A4.1). PEA suppressed swarming most effectively in all media at a concentration of 20 mM however

this concentration was inhibitory to growth of non-swarming strains on SEN medium with 1 % agar (Plate A4.1). PNPG did not inhibit growth and at a concentration of 10 μM prevented swarming on all media. All swarming strains except *V.alginolyticus* UQM 2722 and the non-swarming strain *V.anguillarum* UQM 2771 were induced to form rough colonies in the presence of this inhibitor. This effect was first noted after 4 d and respectively affected 2, 3, and 4, strains at 2.5, 5 and 10 μM PNPG. Plate A4.2 indicates this effect on SET medium with 1 % agar for *V.alginolyticus* UQM 2770 and Σ 200. Gly and EtGl, respectively at maximum tested concentrations of 50 and 60 mM, did not inhibit growth or swarming. No strains grew at concentrations of tannate greater than 20 μM and only two strains *V.alginolyticus* UQM 2722, and Σ 182 were able to grow by 48 h at a media concentration of 20 μM. Strains on this medium did not swarm in the characteristic manner but spread in dendritic processes (Plate A4.2.f). Borate at 25 mM inhibited swarming on SEN but not SET media, growth of non-swarming strains was not inhibited by this chemical (Plate A4.2).

A4.4 Discussion.

Colony diameters on SEN were smaller than SET media and media with 1 % agar had smaller swarming colonies than 2 %. Consequently the most suitable medium for growth without enhancement of swarming was SEN with 1 % agar. Doses of glycerol and ethylene glycol less than 50 and 60 mM respectively, neither inhibited growth nor prevented swarming for short or long periods. Tannate and PEA both inhibited growth and swarming. Tannate may have been active by mordanting growing cells and their flagella. SDS prevented swarming initially; but its utilization and subsequent deactivation by a majority of strains limit its application to short incubations. PNPG 10 μM was active against swarming strains but did not inhibit growth by non-swarming strains although rough colonies were produced by several strains, including *V.harveyi* UQM 2723. The mechanism of this swarming inhibitor was reported as unknown, (Senior 1977), but the rough colony appearance, may be related to binding to specific sites on the cell membrane. Cells were not

tested to determine if the formation of rough colonies was permanent, or reversible, and no cells exposed to PNPG were examined by electron microscopy to check for morphological changes.

Borate ions were not inhibitory to growth, and did not cause visible artifacts. Sykes and Reed (1949) suggested that swarming inhibition in *Proteus* occurred by the formation of complexes between flagellin and borate ions.

A4.5 Conclusions.

Borate and PNPG both seemed suitable media supplements for non-inhibitory prevention of swarming. However because the specific activity of PNPG was unknown and because borate was potentially able to form chemical complexes with substrates to be assayed in phenetic analysis and because non-swarming strains were still potentially cross-feeding (See 2.9.3.6.1) it was decided to prevent swarming by physical rather than chemical means.

APPENDIX 5.

A5.0 Nitrofuran Minimal Inhibitory Concentration Assays upon *Vibrionaceae.*

A5.1 Experimental Design.

The treatment of *Vibrio* disease by antibiotics has become an integral component of fish farm management. The application of antibiotic treatment for management of episodic disease in wild fish stocks is hindered by ecologic and economic considerations, but these do not necessarily preclude chemical control as a potentially valid field disease management option.

Two derivative nitrofurans, nitrofurazone and nifurstyrenate, which had structures which resembled folic acid or PABA inhibitors such as 0/129, sulphmethoxazole and trimethoprim; were seen as potentially able to satisfy economic and disease management criteria. These were evaluated to establish their minimal inhibitory concentrations towards diversely isolated *Vibrionaceae,* mainly from diseased fish and shellfish.

Sodium-enriched Mueller Hinton Broth (SEMHB, 3.4.1.06) was dispensed (200 µl/well) aseptically to eight sterile, lidded micro-titre trays **(Disposable Products)** using a 12 channel micro-pipette **(Flo Laboratories)** and sterile polypropylene tips **(Kartell).** These trays were divided into four sets labeled (A-D) each with two trays. Portions, (100 µl), of 1% alkaline aqueous nitrofurazone, **(Smith, Kline, and French)** in 0.008 N sodium hydroxide in 2% sodium chloride, and 1% aqueous sodium nifurstyrenate **(Ueno Fine Chemicals,** ***Elibazieu)*** in 2 % sodium chloride were respectively dispensed to each of the first rows of trays A and B and trays C and D. A multi-channel pipette was used to make a series of three fold dilutions, ($\log_{10}$ *ca* 0.5), by transferring 100 µl to successive rows in both sets. Growth from 24 h SENA medium cultures primarily from fish pathogens or fish associated species from the *Vibrionaceae* were prepared in 8 ml sterile marine phosphate buffered saline (MPBS 3.4.3.1) to a MacFarland turbidity equivalent of 0.5. The strains examined from (Tables 3.4-5) were *A.hydrophila* UQM 2769, *V.alginolyticus* UQM 2770, *V.anguillarum* UQM 2771, UQM 2843, DA 01,02,03; *V.campbellii* UQM 3061, *V.orientalis* DA 05,06,09,13;

V.tubiashii UQM 2923, *V.damsela* UQM 2853, *V.mimicus* UQM 2954, *V.ordalii* UQM 2890, UQM 2906, DA 12; *V.vulnificus* Biogroup II UQM 2922 and *Vibrio* spp. DA 04,07 & 11.

Culture suspensions were poured to sterile polypropylene troughs **(Robbins)** and 20 µl dispensed to successive columns of nitrofurazone and nifurstyrenate dilution trays. The trays were lidded and incubated 24 h. Growth was scored as positive if turbidity was present and the MIC's expressed as µg/ml in Table A5.2 with abstracted data [Batch 5.] from separate determination of qualitative antibiotic sensitivities and growth at 30°C.

A5.2 Results and Discussion.

The MIC's for nitrofurazone were between 1-2 logs higher than for nifurstyrenate (Table A5.1). Expressed as comparative molar concentrations this difference in activity is increased, but not equilibrated by the ratio of molecular weights i.e. 257/198. The possibility that alkalination of the nitrofurazone stock solution reduced the activity is countered first by the non-stoichometric proportions of alkali and nitrofurazone present in stock solution, and second by pH buffering in SEMHB by its constituent proteins.

While there were consistent patterns for two reference strains of *V.ordalii,* (UQM 2890 and UQM 2906), and between the provincial and type cultures of *V.anguillarum* UQM 2843 and UQM 2771 respectively these patterns were different to those produced by Tasmanian isolates of *V.anguillarum,* DA1, DA2, and DA3 and *V.ordalii* DA 12.

Other variations of profile within species occurred amongst the four strains of the *Vibrio orientalis* DA 05,06,09, and 13 and the atypical *Vibrio* spp. represented by DA 04,07 and 11. For none of the folic acid analogues was there a consistent correlation of activity. Neither were any recognizable correlations seen with novobiocin, penicillin, polymyxin or tetracycline. These unmatched patterns may indicate the limitations of

Table A5.1: MIC's for Nitrofurans, Antibiotic Sensitivities and Growth at 30°C. by Fish Associated Vibrionaceae.

				Attribute.								
Species.	**Nf**	**Ns**	**Ba**	**Pt 10**	**Pt 150**	**Nb**	**Pc**	**Pm**	**Sm**	**Tc**	**Tm**	**30**
A.hydrophila UQM 2769	400	1.2	1	-	-	-	+	-	+	-	+	+
V.alginolyticus UQM 2770	400	1.2	7	-	+	-	+	+	+	-	+	+
V.anguillarum UQM 2771	400	12.5	7	+	+	-	+	-	+	-	+	+
V.anguillarum UQM 2843	400	12.5	1	+	+	-	+	-	+	-	-	+
V.anguillarum DA 1	400	0.38	7	+	+	-	+	+	+	-	-	+
V.anguillarum DA 2	140	0.38	7	+	+	-	+	+	+	-	-	+
V.anguillarum DA 3	400	12.5	7	+	+	-	+	+	+	-	-	+
V.campbellii UQM 3061	400	12.5	7	+	+	-	+	+	+	-	-	+
V.damsela UQM 2853	400	1.2	7	+	+	-	+	+	+	-	-	+
V.mimicus UQM 2954	400	1.2	7	+	+	-	+	+	+	-	-	+
V.ordalii UQM 2890	400	3.9	7	-	+	+	+	-	+	+	-	+
V.ordalii UQM 2906	400	3.9	7	-	+	+	+	-	+	+	-	+
V.ordalii DA 12	1.35	12.5	7	+	+	-	+	-	+	-	-	+
V.orientalis DA 05	400	5	7	+	+	-	+	-	+	-	-	-
V.orientalis DA 06	400	5	7	+	+	-	+	-	+	-	-	-
V.orientalis DA 09	400	3	7	+	+	-	+	-	-	-	-	+
V.orientalis DA 13	140	1.2	7	+	+	-	+	-	+	-	-	+
V.tubiashii UQM 2923	400	12.5	7	+	+	-	+	+	+	-	-	+
V.parahaemolyticus UQM 2776	400	12.5	6	+	+	-	+	+	+	ND	-	+
V.vulnificus II UQM 2922	400	1.2	6	+	+	-	+	+	+	ND	-	+
Vibrio sp. DA 04	140	0.38	7	+	+	-	+	-	+	-	-	-
Vibrio sp. DA 07	140	12.5	7	+	+	-	+	-	+	-	-	-
Vibrio sp. DA 11	400	0.12	7	+	+	-	+	-	+	-	-	+

Nf Nitrofurazone µg/ml.
Ns Nifurstyrenate µg/ml.
Ba Phenotypic Analysis Batch.
Nb Growth 10 µg/ml Novobiocin.
Pc Growth 15 units/ml Benzyl-penicillin
Pt-10 0/129 phosphate 10 µg/ml.
Pt-150 0/129 phosphate 150 µg/ml.
Pm Growth 5µg/ml Polymyxin.
Sm Growth 5µg/ml Sulphamethoxazole.
Tc Growth 10µg/ml Tetracycline.
Tm Growth 10µg/ml Trimethoprim.
30 Growth at 30°C.
ND Not Determined.

qualitative assessments of antibiotic sensitivities. To test the premise that nitrofuran sensitivity was a synergistic feature induced by temperature stress, e.g. of Tasmanian isolates, data for growth at 30°C. was examined. Strains which failed to grow at 30°C. and which consequently may have been stressed at 25°C. were variably sensitive or resistant to both nitrofurans.

These comparative observations of phenotype would suggest that more than one mode of competition or inhibition by the nitrofurans affects bacterial metabolism.

Results indicate a general susceptibility of fish-associated *Vibrionaceae* particularly to nifurstyrenate at doses which may make its use in single applications to RSD affected river systems economically viable. The consequences of such action must be evaluated in the context of the effects of this antibiotic to the ecology of other species, and should make consideration of antibiotics already passed to the environment in sewage.

A5.3 Conclusions.

(i) The activities of nitrofurazone and nifurstyrenate towards *Vibrionaceae* were not correlated.

(ii) *Vibrionaceae* were inhibited more by nitrofurstyrenate than nitrofurazone.

(iii) The activity of neither nitrofuran was consistent within or across species or genera of *Vibrionaceae.*

(iv) Nitrofurans activity towards *Vibrionaceae* was uncorrelated with qualitative doses of folic acid inhibitors or other antibiotics.

(v) Nifurstyrenate may be an effective chemical control measure for episodic RSD disease in river systems if shown not to adversely affect the ecology of non-target species.

APPENDIX 6.

A6.0. Use of Azo-reductase as a Presumptive Indicator of Nitrogen Fixation by *Vibrionaceae.*

A6.1.0. Experimental Design.

The ability of a bacterium to fix dinitrogen may be a valuable marker of its ecological niche because such bacteria, do not need to rely upon proteases for their nitrogen requirements and may form mutualistic rather than commensal or pathogenic symbioses.

Direct assays through the incorporation of ^{15}N, (Fielder and Proksch, 1975) and indirect methods which rely on the reduction of acetylene (Dillworth 1966) are often used to detect and measure nitrogen fixation. Both methods are time consuming and require expensive laboratory equipment. Another method, empirically successful, but unsupported by theory relies upon decolourization of congo red (Harrigan and McCance, 1966), (3.8.2.2.7).

Such an alternate rapid and reliable assay for nitrogen fixation would be a helpful indicator for the determination of ecological role of bacteria. The basis for the acetylene reduction assay reported by Hardy, Holsten, Jackson, and Burns (1968) was the non-specific activity of the nitrogenase enzyme towards carbon as well as nitrogen triple bonds. It was reasoned that the decolourization of congo red, a diazo dye, might also be as a result of this non-specific enzyme.

Activity specifically towards the diazo bond in these dyes i.e. a mono-azo-reductase has been reported e.g. Zimmermann, Kulla and Leisinger (1982). Whether azoreductases might also function as nitrogenase enzymes has not been confirmed, although usage of the dis-azo dye congo red decolourization as an indicator of terrestrial nitrogen fixing bacteria might seem to support this.

The nitrogen fixing bacterium *V.diazotrophicus* was examined to establish if its nitrogenase enzyme might also serve as an azoreductase, and if present

to determine to which dye types, i.e. mono- and dis-azo forms, the greatest activity was detected. Whole cells of this species were exposed to the mono-azo dyes fast blue B (Sigma), fast red TR (Sigma) and fast yellow GC (Sigma) and the dis-azo dyes congo red (Sigma) and trypan blue (BDH) their soluble (zinc) salts were examined periodically spectrophotometrically in buffer solution for decolourization in the absence of any other source of nitrogen. Further, to establish if activity, if any, was as a result of a constitutive or induced enzyme, test media were treated by different inoculum doses.

A6.1.1. Preparation of Absorbance Media.

Freshly autoclaved phosphate buffered glucose salts (PBGS) medium (3.4.4.35) was dispensed in 5ml portions to sterile 16 spectrophotometer tubes. While still warm the medium was overlaid with 1ml sterile mineral oil. Aqueous azo dye solutions were prepared as 10 % FS stock solutions except trypan blue which was prepared as a 2.5 % solution, and 2.5 % congo red which was gamma sterilized. Each dye was added to the top of the oil in three tubes to produce the aqueous concentrations for mono- azo dyes of 1000 mg/l and for dis-azo dyes of 500 mg/l. Excess insoluble salts were allowed *ca* 2h to precipitate.

A6.1.2 Preparation of Inoculum and Incubation.

Growth from a late log phase culture of *Vibrio diazotrophicus* UQM 2780 on SENA was suspended in 5 ml MPBS to a turbidity equivalent of three on the MacFarland scale (Finegold and Baron 1986). The culture suspension was inoculated in 125 and 250 µl portions to sink through the oil layer into each dye. One uninoculated tube of each dye was used as a control. Initial absorbances at 400 and 600 nm (Bausch and Lomb, *Spectronic 20)* were recorded and the tubes were incubated in the dark. Successive absorbance readings were recorded at both wavelengths over 79 d. All measurements were through unmixed buffered dye solution.

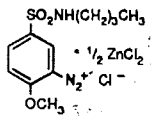
A6.1.3 Analysis of Results.

Each set of data was comparatively examined by linear regression analysis of absorbance against $\log_{10}$ of time. The slopes of these lines were compared by calculating the *t*-statistic for overlapping lines and comparing this with the tabulated value for 8 degrees of freedom. Supplementary comparisons were made for congo red and trypan blue treatments, with an untransformed timescale of 11 days (7 degrees of freedom).

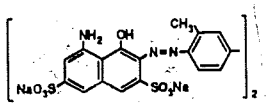
A6.1.4 Results.

All tubes formed a precipitant layer of insoluble phosphate onto which dyes were adsorbed as incubations progressed. The regression lines calculated, and comparative *t*-statistics are as shown in Table A6.1. Figures A6.1-3 are plots of the absorbance for fast yellow GC, fast red TR and trypan blue at 400 nm for inoculated and uninoculated control tubes against log time for up to 79 days. Figures A6.4 and A6.5 are plots for absorbance at 600 nm of tubes containing congo red and trypan blue over the same period. In all tubes containing bacteria the absorbance declined rapidly for up to 11 days and then was observed to stabilize or increase slightly. Linear regressions to the data were also determined for this initial period and the slopes compared using the *t*-statistic. Significant differences were found only for trypan blue and congo red at 600 nm (Table A6.1). For both of these dis-azo dyes neither of the slopes or regression lines from different inoculum doses were significantly different from each other; (Congo Red t=1.31 i.e. < $t_{tab.\ 0.05}$ 8 d.f. = 1.8 and Trypan Blue t = 1.27 i.e < 1.8). But when calculations included the origin and were based upon an untransformed timescale, truncated to include only the first 11 days, a significant difference was observed between dose-groups of congo red ($t_{calc.}$ 2.69 > $t_{tab.\ 0.05}$ 7d.f. = 1.9) in addition to the previously mentioned difference with the control. For trypan blue a significant difference existed between inoculum-treatments, ($t_{calc.}$ 2.72 > 1.90), but the slopes for neither of the 2 inocula was significantly different from the control ($t_{calc.}$ = 1.02

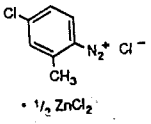
Figure A6.1: The Chemical Structures and Absorbance Plots for Decolourization of Mono-and Dis-Azo Dyes Against Log_{10} Time: i-iii; Fast Yellow GC, Fast Red TR and Trypan Blue at 400 nm, iv-v; Congo Red and Trypan Blue at 600 nm; and vi, Fast Blue B.



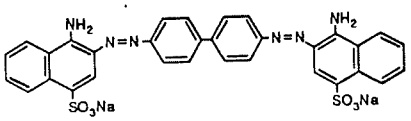
(i) Fast Yellow GC



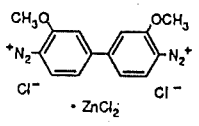
(iii) Trypan Blue



(ii) Fast Red TR



(iv) Congo Red



(vi) Fast Blue B

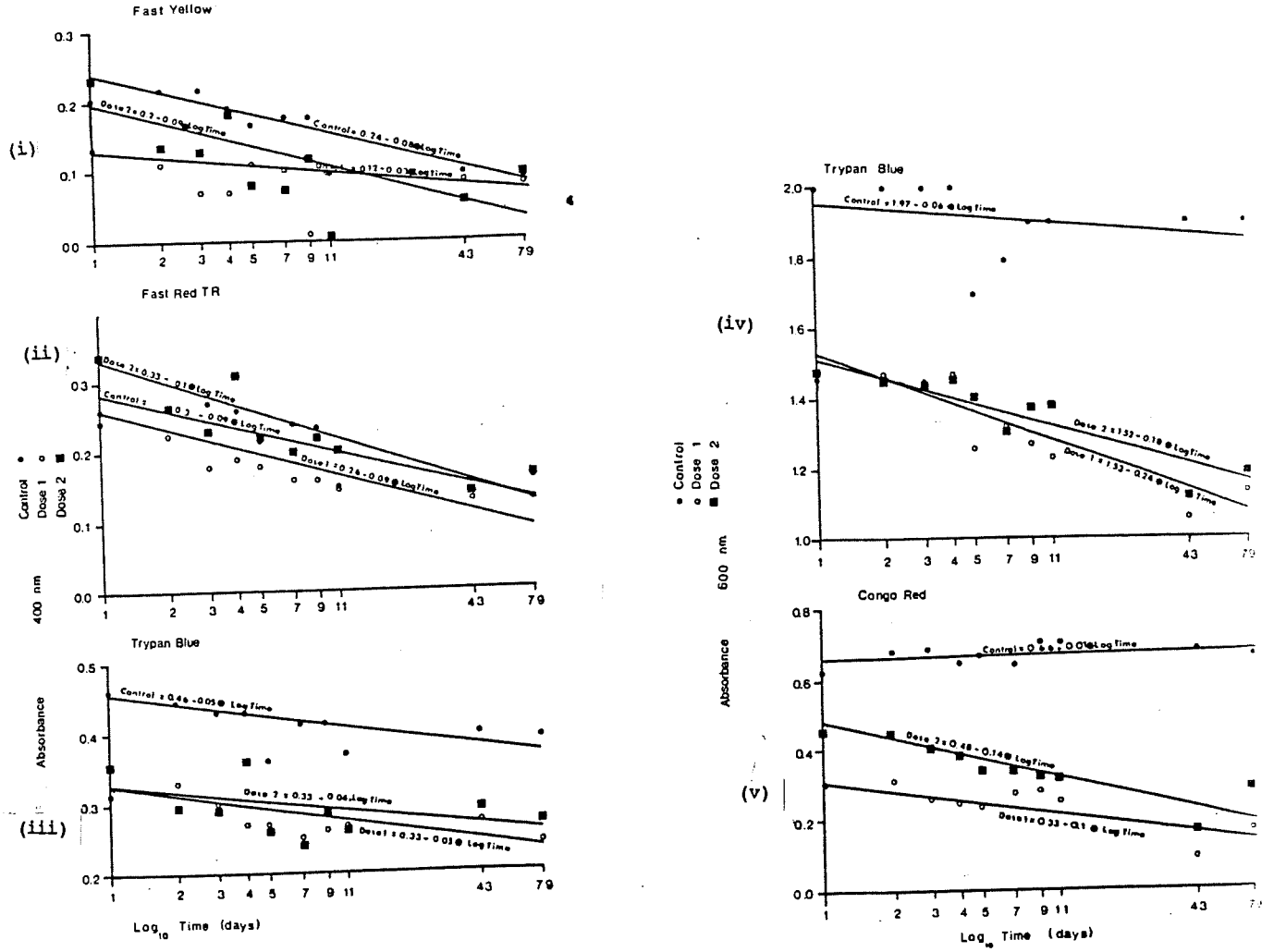


Table A6.1: Comparative Regression Equations and *t*-Statistics for Mono- and Dis-Azo Dye Decolourization Slopes at 400 and 600 nm.

			t- Calc.,(d.f.)
400 nm Fast Blue B	Control	$x = 0.18 - 0.08*\text{Log}_{10}$ Time.	x * y *t* = 1.77,(9)
Dose 1	0.12 (ml)	$y = 0.03 + 0.01*\text{Log}_{10}$ Time.	x * z *t* = 1.23,(9)
Dose 2	0.25 (ml)	$z = 0.06 - 0.03*\text{Log}_{10}$ Time.	y * z *t* = 0.63,(9)
600 nm Fast Blue B	Control	Absorbance too low.	
Dose 1	0.12 (ml)	Absorbance too low.	
Dose 2	0.25 (ml)	Absorbance too low.	
400 nm Fast Red TR	Control	$x = 0.30 - 0.09*\text{Log}_{10}$ Time.	x * y *t* = 0.57,(9)
Dose 1	0.12 (ml)	$y = 0.26 - 0.10*\text{Log}_{10}$ Time.	x * z *t* = 0.81,(9)
Dose 2	0.25 (ml)	$z = 0.33 - 0.09*\text{Log}_{10}$ Time.	y * z *t* = 0.36,(9)
600 nm Fast Red TR	Control	Absorbance too low.	
Dose 1	0.12 (ml)	Absorbance too low.	
Dose 2	0.25 (ml)	Absorbance too low.	
400 nm Fast Yellow GC	Control	$x = 0.24 - 0.08*\text{Log}_{10}$ Time.	x * y *t* = 0.42,(9)
Dose 1	0.12 (ml)	$y = 0.20 - 0.09*\text{Log}_{10}$ Time.	x * z *t* = 1.65,(9)
Dose 2	0.25 (ml)	$z = 0.12 - 0.03*\text{Log}_{10}$ Time.	y * z *t* = 1.65,(9)
600 nm Fast Yellow GC	Control	Absorbance too low.	
Dose 1	0.12 (ml)	Absorbance too low.	
Dose 2	0.25 (ml)	Absorbance too low.	
400 nm Congo Red	Control	Absorbance too high.	
Dose 1	0.12 (ml)	Absorbance too high.	
Dose 2	0.25 (ml)	Absorbance too high.	
600 nm Congo Red	Control	$x = 0.66 - 0.01*\text{Log}_{10}$ Time.	x * y ***t* = 4.56,*** (9)
Dose 1	0.12 (ml)	$y = 0.48 - 0.14*\text{Log}_{10}$ Time.	x * z ***t* = 5.76,*** (9)
Dose 2	0.25 (ml)	$z = 0.33 - 0.10*\text{Log}_{10}$ Time.	y * z *t* = 1.31,(9)
400 nm Trypan Blue	Control	$x = 0.46 - 0.05*\text{Log}_{10}$ Time.	x * y *t* = 0.16,(9)
Dose 1	0.12 (ml)	$y = 0.33 - 0.05*\text{Log}_{10}$ Time.	x * z *t* = 0.35,(9)
Dose 2	0.25 (ml)	$z = 0.33 - 0.04*\text{Log}_{10}$ Time.	y * z *t* = 0.55,(9)
600 nm Trypan Blue	Control	$x = 1.97 - 0.06*\text{Log}_{10}$ Time.	x * y ***t* = 2.74,*** (9)
Dose 1	0.12 (ml)	$y = 1.53 - 0.24*\text{Log}_{10}$ Time.	x * z ***t* = 1.96,*** (9)
Dose 2	0.25 (ml)	$z = 1.52 - 0.18*\text{Log}_{10}$ Time.	y * z *t* = 1.27,(9)
600 nm Congo Red	Control	x = 0.65 + 0.004*Time.	x * y ***t* = 2.74,*** (7)
Dose 1	0.12 (ml)	y = 0.46 - 0.02*Time.	x * z ***t* = 2.69,*** (7)
Dose 2	0.25 (ml)	z = 0.30 - 0.01*Time.	y * z ***t* = 5.54,*** (7)
600 nm Trypan Blue	Control	x = 1.99 - 0.04*Time.	x * y *t* = 1.02,(7)
Dose 1	0.12 (ml)	y = 1.51 - 0.03*Time.	x * z *t* = 0.51,(7)
Dose 2	0.25 (ml)	z = 1.46 - 0.01*Time.	y * z ***t* = 2.72,*** (7)

$|t\text{-tab.}|^{9}_{(0.05)} = 1.83$ $|t\text{-tab.}|^{9}_{(0.05)} = 2.82$

$|t\text{-tab.}|^{7}_{(0.05)} = 1.89$ $|t\text{-tab.}|^{7}_{(0.05)} = 3.00$

Note: Figures for *t*-calc. in **bold type are significant (5%)**, in ***bold italic highly significant (1%)***.

and 0.512 respectively < 1.89). Calculations for the mono-azo dyes fast red, fast yellow, and fast blue B, revealed no significant results.

A6.1.5 Discussion.

No significant differences were seen in decolourization of solutions of the mono-azo dyes between inoculated and uninoculated samples, and between-samples receiving different doses of inoculum.

Results for dis-azo dyes at 600 nm indicated some significant differences between inoculated and uninoculated tubes but only in one case, (congo red observed at 600 nm in a truncated analysis), was there a statistically significant difference corresponding to a dose effect i.e. a larger inoculum produced a greater decolourization. These results may indicate that the inoculated bacteria did not grow in PBGS medium and decolourization resulted only from nitrogenase enzyme present in the inoculum i.e. nitrogenase enzyme of *V.diazotrophicus* is constitutive. In assays continued beyond 11 days, congo red which had been precipitated from solution initially may have continued slowly to dissolve back to an equilibrium concentration after enzyme activity had ceased yielding negative results.

The greater activity of the azo-reductase enzyme towards dis-azo dyes may have been as a result of some "electron-donor" effect which isolates the diazo bonds in dis-azo dyes to a greater extent than in mono-azo dyes.

A6.2.0 Comparative Absorbance Profiles.

A6.2.1 Experimental Design.

To determine whether decolourization was as a result of chemical alteration or from precipitation, the visible absorbance spectra of congo red in PBGS medium with and without inoculation of *V.diazotrophicus* UQM 2780 (prepared by L.Brancato) were scanned by a **Hitachi** *150-20* over the range 325-700 nm after 14 d incubation and after standing in a waterbath at 60°C. 30 min to redissolve precipiated dye. These absorbance scans, with peaks and inflection points marked, appear in Figure A6.6.

A6.2.2 **Results.**

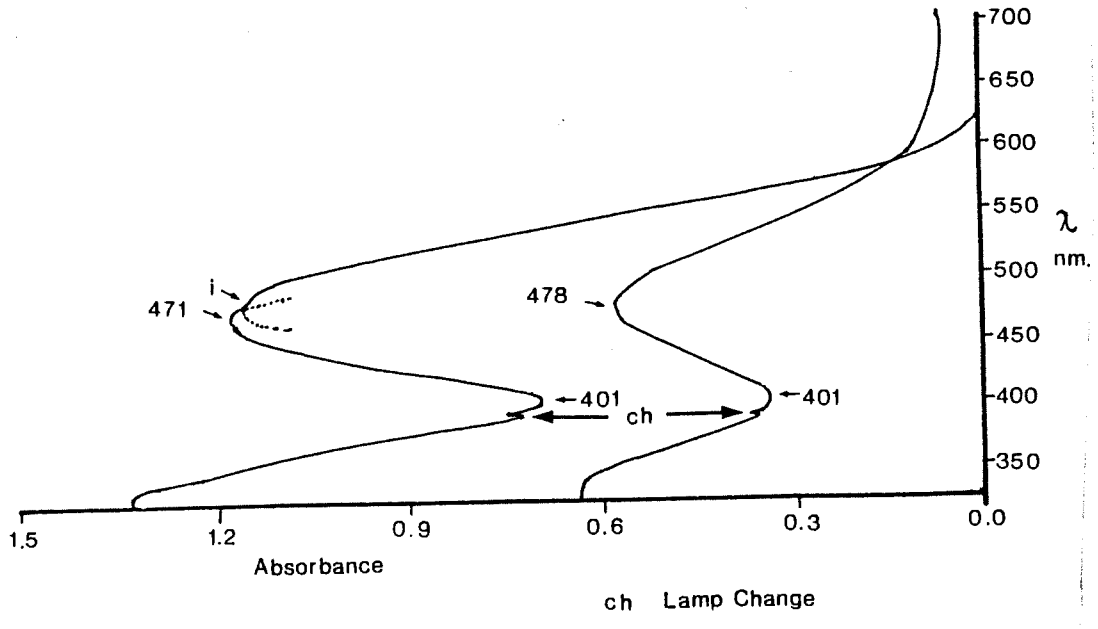
Absorbance profiles for treated and untreated samples were similar but had distinct peaks and troughs. The absorbance spike at 390 nm present on both curves corresponds with the change over from UV to incandescent light sources. The control sample had an absorbance peak of 1.2 at 471.2 nm and a trough at 401.2 nm and a second minor inflection point at *ca* 480 nm. The treated sample had a peak of 0.56 at 476 nm and a trough at 402.0 nm, no similar minor inflection point was visible.

A6.2.3 **Discussion.**

Because *V.diazotrophicus* UQM 2780 did not show any activity towards the cyclic-compounds, benzoate, hydroxybenzoate, or phenylacetate in phenotypic screening [Batches 2. & 7.]; it is likely that the difference in the treated sample resulted from activity of this species towards the N=N bonds.

The absorbance peak for aqueous Congo Red (*Merk Index,* 1983) is 488 nm at pH 7.3. Both the control and test absorbance peaks samples here were found to differ from this, probably because of pH or salinity differences.

Figure A6.6: Comparative Absorbance Scans of Congo Red after 14 d in PBGS Medium for and Uninoculated Control and a Decolourizing and for *V.diazotrophicus* UQM 2780.



The lower absorbance, and absence of the minor inflection point in the inoculated tube compared with the control indicate a specific activity of *V.diazotrophicus* towards only one component of this dye.

If the absorbance profile of the dye is considered as a composite produced from both of the congo red molecule's chromophores each with closely overlapping peaks, as indicated by the broken lines in Figure A6.2 the differences between-profiles of inoculated and uninoculated tubes and previous results suggesting *V.diazotrophicus* has activity towards dis-azo dyes but not mono-azo dyes may be explained.

The shift in absorbance peak and absence of inflection point in the inoculated sample and 50 % lower absorbance in the inoculated tube than in the uninoculated tube seems to support the earlier premise that activity is towards only the first of a dis-azo dye's chromophores and suggest the later exclusive presence of a mono-azo endproduct of this activity. This could be tested by more sophisticated spectroscopy or perhaps by comparison of absorbance profiles with other mono-azo dyes.

A6.3.0 Comparative Determination of the Nitrogen Fixation by Congo Red Reduction and Acetylene Reduction.

A6.3.1 Experimental Design.

Rather than to pursue the chemical species produced as a result of exposure of the dis-azo dye to these bacteria it was decided to evaluate strains showing both positive and negative results for azo-reductase, with the acetylene reduction technique. OTU's assayed in Batches [7. & 8.] were examined for their ability to decolourize congo red in the medium as described above with absorbance measured only at 600 nm initially and 28 d post-inoculation after treatment at 80°C. 30 min. This wavelength was remote from the absorbance maximum of congo-red as indicated in Figure A6.6 however the absorbance-relativities between the proposed dis-azo and depleted mono-azo dyes may not be severely affected by reading at this wavelength even when a relatively insensitive spectrophotometer *(Spectronic 20)* was used.

From these, 10 OTU's, 7 positive and 3 negative by this method, *[A.hydrophila* : Stn 3310; *V.alginolyticus* : Stn 3190, 3380, 3960 and Stn 5000; *V.natriegens* : Stn 4800; *Vibrio fluvialis* I & II : Stn 4631, Stn 5650; *V.fluvialis* III : Stn 5800 & Stn 5810]; were comparatively assayed by acetylene reduction. Duplicates of *V.diazotrophicus* UQM 2780 were included one to serve as a positive control and the other as a blind (operator) positive control. *V.campbellii* UQM 2779, (Table 3.4), was used as a negative control for acetylene reduction but was not assayed by dye decolourization.

Five strains from above were also examined by L.Brancato (LB) at 510 nm after 14 d as in (A6.2.1) and a pooled comparison was made between these and acetylene reduction results.

A6.3.2 Acetylene Reduction Method.

A6.3.3 Preparation of Aerobic Support Medium.

MPBGS medium (3.4.4.35) modified to contain 1 % agar (Oxoid L 28), 0.2 % sodium sulphate *(Ajax)* and with 2 % maltose *(Sigma)* substituted for glucose, was sterilized and the separate salt solution (a) and nutrient, salt and agar solution (b) were combined. Filter sterilized pyridoxal hydrochloride (Sigma) was added to a final concentration of 5 mg/l and 10 ml aliquots of the complete medium were dispensed aseptically into 25 ml sterile bottles, capped with injection stoppers, (William Freeman and Co, *Suba-Seals*$^{r.}$), cooled to gel as slopes, inoculated with 0.1 ml of a late log phase SEN broth cultures of cultures above, and stored at 4°C. for 4 h.

A6.3.4 Acetylene Reduction Analysis Protocol.

Analyses were conducted by M.O'Donahue as follows: Into each inoculated bottle and two uninoculated controls 10-20 % head space of acetylene was created. After mixing by returning 1 ml 3 times, 0.5 ml samples of the headspace were withdrawn to 5 ml evacuated blood samplers (Terumo, *Vacuutainers).* The tubes were incubated 4 d at 25°C. and 0.5 ml sub-sampled

into a second series of blood samplers and directly to a gas chromatograph (Shimadzu *4ABPS)* with *ca* 1 m, 0.5mm diameter stainless steel column packed with 80-100 mesh separation medium, (Waters Associates, *Porapak N).* Column temperatures were 80°C. at injection port and in the column, and 150°C. at the detector. All gases were C.I.G., *High Purity* grade, Flow rates were air 300 ml/min, Hydrogen 45 ml/min and Nitrogen 60 ml/min). Samples for which the height of ethylene peaks was greater than peak of the lowest positive control samples were scored as positive. Comparative results shown in Table A6.2 were tested for conformity with the χ^2 - distribution.

Table A6.2: Comparative Results for Azoreductase and Acetylene Reduction by *Vibrionaceae.*

Batch	Accession Code.	Species	Absorbance Reduction Congo Red Score.	Acetylene Reduction.
7	UQM 2780	*V.diazotrophicus*	+	+
LB	UQM 2780	*V.diazotrophicus*	+	+
ND	UQM 2779	*V.campbellii*	ND	+
7	Stn 3310	*A.hydrophila*	+	+
7	Stn 3190	*V.alginolyticus*	+	+
8	Stn 5000	*V.alginolyticus*	+	+
LB	|		+	
7	Stn 3380	*V.alginolyticus*	+	+
LB	|		+	
8	Stn 3960	*V.alginolyticus*	+	-
8	Stn 5650	*Vibrio fluvialis* I & II	-	+
8	Stn 4631	*Vibrio fluvialis*	-	+
8	Stn 5800	*V.fluvialis* III	+	+
8	Stn 5810	*V.fluvialis* III	-	+
LB	|		+	
7	Stn 4800	*V.natriegens*	-	-
LB	|		+	

A6.3.5 Results.

V.campbellii UQM 2779, and other *Vibrio* spp. (Table A6.2) apart from *V.diazotrophicus* produced positive results by both assay methods. A *ca* 70 % correlation was found between acetylene reduction and dye decolourization procedures. For *V.natriegens* Stn 4800 negative results were recorded by both

procedures but this result was not verified by the other azide reductase operator. The Yates corrected χ^2 was calculated as 3.79 (1 d.f.) (<3.84) and consequently indicated no significant difference between activity of azoreductase and detected acetylene reductase activity.

A6.3.6 Discussion.

Results from comparative analysis suggest that *Vibrio* strains which produce azoreductase also produce acetylene reductase. Further assay by mass spectroscopy is required to ascertain whether or not azoreductase and nitrogen reductase are the different measures of the same feature.

The distribution of nitrogen fixation amongst other species of *Vibrionaceae* apart from *V.diazotrophicus* e.g. for *V.campbellii* has already been reported, (West *et al.,* 1985; Uradaci *et al.,* 1988) indicating the feasibility of the acetylene reduction assay result for *V.campbellii* UQM 2779 and other species being correct.

A6.4 Conclusion.

The results obtained are tentative but support the possibility azo-reductase asays might also indicate nitrogen fixation. It was shown that there was no detectable activity by the nitrogen fixing bacterium *V.diazotrophicus* towards mono-azo dyes, but some activity was detected towards dis-azo dyes and a dose dependent effect was found in activity towards congo red. A correlation was found between putatively nitrogen fixing species detected by this method and those confirmed by the acetylene reduction procedure. It was observed that dye solution exposed to *V.diazotrophicus* exhibited a absorption spectrum which may be that of a mono-azo dye.

While the spectrophotometric procedure developed and tested as above was successful in most cases in detecting nitrogen reduction, the poor, *ca* (70%), correlation of results with the acetylene reduction procedure indicate its use, as presented above, only as a presumptive assay.

Problems associated with the method were related to dye precipitation and to sampling of absorbance at a relatively insensitive point in the congo red absorbance curve. Assuming that azo-reductase is a constitutive enzyme media could be instead prepared to limit "salting out", i.e. by using only MPBS to maintain pH control, and prepared at a lesser absorbance at a lower wavelength i.e. *ca* 0.3 absorbance units at 475 nm by adding 250 μl inoculum as above but reading after only 14 d.

If no activity is detected in these starvations conditions then the enzyme is most probably induced, and a universal carbon-source such as glucose or maltose may need to be incorporated in the medium to provide energy for reordering of cellular enzyme production, with this come concomitant requirements for additional buffering to counter acid production.

APPENDIX 7.

A7.0 Electron Microscopy.

A7.1 Experimental Design.

Baumann and Schubert (1984) used the presence of sheathed polar flagella on *Vibrio* spp. as a primary criterion for their differentiation from species of *Photobacterium.* While the presence of sheathed flagellae is easily discerned by negative stains the clear electron-micrographic resolution of cells from marine *Vibrios* is frustrated by two features. The first stems from requirements of these species for sodium to maintain membrane integrity, (Unemoto, Tsuruoka and Hayashi, 1973). If cells are stained in the absence of sodium the possibility exists that surfaces visualized are induced as a result of osmotic stress; if stained in the presence of sodium chloride a mat of salt crystals appears on support films to disrupt preparations and obscure observations.

The second feature of these bacteria is occurrence of a layer of electron-dense extracellular slime. Allen and Baumann (1971) cleared these slimes from negative stains by using 0.4 % sucrose as a wetting agent. No acknowledgment was made to the possibility that the cells were subjected to osmotic stress by this procedure. Gregory and Pirie (1973) and J.A. Fuerst (pers. comm.) advocated the use of bacitracin as a wetting agent for the examination of such electron dense specimens.

In the present work a comparison was made of differing protocols which incorporated bacitracin or sucrose as wetting agents, in the presence of different negative stains with and without osmotic balancing.

A7.1.1 Electron Microscopy Reagents.

A7.1.1.1 Negative Stains.

(1) *Dodeca*-tungstophosphoric Acid, (1% aq.) (PTA) ($24WO_3 .2H_3PO_4 .48H_2O$) (Ajax AR) adjusted to pH 6 with 1 N sodium hydroxide.

(2) Ammonium Molybdate, (1%) $(NH_4)_6Mo_7O_{24}.4H_2O$ (Ajax AR) adjusted to pH 4.0 with 1 N hydrochloric acid in 0.334 M sodium chloride. (Molybdate/Saline).

(3) Uranyl Acetate, (1% aq.), $UO_2(OOCCH_3)_2.2H_2O$ (BDH-Analar) adjusted to pH 4.0 with 1 N acetic acid. (UAc)

(4) Uranyl Acetate, (2%), pH 4.0 in 0.295 M sodium chloride (UAc/Saline).

A7.1.1.2 Washing Reagents.

(1) Bacitracin (Sigma 74 200 units/g) 10 mg/ml (aq).

(2) Sucrose, 1% (w/v) (aq.).

A7.1.1.3 Suspension Buffers.

(1) NaCl, 2 %.

(2) NaCl, 2 % in 25 μg/ml Bacitracin.

(3) NaCl, 2 % in 250 μg/ml Bacitracin.

A7.1.2 Preparation of Grids.

Copper grids, 200 mesh, (Polaron Equipment) were prepared by Ms Jane Westcott as follows: Grids were mounted on a 1mm wire mesh in a bath containing 1% (w/v) cellulose nitrate in isoamyl acetate. The bath was drained, and the mesh dried in a lidded glass petridish, to leave cellulose nitrate grid films.

A7.1.3.1 Negative Staining.

Grids were held by forceps, and a drop (0.05 ml) of culture suspension was placed on the grid film. Cells were stained by exposure to a drop of negative stain *ca* 30 secs, and excess fluid drawn into a blotting paper wick. Preparations were mounted on a stage and sealed in the evacuated chamber of Hitachi H-800 electron microscope and examined by TEM at magnifications of 10 000 - 20 000.

A7.1.3.2 Cell Cultures.

Cells preparations were made from 18-24 h cultures on SENA medium.

A7.1.3.3 Staining Protocols.

A7.1.3.3.1 Phosphotungstic Acid.

Growth from cultures of *V.anguillarum* UQM 2771, and UQM 2843; was suspended in 2% saline and stained with 1% PTA.

A7.1.3.3.2.1 Sodium Chloride (2 %)/Bacitracin (25 µg/ml) with Uranyl Acetate and Ammonium Molybdate.

Growth of *V.anguillarum* UQM 2771 was harvested into 2 tubes containing the buffers from A7.1.1.3.1-2. and stood 2 h. Cell suspension from the first tube were deposited on the grid membrane as above, washed with sucrose and stained with UAc. Preparations from cells soaked in 25 µg/ml bacitracin were stained by Molybdate and UAc/Saline.

A7.1.3.3.2.2 General Evaluation of Bacitracin/Molybdate/Saline Staining.

Consequent to A7.1.3.3.2.1, *Aeromonas hydrophila* UQM 2768 *Vibrio cholerae* UQM 2772, UQM 2773, *V.fluvialis* UQM 2774, *V.parahaemolyticus* UQM 2776, *V.diazotrophicus* UQM 2780, *V.natriegens* UQM 2782, *V.nereis* UQM 2783, *V.nigripulchritudo* 2784, *V.pelagius* UQM 2785, and *V.harveyi* UQM 2855, were washed with the second suspension buffer (A7.1.1.3.2) and stained with molybdate/saline.

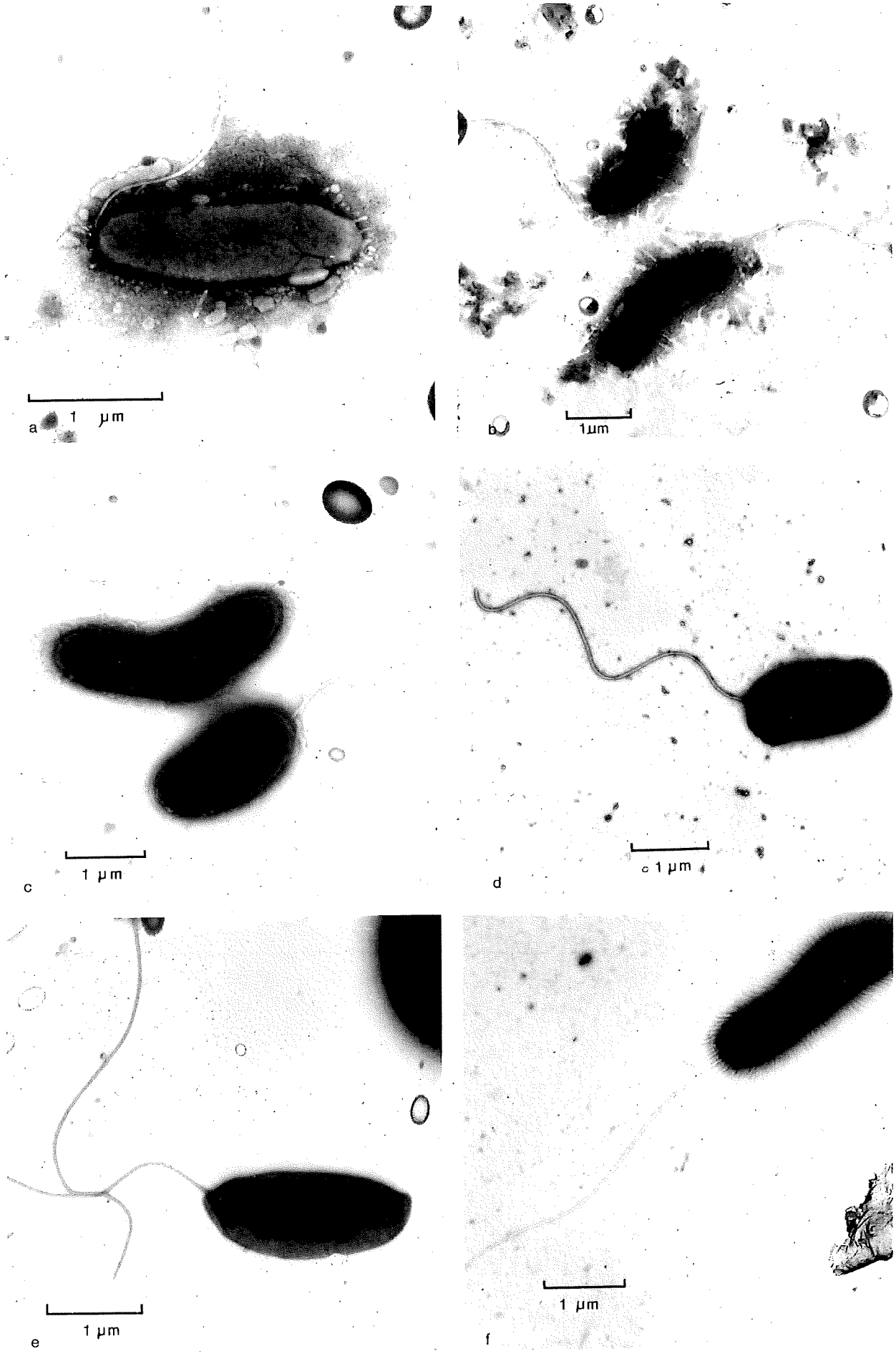
A7.1.3.3.3 Sodium chloride 2 %, in 250 µg/ml Bacitracin with Uranyl Acetate.

Growth from *Vibrio zobellii* UQM 3028 and UQM 3029 was suspended into 2 ml sterile 2 % NaCl in 250 µg/ml Bacitracin, stood for 90 minutes, and stained with UAc/Saline.

A7.2 Results and Discussion.

Cells of *V.anguillarum* UQM 2771 and UQM 2843 (Plate A7.1 a-b) stained well by PTA but various abberations occurred. The surface of films surrounding cells was grainy with an uneven texture on both preparations. This effect was most prominent at the edges of cells where dendrites occurred resulting

Plate A7.1.(a-f) Electron Micrographs of *Vibrionaceae,* respectively a,b *V.anguillarum* UQM 2771, UQM 2843 (PTA); c, UQM 2771 25 µg/ml bacitracin/(molybdate/saline); d, UQM 2771 25 µg/ml bacitracin-(UAc/saline); e, UQM 2771 sucrose-UAc; f, *A.hydrophila* UQM 2769 µg/ml bacitracin-(molybdate/saline).


1 µm
a
1µm
b
1 µm
c
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d
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e
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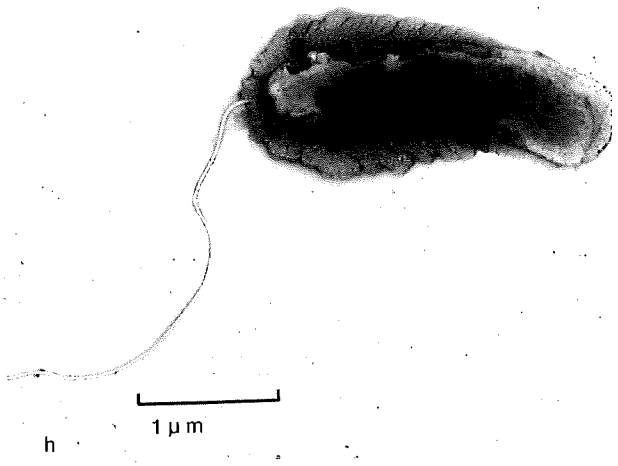
f

Plate A7.1.(g-l) Electron Micrographs of *Vibrionaceae*, 25 µg/ml bacitracin-(molybdate/saline); g,h *Vibrio cholerae* UQM 2772, UQM 2773; i, *V.fluvialis* UQM 2774; j, *V.parahaemolyticus* UQM 2776; k, *V.diazotrophicus* UQM 2780; l, *V.natriegens* UQM 2782.

g



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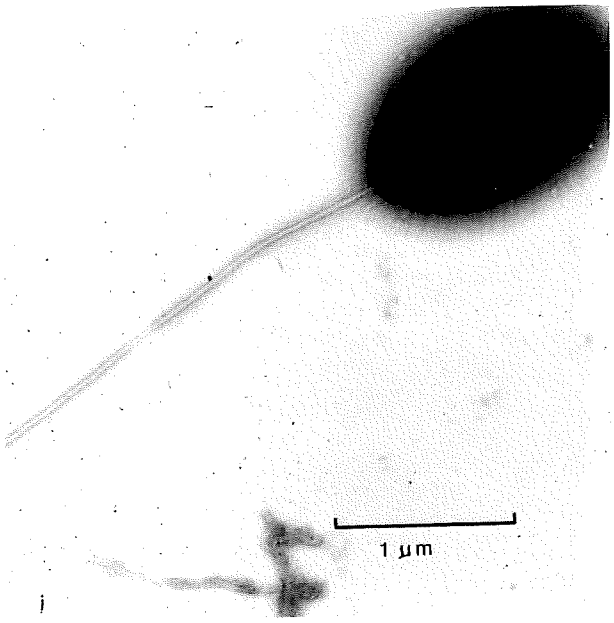

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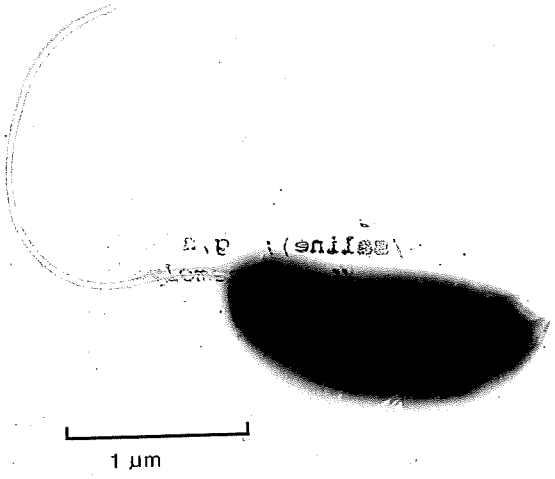

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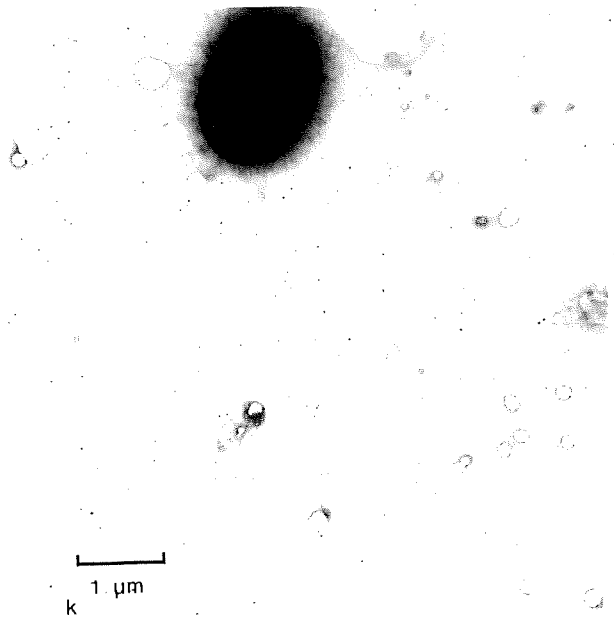

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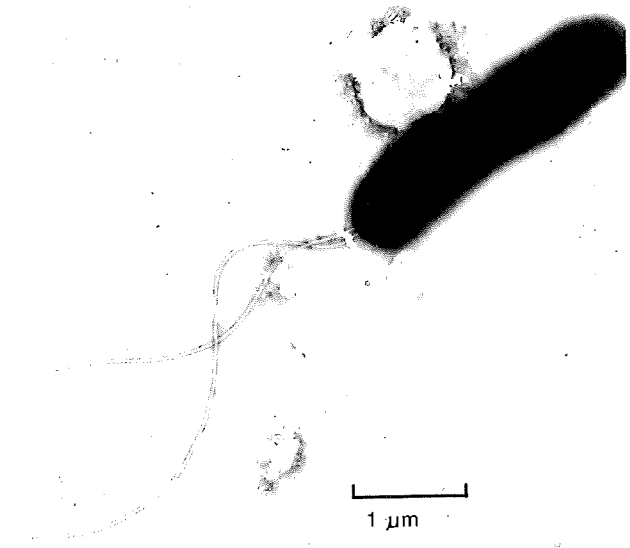

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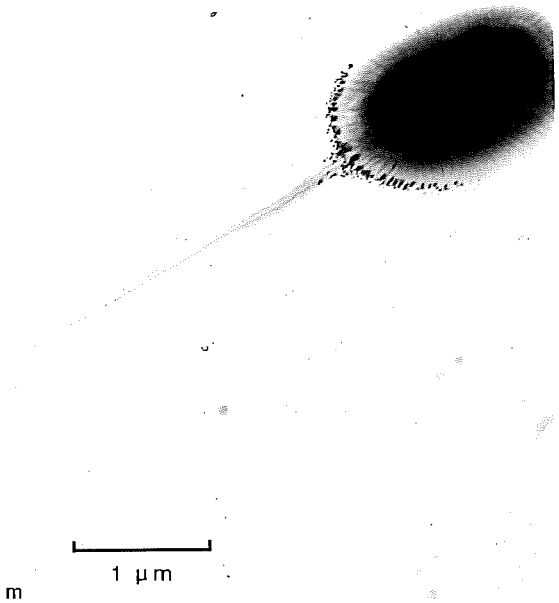
Plate A7.1.(m-r) Electron Micrographs of *Vibrionaceae;* m-p, 25 µg/ml bacitracin-(molybdate/saline); m, *V.nereis* UQM 2783, n, *V.nigripulchritudo* 2784; o *V.pelagius* UQM 2785; p, *V.harveyi* UQM 2855; (q,r, 250 µg/ml bacitracin-(UAC/Saline)) *V.zobellii* UQM 3028, UQM 3029.



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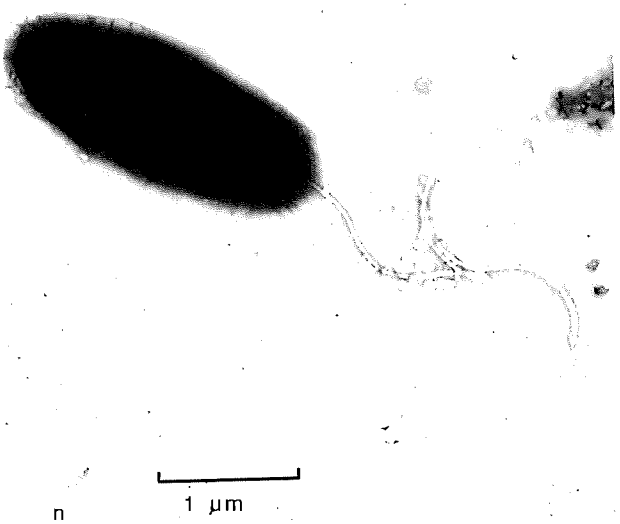

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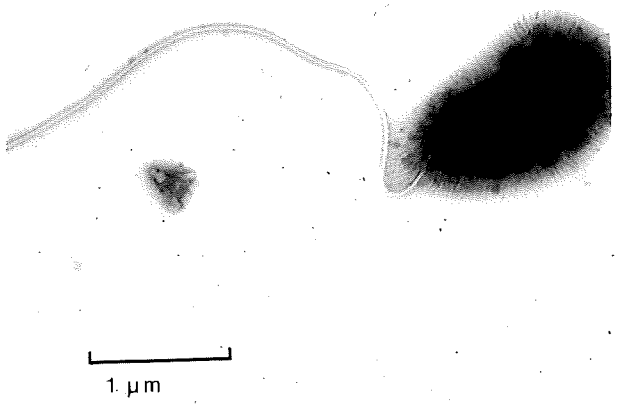

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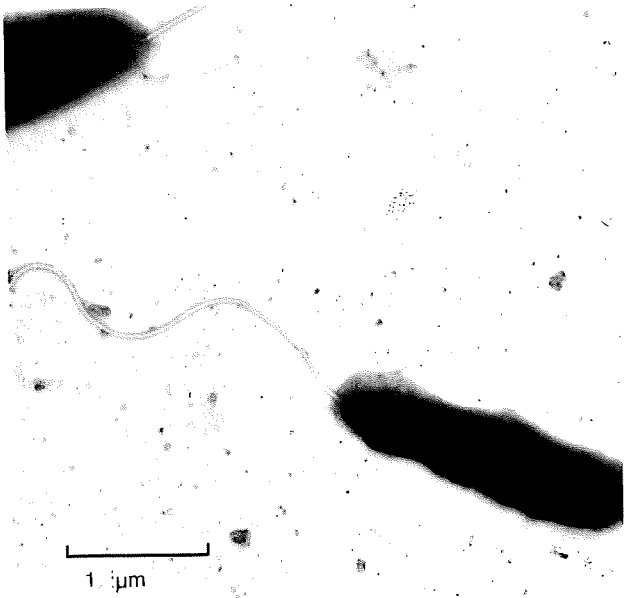

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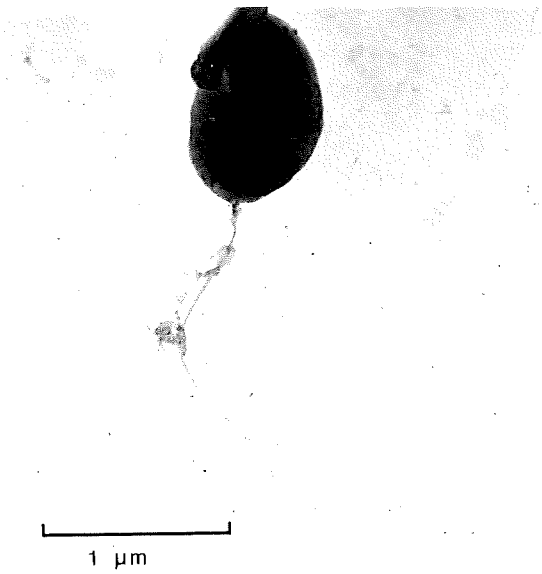

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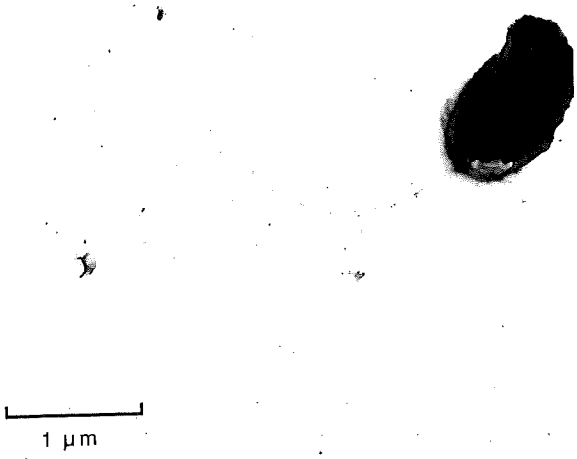


r

presumably from uneven drying. Surface slimes were present but these were not stained heavily by PTA. Surface outgrowths, were present to a variable extent in all preparations.

Preparations of *V.anguillarum* UQM 2771 soaked in bacitracin or rinsed in 1 % sucrose (Plates A7.c,d;e) prior to staining were cleaner and largely free from the cell and grid surface artifacts mentioned above. Cells stained with 1 and 2 % UAc(/Saline) had better tonal qualities than those stained with Molybdate (Plates A7.d,e;c respectively), particularly for resolution of flagella; but cell envelopes of *V.anguillarum* stained by UAc and UAc/Saline were collapsed. Cells washed by sucrose were cleaner than those treated by bacitracin but the collapse of cells with this washing agent seemed more severe than with 2% UAc/Saline. This may have been because removal of slime coats renders cells more porous, or because sodium ions previously binding cell membranes are dispersed into solution, with sucrose consequently making the cells more fragile, (cf. Tamura *et al.*, 1976, (2.10.1). Because of collapse of cells under UAc but not with the combination Bacitracin/Molybdate/Saline this protocol was further evaluated. Plates A71.f-p were respectively of the following cultures *Aeromonas hydrophila* UQM 2768, *Vibrio cholerae* UQM 2772, UQM 2773, *V.fluvialis* UQM 2774, *V.parahaemolyticus* UQM 2776, *V.diazotrophicus* UQM 2780, *V.natriegens* UQM 2782, *V.nereis* UQM 2783, *V.nigripulchritudo* 2784, *V.pelagius* UQM 2785 and *V.harveyi* UQM 2855. While no significant surface outgrowths were seen under this protocol, bacitracin did not remove slime in all cases. This may have been due to different quantities of slime produced by cells of different culture age or possibly slimes chemically differed between species. Further, the flagella wavelengths of some species stained under this protocol seemed atypical e.g. *V.fluvialis*, *V.nigripulchritudo* and *V.cholerae* UQM 2772.

V.zobellii UQM 3028 and UQM 3029 stained after soaking in 250 µg/ml bacitracin did not have cellular envelopes which collapsed upon treatment with UAc/Saline. The slime coat partially sloughed allowing estimation of thickness to i.e. 1.5 times that of the sheathed flagellum.

A7.3 Summary and Conclusion.

Negative stains in isotonic solution reduced the incidence of surface outgrowths in *V.anguillarum*, and treatment by 25 µg/ml bacitracin and 2 % sucrose successfully removed surface slimes from late log phase *V.anguillarum* grown on SENA medium. However the treatment and staining protocol appropriate for this species was not suitable for most other species of *Vibrio*.

APPENDIX 8.

A8.0 Evaluation of Two Commercial *Vibrionaceae* Identification Schemes.

A8.1 Experimental Design.

Detailed numerical analysis is not practical for rapid diagnosis of bacterial species. An option now used for the majority of commonly encountered clinical bacterial identifications are miniaturized commercial diagnostic arrays with corresponding species profile registers. The usefulness of these systems to environmental or even medical *Vibrionaceae* has not been thoroughly examined, (See 2.8).

Commercially prepared diagnostic testing schemes prepared by **Analytab Products, (API** *20 NE),* and **Eiken Chemical Company, (Eiken** *Systek No.1),* were compared for their recognition of *Vibrio* species. The **API** scheme was designed to identify Gram-negative oxidative bacteria and the **Eiken** to identify *Enterobacteriaceae.* The makers of both schemes claim that their products can also diagnose common species from the *Vibrionaceae* especially when supplementary tests for tolerance to sodium chloride and 0/129 phosphate are conducted.

The API scheme is claimed to recognize profiles generated by *Aeromonas hydrophila, Plesiomonas shigelloides, Vibrio cholerae, V.parahaemolyticus, V.alginolyticus,* and *V.vulnificus.* Eiken claim that their scheme will also resolve the species *A.sobria, V.metschnikovii, V.mimicus,* and *V.fluvialis.*

Not addressed in previous evaluations of such schemes (2.8.1) are the fundamental sodium and temperature requirements of some *Vibrionaceae* for growth; and correspondence of results between miniaturized and standard phenotypic tests. Ideally, and depending upon how well test characters are chosen such schemes should produce identical or very similar profiles for the same species and different profiles for different species. Even if the chosen characters are not ideal, if tests are used with an adequate database, (profile register); then strains will be identified to the maximum resolution of test characters, i.e. to a group of species, genus etc.

Sufficient strains to gauge test variation within- and between- species were selected, (Tables 3.4-5,7), so that real, (theoretical, unkeyed), and apparent, (keyed), resolutions of these schemes would be demonstrated. Tests were conducted at temperatures and salinities known to support all strains.

After tests were conducted, the COMP program (2.8.1.2) was operated to sequentially list the most differential characters amongst the *Vibrionaceae,* and to determine how many of these characters were also in the commercial schemes. This program differs from the *GBEST* program of Bryant *et al.,* (1986) by the capacity to sequentially rank the differential quality of characters.

A8.1.1 API *20 NE.*

The API *20 NE* (Plate A8.1, top) system requires preliminary, separate observation of the oxidase reaction and then inoculation and incubation of a wafered tray with 20 test cupules comprising eight tests for enzyme production, (nitrate reduction, indole production, acid from glucose, arginine dihydrolase, urease, gelatin hydrolysis and β-galactosidase); and 12 carbon assimilation tests in vitamin enriched medium, for glucose, arabinose, mannose, mannitol, *n*-acetyl-glucosamine, maltose, gluconate, caprate, adipate, malate, citrate, and phenylacetate.

Cultures of 21 strains of *Vibrio* spp. from (Table 3.4-5) were taken from storage, grown for 24 h in SETSB and verified as pure by streaking to SEHIA and incubating a further 24 h.

Colonies were picked, from pure cultures the oxidase reaction scored as in (3.8.2.2.4.3) and cell suspensions were prepared in 2 ml 0.85 % sterile saline in 12 x 75 ml capped tubes (Kimble) to a turbidity equivalent of 0.5 on the MacFarland scale.

Aliquots of cell suspensions were used to fill test-kit cupules. Cupules for glucose fermentation, arginine dihydrolase and urease were additionally overlaid with sterile mineral oil. A loopful of each inoculum suspension was

streaked to separate plates of SEHIA medium to confirm purity as above. Inoculated trays were incubated at 30°C. and results recorded after 48 h. In some cases it was necessary to re-inoculate glucose fermentation cupules before acid production was demonstrated. Reagents used to test for nitrate reduction and indole production were respectively prepared as in (3.4.4.07.1) and (3.4.4.05.1).

Results for carbon assimilation were scored as positive if more than a slight turbidity was present. Uncoded data is in Table A8.1. For profile identification, results were coded in seven groups of three octal numbers and were compared with the API reference library.

A8.1.2 Eiken *Systek No. 1.*

The Eiken *Systek No. 1* test array, (Plate A8.1, bottom), comprises 20 wells and 5 test strips. The characters tested in the system are for cytochrome oxidase, nitrate reduction, production of sulphide (ferrous indicator), β-galactosidase, indole pyruvic acid, acetoin, indole (l-tryptophan) and urease, citrate and malonate utilization; lysine, ornithine, and arginine decarboxylases, and fermentation of the carbohydrates glucose, mannitol, adonitol, arabinose, inositol, rhamnose, sorbitol, maltose, and sucrose (phenol red indicator).

Cultures of 47 *Vibrionaceae* strains from (Tables 3.4-5,7) were removed from storage and verified as pure on SENA. Suspensions in 4.5 ml sterile 2 % saline were prepared to a turbidity equivalent of 3 on the MacFarland scale. Inocula were streaked onto SENA to test purity. The remainder of the inoculum was transferred to the tray inoculation trough, rocked longitudinally and tilted to rehydrate and inoculate test wells as in the maker's instructions. Wells for decarboxylase and urease tests were overlaid with sterile mineral oil. Trays were covered and incubated at 25°C. for 24 h. Oxidase tests were performed on supplied test-kit strips but scored after 10 seconds rather than 1 minute as set out by the maker. A drop (0.05 ml), 40% potassium hydroxide was added to well 4 and supplied test strips for

ONPG, (yellow positive); indole pyruvic acid (red brown positive), acetoin and indole (red positive from l-tryptophan); were immersed in wells, 2-5 and read immediately except for acetoin which was read after after 15 minutes. Results for the other biochemical tests were scored after 24 h and coded as for API kits. Uncoded data is in Table A8.2.

A8.1.3 Analysis Procedures.

A8.1.3.1 API and Eiken Schemes.

Comparative results from characters in the test schemes were checked where possible against results from first replicates of standard bench tests from (3.8.3-6); Disk_2 \APENDICES\APNDX_ 12 and transferred as listed in Tables A8.1-2 to the hybrid data-files API20NE.BEN and SYSTEK.BEN in the sub-directory APNDIX_08). Comparative data was pooled within taxa and the correspondance (%), calculated (Table A9.3). Table A9.3 also indicates whether the profiles generated were keyed or unkeyed by the profile registers and is further scored according to the resolution of keyed or unkeyed (apparent and real) identifications (by indicating the taxonomic level at which the test profile might be confused with that of another taxon i.e. species (S), genus (G), family (F) or order (O) so that coincidental identifications by these schemes are indicated).

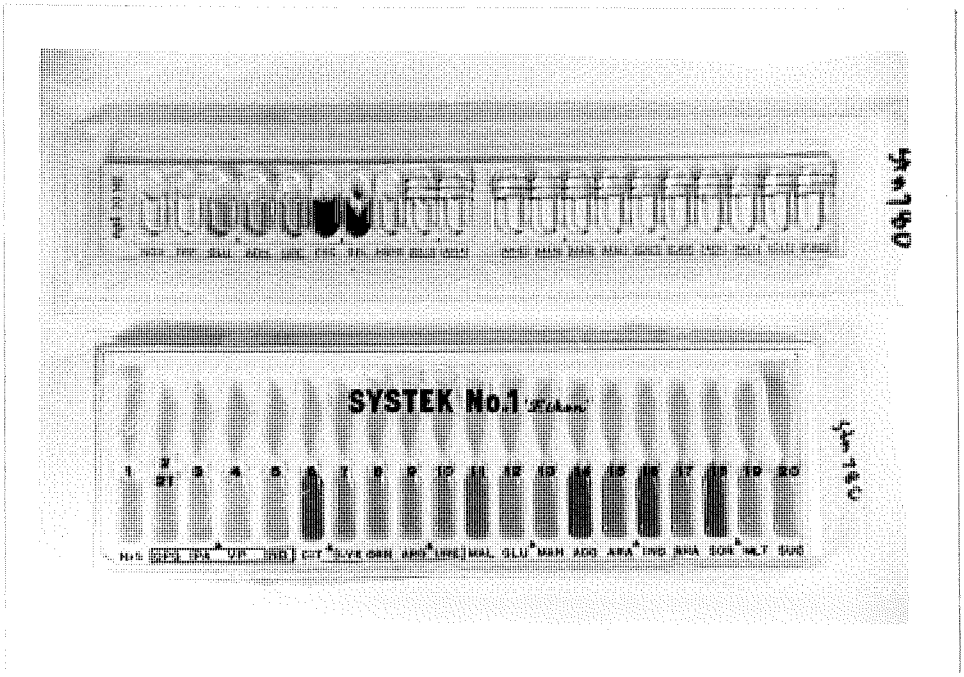


Plate A8.1: Top; API *20 NE,* and Bottom; Eiken *Systek No. 1,* Test Arrays.

A8.1.3.2 *Vibrionaceae* Diagnostic Features.

The *COMP* program (2.8.1.2) was run and nulls were inserted at the first 27 prompts so that a serial list of diagnostic features for the *Vibrionaceae* was derived. These were compared with tests contained in commercial schemes.

A8.2 Results and Discussion.

In the API array only the nitrate reduction test was uniformly positive, however the result was incorrect for *V.metschnikovii*. In the Eiken array, sulphide and indole pyruvic acid tests were uniformly negative. Test agreement for strains with earlier phenetic analysis ranged from *ca* 43 to 90 % for the API system and 53 to 100 % for the Eiken system. The indole tests for both schemes were more sensitive than the bench test used.

The API array required heavy inoculation to prevent false negative results for glucose fermentation. Similarly neither array detected all arginine dihydrolase (ADH) positive strains e.g. API, failed to detect the reaction by *V.metschnikovii* and Eiken by *V.diazotrophicus* UQM 2780. Different strains within species, e.g. *V.harveyi,* in both arrays generated profiles as varied as those produced between species.

Only one of the twenty-one strains tested was coded for in the profile register for the API system and this was misidentified *[V.fluvialis* as *A.hydrophila].* Only 4 correct identifications were made of the twenty-two profiles recognized from forty-seven strains tested by Eiken system. These were strains of *A.hydrophila* and *V.cholerae*. Profiles generated even by type strains of other makers' designated target species were not recognized by either scheme. Both systems failed to distinguish *A.hydrophila* from species of *Vibrionaceae* which produced ADH. Eiken misidentifications resulted from confusion of oxidase-negative species of *Enterobacteriaceae* and *Vibrionaceae* and of sucrose positive and negative species of *Vibrio* with *V.cholerae* and *V.mimicus*.

With the API scheme less than 70 % of tests were correspondent to bench tests for *V.anguillarum, V.cholerae, V.metschnikovii* and *V.nigripulchritudo*

Table A8.1 : API *20 NE* Profiles from 21 *Vibrionaceae* Strains.

		Nitrate reduction	Indole production	Glucose	Arginine dihydrolase	Urease	Aesculin hydrolysis	Gelatin hydrolysis	PNPG	Glucose	Arabinose	Mannose	Mannitol	N-Acetyl-glucosamine	Maltose	Gluconate	Caprate	Adipate	Malate	Citrate	Phenylacetate
Aeromonas hydrophila	UQM 2768	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A.hydrophila	UQM 2838	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Vibrio anguillarum	UQM 2628	+	+	+	-	-	-	+	+	+	-	+	+	+	+	+	-	-	+	-	-
V.anguillarum	UQM 2843	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-
V.campbellii	UQM 2744	+	-	+	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-
V.cholerae	UQM 2732	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+
V.costicola-like	Stn 273	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V.fluvialis	UQM 2774	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
V.harveyi	UQM 2781	+	+	(+)	-	-	+	+	+	+	-	+	+	-	+	+	-	-	+	+	-
V.harveyi	Stn 47	+	+	(+)	-	-	-	+	-	+	+	+	+	+	+	+	-	+	+	+	-
V.harveyi	UQM 2839	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	-	-	+	+	-
V.harveyi	UQM 2855	+	+	+	-	-	+	+	-	+	-	+	+	+	+	+	-	+	+	+	+
V.harveyi	ϕ 29	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+	+	-
V.metschnikovii	UQM 211	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-
V.natriegens	UQM 2782	+	-	+	-	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+
V.nigripulchritudo	UQM 2784	+	-	(+)	-	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	-
V.orientalis	UQM 3012	+	-	(+)	-	-	-	+	+	-	-	-	-	-	-	+	-	-	+	+	-
V.parahaemolyticus	UQM 2776	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	+	+	+	-
V.vulnificus	UQM 2729	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+	-
V.vulnificus	UQM 2740	+	+	+	-	-	-	+	-	+	+	+	+	+	+	+	-	+	+	+	-
V.vulnificus	UQM 2745	+	+	(+)	-	-	-	+	-	+	+	+	+	+	+	+	-	+	+	+	+

(+) Denotes weak reaction.

Table A8.2 : Eiken *Systek No. 1* Profiles from 47 *Vibrionaceae* Strains.

		Oxidase	Sulphide	ONPG	Indpyruvic acid	Acetoin prod.	Indole prod.	Citrate ut.	Lysine decarb.	Ornithine decarb.	Arginine dihydr.	Urease prod.	Malonate ut.	Gluconate acid	Mannitol acid	Adonitol acid	Arabinose acid	Inositol acid	Rhamnose acid	Sorbitol acid	Maltose acid	Sucrose acid	Nitrate reduction
Aeromonas hydrophila	UQM 2769	+	-	+	-	-	+	-	+	-	+	-	-	+	+	-	-	-	-	-	+	+	+
A.hydrophila	UQM 2838	+	-	+	-	-	+	-	-	-	+	-	-	+	+	-	-	-	-	-	+	+	+
A.hydrophila	Stn 3230	+	-	+	-	+	+	-	+	-	+	-	-	+	+	-	-	-	-	-	+	+	+
A.hydrophila	Stn 7770	+	-	+	-	-	+	-	+	-	+	-	-	+	+	-	-	-	-	-	+	-	+
A.hydrophila	Stn 7840	+	-	+	-	-	+	-	-	-	-	-	-	+	+	-	+	-	-	-	+	+	+
Photobacterium-like.	Stn 3060	+	-	+	-	-	+	-	-	-	-	-	-	+	+	+	+	-	+	-	+	+	+
Vibrio alginolyticus	UQM 2770	+	-	-	-	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	+	+	+
V.alginolyticus	Stn 3450	+	-	-	-	-	+	-	+	-	-	-	-	+	+	-	+	-	-	-	+	-	+
V.anguillarum	UQM 2628	+	-	+	-	+	+	-	-	-	-	-	-	+	+	+	+	-	-	+	+	+	+
V.anguillarum	UQM 2771	+	-	+	-	+	+	-	-	-	+	-	+	+	+	-	+	-	-	+	+	+	+
V.anguillarum	UQM 2843	+	-	+	-	+	+	-	-	-	+	-	-	+	+	-	+	-	-	+	+	+	+
V.campbellii	UQM 2779	+	-	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	-	-	+	-	+
V.campbellii	UQM 2744	+	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-	+	+	+
V.campbellii	Stn 7000	+	-	+	-	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	+	-	+
V.carchariae	UQM 2849	+	-	+	-	-	+	-	+	+	-	+	-	+	+	-	-	-	-	-	-	+	+
V.carchariae	Stn 6550	+	-	-	-	-	+	-	+	+	-	+	-	+	-	-	-	-	-	-	+	-	+
V.carchariae/V.harveyi	Stn 7360	+	-	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	-	+	+	-	+
V.carchariae/V.harveyi	Stn 7460	+	-	-	-	-	+	-	+	-	-	-	-	+	+	-	-	+	+	+	+	+	+
V.carchariae/V.harveyi	UQM 2855	+	-	-	-	-	+	+	+	+	-	+	-	+	+	-	-	-	-	+	+	+	+
V.cholerae	UQM 2772	+	-	+	-	+	+	-	+	+	-	-	-	+	-	-	-	-	-	-	+	+	+
V.cholerae	UQM 2773	+	-	+	-	+	+	-	+	+	-	-	-	+	+	-	-	-	-	-	+	+	+
V.diazotrophicus	UQM 2780	+	-	+	-	-	+	+	-	-	-	-	-	+	+	-	+	-	+	-	+	+	+
Vibrio fluvialis	UQM 2774	+	-	+	-	-	-	-	-	-	+	-	-	+	+	-	+	-	-	-	+	+	+
V.fluvialis	Stn 1640	+	-	+	-	-	+	+	-	-	+	-	-	+	+	-	+	+	+	+	+	+	+
V.fluvialis	Stn 1670	+	-	+	-	-	+	-	-	-	+	-	-	+	+	-	+	-	-	-	+	+	+
V.furnissii	UQM 2775	+	-	+	-	-	+	-	-	-	+	-	-	+	+	-	+	-	-	-	+	+	+
V.gazogenes	UQM 2840	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	+
V.harveyi	UQM 2724	+	-	-	-	-	+	+	+	-	-	+	-	+	+	-	-	-	-	-	+	+	+
V.harveyi	Stn 182	+	-	-	-	-	+	-	+	+	-	-	-	+	+	-	-	-	-	-	+	+	+
V.harveyi	Stn 200	+	-	-	-	+	+	-	+	+	-	-	-	+	+	-	-	-	-	-	+	+	+
V.harveyi	UQM 2781	+	-	+	-	-	+	+	+	+	-	-	-	+	+	-	-	-	-	-	+	+	+
V.harveyi	UQM 2723	+	-	-	-	-	+	+	+	+	-	+	-	+	+	-	-	-	-	-	+	+	+
V.harveyi	ϕ 29	+	-	-	-	-	+	-	+	+	-	+	-	+	+	-	-	-	-	-	+	+	+
V.harveyi	UQM 2839	+	-	+	-	-	+	+	+	+	-	-	-	+	+	-	-	-	-	+	+	+	+
V.harveyi	Stn 6390	+	-	+	-	-	+	-	+	-	-	-	-	+	+	-	+	-	+	-	+	-	+
V.metschnikovii	UQM 211	-	-	+	-	+	+	-	-	-	+	-	-	+	+	+	-	+	-	-	+	+	-
V.natriegens	UQM 2782	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	+	-	+	+	+
V.nereis	UQM 2783	+	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	+
V.nigripulchritudo	UQM 2784	+	-	-	-	-	+	-	-	-	-	-	+	+	+	-	+	-	+	-	+	-	+
V.orientalis	Stn 6690	+	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	+
V.parahaemolyticus	UQM 2776	+	-	-	-	-	+	-	+	+	-	+	-	+	-	-	+	-	-	-	+	-	+
V.parahaemolyticus	Stn 6410	+	-	+	-	-	+	-	+	-	-	-	-	+	-	-	+	-	+	-	+	-	+
V.pelagius	Stn 7650	+	-	+	-	-	+	-	+	+	-	-	-	+	+	-	+	-	+	-	+	-	+
V.vulnificus	UQM 2778	+	-	-	-	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	+
V.vulnificus	UQM 2740	+	-	-	-	-	+	-	+	+	-	-	-	+	+	-	-	-	-	-	+	-	+
V.vulnificus	UQM 2743	+	-	+	-	-	+	-	+	-	-	-	-	+	+	-	-	-	-	-	+	-	+
V.vulnificus	Stn 7510	+	-	+	-	-	+	-	+	-	-	-	-	+	+	-	-	-	-	-	+	-	+

Table A8.3 : Comparative Evaluation of Rapid Identification Schemes.

Species Examined.	Number of Strains.	API 20 NE. Resolution. Keyed *	API 20 NE. Resolution. Unkeyed	API 20 NE. (%) Congruence. (17 - 21 Tests).	Number of Strains.	Eiken Systek No. 1. Resolution. Keyed	Eiken Systek No. 1. Resolution. Unkeyed	Eiken Systek No. 1. (%) Congruence. (10 - 17 Tests).
Aeromonas hydrophila	2	-	F	(85)	5	2S,3G	F	(81)
Photobacterium sp.	-	-	-	-	1	O	O	(64)
V.alginolyticus	-	-	-	-	2	-	G	(80)
V.anguillarum	2	G	F	(53)	3	F,2O	F	(56)
V.campbellii	-	-	-	-	3	-	G	(82)
V.carchariae	-	-	-	-	2	-	G	(81)
V.carchariae/V.harveyi	-	-	-	-	3	-	G	(83)
V.cholerae	1	-	G	(56)	2	2S	G	(73)
V.costicola-like	1	-	G	(84)	-	-	-	-
V.diazotrophicus	-	-	-	-	1	O	F	(90)
V.fluvialis	1	-	F	(81)	3	2F,O	F	(85)
V.furnissii	-	-	-	-	1	F	F	(91)
V.gazogenes	-	-	-	-	1	-	O	(82)
V.harveyi	5	-	G	(79)	8	-	G	(77)
V.metschnikovii	1	-	G	(65)	1	-	O	(95)
V.orientalis	1	-	F	(79)	1	O	F	(100)
V.natriegens	1	-	G	(90)	1	-	G	(64)
V.nigripulchritudo	-	-	G	(43)	1	-	G	(91)
V.nereis	-	-	-	-	1	-	F	(95)
V.parahaemolyticus	1	-	G	(90)	2	-	G	(89)
V.pelagius	-	-	-	-	1	-	G	(55)
V.vulnificus	3	-	G	(84)	4	3G	G	(86)

* (S) Species, (G) Genus, (F) Family, (O) Order.

With the Eiken scheme *Photobacterium*-like, *V.anguillarum, V.natriegens* and *V.pelagius* strains were less than 70 % correspondent with bench test results.

Twenty-seven sorted diagnostic tests for the most differential resolution (numbered) of presumptive *Vibrionaceae* yielded from the COMP program were; arginine (2), lysine (13) and ornithine (5) decarboxylases, separate utilization of DL-hydroxybutyrate (3), trehalose (4), n-acetyl glucosamine (16), d-glucose (12), l-arabinose (14), maltose (17), propanol (20), glucuronate (21), gluconate (22), leucine (25) and fructose (27), hydrolysis of starch (1), gelatin (6), DNA (9), lecithin (11), xanthine (26), production of acetoin (15), acid from arabinose (23), swarming (7), growth

in the presence of 50 i.u. polymyxin (19), growth in the absence of sodium chloride (8), and growth at 4°C. (10). The API scheme included six (2,6,14,16,17 & 22) of these features and the Eiken only five (2,5,13,15, & 23). Both the API and Eiken schemes included eleven tests equivalent to those in *Vibrionaceae* probability matrix (Table 3.26).

A8.3 Conclusion.

Both systems were mechanically well designed, easy to inoculate and interpret but the addition of salt, and lowering of incubation temperature did not make diagnostic schemes for terrestrial bacteria suitable for the accurate resolution of either medical or environmental *Vibrionaceae.* Both were inadequate for the resolution of any species with confidence and there were large, consistencies of results between-systems and when compared with standard bench tests e.g. *V.natriegens* and *V.metschnikovii.* Maker's suggestions for supplementary tests such as for growth in the presence of 0/129 and 6 % saline may help with the differentiation of *A.hydrophila* from ADH positive *Vibrio* spp. but do not assist with further resolution of species within this now substantial group (See 2.7.2.5.1). Most tests in these arrays are non-discriminatory and show variability rather than stability of profiles within species. The indole test by using tryptophan became too sensitive and failed to distinguish weak and strong indole producers. The diagnostic schemes based on the arrays may seem relevant only if a small number of species is examined, but with any number of strains from the same species this inadequacy becomes apparent. An introduction by the makers of more strains to the profile registers would cause identifications to approximate the earlier-listed theoretical resolution for species and as such would provide users a more responsible indication of other species with similar profiles. It would be possible to increase resolution of these schemes by introducing further supplementary-tests, but to do so would not be cost- effective. For less than the time and cost of pooling both schemes more effective diagnoses could be performed in the array from (3.8.6.5) in 48- well tissue culture trays.

APPENDIX 9.

A9.0 Determination of *Vibrionaceae* DNA Base Composition and DNA Relatedness.

A9.1 Experimental Design.

Some phenetic strain assignments were secondarily checked by thermal examinations of DNA to determine G + C base ratios and DNA relatedness. Time restricted such assays to strains which were phenetically similar to known fish pathogens, or which were distinct from previously described species.

A9.1.1 Bacterial Cultures.

DNA was extracted from the following type or reference cultures with origins as listed in Tables 3.4-5: *Escherichia coli* UQM 1803, *V.anguillarum* UQM 2771; Σ 1017 = UQM 2843; *V.campbellii* UQM 2779, *V.carchariae* UQM 2920; *V.natriegens* UQM 2782, *V.nereis* UQM 2783 and *V.pelagius* UQM 2785. Cultures from this study with their tentative assignments were (?) *Vibrio carchariae ?*, Σ 36 = UQM 2849, Stn 6660 = UQM 3010; Stn 6670 = UQM 3011, *Vibrio harveyi ?* sp. Stn 6550; Stn 6551; *V.natriegens ?* Stn 1010 = UQM 3077; *V.tubiashii ?* B 1, UQM 3012; *V.vulnificus ?* Stn 3010; *Vibrio* sp. Stn 750 = UQM 3027; *Vibrio* sp. Stn 760 = UQM 3028; *Vibrio* sp. Stn 770 = UQM 3029; *Vibrio* sp.

A9.1.2 Extraction of DNA.

DNA from cultures of bacteria, verified as pure after growth in 150 ml SENB, in shaking culture at 28°C. for 18 h, was extracted by the method of Marmur and Doty (1962) with modifications by Johnson (1981) and Sly, Blackall, Kraat, Tian-Shen, and Sangkhobol (1986) as follow: Culture suspensions were centrifuged for 15 min at 1 500 g at 4°C., **(International B 20).** The supernates were discarded and the pellets from each tube resuspended in *ca* 1 ml 0.15 M saline/0.01 M EDTA pH 8.0 and transferred to a 10 ml graduated polypropylene centrifuge tube **(Johns)** and made to 4ml. Sodium dodecylsulphate (SDS, 20%), (0.5ml), was added to each tube, mixed and these incubated overnight at 37°C. If necessary a further aliquot of SDS was added and tubes reincubated until suspensions were translucent. A volume

of 5 M sodium perchlorate corresponding to 25% of the volume of lysate in each tube was added, followed by an equal volume of chloroform/isoamyl alcohol prepared in a ratio of (24:1 v/v). Preparations were placed on a rotating mixer (Clements) at 100 rpm for 6-12 h, and centrifuged 30 min at 1 000 g. Transparent supernates were transferred with a wide-mouthed pipette to new tubes, re-extracted with solvent, and centrifuged again. These steps were repeated until no protein was deposited at the aqueous/organic interface (usually 1-3 times).

Partially purified DNA solutions were transferred to sterile wide mouthed 20 ml bottles and overlaid with absolute ethanol chilled to -20°C. DNA which precipitated was spooled from the interfaces onto glass rods and allowed to air dry. These were placed in 3ml 0.1 X standard saline citrate (SSC) until DNA redissolved, and treated with 30 µl protease (1 mg/ml) (Sigma) and incubated 37°C., 30 min, before adding 30 µl RNA'ase (Sigma), (2 mg/ml), and incubating for a further 30 min. An equal volume of chloroform/isoamyl alcohol was added, mixed and centrifuged as above repeatedly, until no further sediment was seen at the interface. The supernate was again withdrawn, precipitated with ethanol, spooled dried and redissolved in 0.1 X SSC. Repurified DNA solutions were placed in dialysis sacs (Union Carbide MW 12 000) and suspended in 1 l 0.1 X SSC 12 h at 4°C. Dialyzed DNA was transferred to sterile 10 X 75 mm borosilicate tubes covered with wax film and stored at -20°C.

A9.2.0 Determination of Mole % G +C DNA.

A9.2.1 Experimental Design.

Determination of mole % G + C of DNA by the indirect method of Marmur and Doty (1962) relies on the linear correlation between the DNA melting temperature and its base composition in constant ionic strengths of suspending buffer usually 0.1 X SSC (for melting temperatures < 80°C.).

The problem with any such method is that they can only be as accurate as their standards and calibration curves. This has been tacitly ignored in the literature, where published values reviewed by Hollander and Pohl,

(1979) for *E.coli* ranged between 48.9-52.1 mole % G + C i.e. there was confusion with the actual value of G + C of reference DNA. Later direct determinations of individual bases by HPLC, Tamaoka and Komagata (1984), has shown that G + C values for 2 non-type cultures of *E.coli* were in the range 51.5-7. These results are most consistent with those reported earlier by Star and Mandel (1969), who suggested a value of 51.7 for the type culture, (ATCC 11775 = UQM 1803), and which has been empirically endorsed as a reliable accurate by Sly *et al.,* (1986).

Further potential sources of error can arise from small differences in buffer concentrations between-samples and also from the potentially slightly non-linear relationship between melting temperature and DNA base composition. Both of these pretexts are trivial if the unknown DNA has a base composition similar to that of the reference preparation, but for *Vibrionaceae* where base ratios range between *ca* 40 and 60 they may be highly significant.

To accommodate these possibilities base compositions of unknowns were inferred by correlation with an internally constructed regression line between two standards in a constant buffer strength *ca* 0.1 X SSC.

A9.2.2 Methods.

DNA preparations from two unknowns, and a reference strain with known G + C base ratio, *(E.coli* UQM 1803, *V.natriegens* 2782, or *V.campbellii* UQM 2779) were removed from storage and thawed. Absorbances at 260 nm **(Gilford 2600),** were adjusted to *ca* 0.5 units by dilution with 0.1 strength SSC and increases in absorbance of preparations as denaturation occurred were plotted **(Hewlett-Packard 7225 A)** while temperature was incremented by a thermoprogrammer **(Gilford 2527)** from 25°C. at 0.5°C./min. over a period of 130 minutes. The 2nd derivative plots, which accurately approximate denaturation temperature ($T_{m.}$), Sly *et al.,* (1986) were produced by the spectrophotometer. A linear regression equation was calculated which incorporated the average $T_{m.}$ of *E.coli* (5 determinations) with that of the

most replicable *Vibrio* standard, *V.natriegens*, (4 determinations) and adopted the correspondent mole % G + C base ratios of 51.7 (Starr and Mandel 1969) and 46.3 (Baumann, Baumann, Bang and Woolkalis 1980) respectively. The $T_{m.}$ values for other cultures were used by substitution into this equation to infer correspondent mole % G + C base composition of DNA's.

A9.2.3 Results and Discussion.

The regression equation calculated for determination of mole % G + C of DNA was as follows:

$$\text{Mole \% G + C} = 2.077T_{m.} - 108.6$$

Calculated values for reference and unknown strains and comparative values from the literature are in Table A9.1. Values with the exception of *V.nereis* are less than those previously reported. With the exception also of *V.carchariae* which was found to have a lower rather than similar G + C content to *V.harveyi*, the same relativities existed between species i.e. *V.anguillarum* had a lower G + C ratio than *V.natriegens*. This may be as a result of the internal regression calibration procedure, or it may simply reflect the depression of melting temperatures in 0.1 X SSC, and the consequent increased difficulty in accurately measuring *Vibrionaceae* DNA base composition. Supporting this thesis are the comparative HPLC results of Tamoaka *et al.*, 1984 for *Alteromonas espejiana* showing the G + C content to be 2 mole % less than reported by Chan, Baumann, Garza and Baumann (1978) by thermal denaturation i.e. 41 rather than 43 mole %.

Grimes *et al.*, (1984) used regression analysis to determine mole % G + C of *V.damsela* by polyacrylamide gel electrophoresis, and also found lower results than predicted by the single standard $T_{m.}$ method (38.9 cf. 7 determinations "in the 40 % range").

Table A9.1: Mole % G + C DNA for *Vibrionaceae* Unknowns and Reference Preparations.

Species.	Laboratory Code.	Accession Number.	Number of Replicates.	Mole % G + C. T_m.	Published.	Ref.
**E.coli*	NA	UQM 1803	(5)	51.7 ± 1.1	51.7	1
V.vulnificus	NA	ATCC 27562		ND	47.2	2
**V.nereis*	NA	UQM 2783	(1)	47.6	46.4 ± 0.3	2
**V.natriegens*	NA	UQM 2782	(4)	46.3 ± 0.3	46.3 ± 0.1	2
**V.campbellii*	NA	UQM 2779	(4)	45.1 ± 0.9	46.5 ± 0.4	2
**V.harveyi*	NA	ATCC 14126		ND	46.4 ± 0.4	2
V.anguillarum	Σ 1017	UQM 2843	(1)	43.1	NA	
**V.anguillarum*	NA	UQM 2771	(1)	42.9	45.4	2
**V.carchariae*	NA	UQM 2920	(1)	41.9	46.3	3
V.carchariae ?	Σ 36	UQM 2849	(2)	41.9 ± 0.0	NA	
V.tubiashii	NA	ATCC 19109		ND	45 ± 0.7	6
V.orientalis	NA	ATCC 33934		ND	45.4	5
V.vulnificus	Stn 3010		(1)	46.1		
V.harveyi	Stn 6551		(1)	45.0		
V.carchariae	Stn 6670	UQM 3011	(1)	44.0		
V.carchariae	Stn 6660	UQM 3010	(1)	43.0 ± 1.0		
V.carchariae	Stn 6650		(1)	41.9		
V.carc/V.harv	Stn 6550		(1)	41.9		
V.tubiashii	B 1	UQM 3012	(6)	43.3 ± 1.0		
V.natriegens	Stn 1010	UQM 3077	(1)	36.6		
Vibrio sp.	Stn 760	UQM 3028	(2)	45.6 ± 1.5		
Vibrio sp.	Stn 750	UQM 3027	(2)	44.9 ± 1.6		
Vibrio sp.	Stn 770	UQM 3029	(2)	42.4 ± 0.5		

* Denotes Type Culture.

References.

1 Starr and Mandel (1969).
2 Baumann *et al.*, (1980).
3 Grimes *et al.*, (1984).
4 Guerinot *et al.*, (1982).
5 Yang *et al.*, (1982).
6 Hada *et al.*, (1983).

Consequently it would seem that by the use of an internally calibrated regression line and the adoption of an *E.coli* standard as 51.7 mole % generates results which are more consistent with other methods than T_m. results derived from only a single standard. The corollary from this is that the earlier result of Grimes *et al.*, (1984) for G + C content of *V.carchariae* UQM 2920 was likely affected by skewing as minimized in these analyses. The G + C ratios of the strain UQM 2843 phenetically identified as *V.anguillarum;* and the strains Σ 36 = UQM 2849, Stn 6650 and Stn 6660 = UQM

3010 and Stn 6670 = UQM 3011 phenetically identified as *V.carchariae*; were within 2 mole % of G + C values of respective type cultures. When the downward shift to G + C ratios obtained by this analysis method is considered, the strains Stn 6551 phenetically identified as *V.harveyi*, B 1 = UQM 3012 phenetically identified as *V.tubiashii*, and Stn 3010 phenetically identified as *V.vulnificus* correspond respectively to reports by Baumann *et al.*, 1984, Hada *et al.*, (1984) and Baumann *et al.*, 1980. The similarity of published base ratios for *V.orientalis* and *V.tubiashii* in the context of high phenetic similarity might seem to endorse the view that these species are the same.

While the differentiation of *V.carchariae* from *V.harveyi* is difficult by phenotypic procedure (3.8.7) G + C ratios between the two species have been shown here to be quite distinct and a useful differential criterion.

Similarly the strain UQM 3077 phenetically resembling *V.natriegens* had a G + C ratio less than expected. The extent of the deviation of the G + C ratio from all other species tested indicates a that it should be assigned to a new genus. G + C ratios for strains from the decarboxylase negative *Vibrio* sp. strains Stn 750-770 = UQM 3027-9 were in the range 42-44.5 i.e. lower than *V.natriegens* and *V.campbellii* but greater than *V.anguillarum*.

A9.2.4 **Summary.**

Based upon regressive determinations of mole G + C, values obtained here were lower than reported by e.g. Baumann *et al.*, (1984) using single reference ($T_{m.}$ and $B_{d.}$) techniques; but between-species relativity was consistent with that earlier work. There was further indication of other corresponding lower results derived by an electrophoretic method, also calibrated by a regression line rather than from a distant single reference strain as has previously been the general habit when G + C determinations are conducted.

The strains for UQM 3077 (phenetically similar to *V.natriegens)* and the *Vibrio* sp. UQM 3027-9 were sufficiently different from other *Vibrio* spp.,

phenetically and/or genotypically to warrant assignment as separate species.

Determination of DNA homology between type strains of *V.orientalis* and *V.tubiashii* would clarify the taxonomic status of this later-described species, which is phenotypically similar and which has a similar DNA base ratio to *V.orientalis.*

A9.3 DNA/DNA Homology.

A9.3.1 Experimental Design.

The phenetically distinct decarboxylase-negative phenon described earlier (3.8.6.3.2.3.4), which was found in A9.2 to have G + C base ratios between type cultures of *V.anguillarum* and *V.natriegens* was examined for its DNA relatedness to some of the other species from the *Vibrionaceae* reported as being decarboxylase negative (West *et al.,* 1985). The strain UQM 3027, which had a DNA base ratio intermediate between UQM 3028 and UQM 3029, was used as a reference in comparisons.

The following hybridizations were made, for *Vibrio* sp. UQM 3027 X [UQM 3027, UQM 3028, UQM 3030 (duplicates), *E.coli* UQM 1803, *V.campbellii* UQM 2779, *V.natriegens* UQM 2782, *V.nereis* UQM 2783 and *V.pelagius* UQM 2785] using the renaturation procedure of Huss, Festyl and Schleifer (1983).

The absorbance of DNA samples extracted from test and reference cultures in A9.1.1 was measured at 260 nm (Gilford 2600) and diluted with 2 X SSC to *ca* 0.50 absorbance units. DNA from these was sheared in a French press (American Instrument Co.) three times at 140 000 KPa. Between preparations the pressure cell was washed with 2 changes of distilled water and then rinsed with 2 X SSC. Resultant sheared preparations were dialyzed in one l 2 X SSC, 16 h at 4°C., and stored in sterile bottles at -20°C.

For each determination two DNA samples (1 & 2) were removed from storage and thawed. Cuvette 1, the absorbance blank, was filled with 2 X SSC. Cuvettes 2-4 respectively were loaded with DNA from sample 1, 2, and 1 with 2 (1:1). These were placed into the spectrophotometer and the thermal programmer set to 99°C. for 5 minutes or until no further increase in absorbance was

observed. The temperature was rapidly cooled to the optimal renaturation temperature as calculated by substitution into the equation of Gillis, De Ley and De Cleene (1970):

$$0.51 \text{ X } \%(G + C)_{DNA\ 1} + 47.0.$$

This temperature (70°C.) was maintained for 40 minutes and the absorbance plotted against time as in (A9.2). The decrease in absorbance over a fixed period, "D", (e.g. 30 min) was measured for each of the DNA samples, and homology determined by substitution into the formula below:

$$\% \text{ Homology} = \frac{100 \text{ X } (4D_{mixture} - (D_{DNA1} + D_{DNA2})}{2 \text{ X } \sqrt{(D_{DNA1} \cdot D_{DNA2})}}$$

Results were tabulated in the range (30 to 100 %) of the reported resolution (Huss *et al.*, 1983).

A9.3.2 Results and Discussion.

Results for DNA renaturations and hybridizations are shown in Table A9.2. From the duplicate hybridizations, UQM 3027 X UQM 3030 and UQM 3027 X UQM 3027, results indicated an experimental error of ± 3 %, and tendency towards overestimated (i.e. > 100%) homology. All strains tested from the

Table A9.2 : DNA Homology, (%), Between UQM 3027 and Selected Strains.

Species	Laboratory Code.	Accession Number.	% Homology.
Vibrio sp.	Stn 750	UQM 3027	106
Vibrio sp.	Stn 760	UQM 3028	100
Vibrio sp.	Stn 780	UQM 3030	101 ± 3
V.campbellii		UQM 2779	< 30
V.natriegens		UQM 2782	< 30
V.nereis		UQM 2783	< 30
V.pelagius		UQM 2785	< 30
E.coli		UQM 1803	< 30

decarboxylase-negative phenon, because they were more than 90 % homologous, can be seen to come from the same species. Optimal renaturation conditions (70°C.) did not enhance hybridization of DNA from the strain UQM 3027, to less related species, from *Vibrionaceae* or *E.coli*, to the extent where comparative relatedness could be assayed by this method because all other associations were less than 30 %.

A9.3.3 Conclusion.

The thermal renaturation method for DNA hybridization showed the decarboxylase negative phenon to be a genotypically cohesive taxon, distinct from *E.coli* and four previously described species of *Vibrionaceae*. The range of resolution, (30-100%), was not sufficient to indicate relatedness of this new species to any of the other species assayed. Incomplete departmental co-operative work to establish by thermal methods relatedness between this species and *V.diazotrophicus* UQM 2780, has shown that this species has a G + C content higher than *V.zobellii*. Enquiries regarding this work should be directed to Dr.L.I.Sly.

A9.4 Conclusion.

The combination of thermal methods for determining G + C and DNA hybridization is suitable for the resolution of separate species and testing phenetic classifications but the narrow resolution of the thermal renaturation method makes examination of between-species-relationships more difficult than traditional radio-labeling techniques.

APPENDIX 10.

A10.0 Parsimony Analysis of Abstracted Ribosomal RNA Sequence Data.

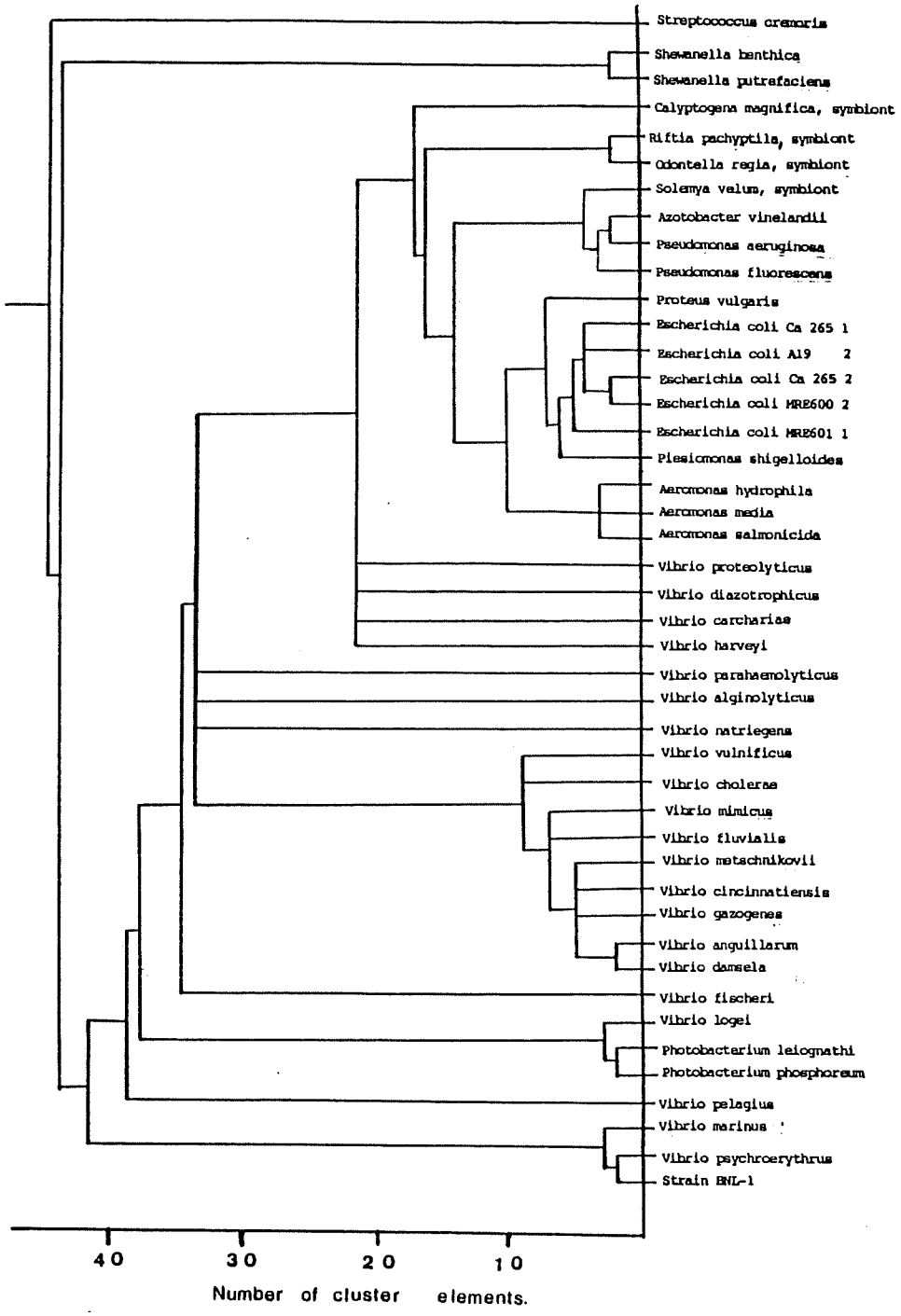
A10.1 Experimental Design.

Nearhos and Fuerst (1987) analyzed 5S rRNA sequence data of MacDonell and Colwell (1985) to evaluate the potential of a conventional distance matrix sorting procedure to produce phylogenetic analyses. Results were generally consistent or complimentary with current classification of the *Vibrionaceae* but did not support the proposed combinations *Listonella anguillara, L.damsela* and *L.pelagica*. The confidence of these findings was moderated by the possibly spurious placement of *A.salmonicida* amongst *Vibrio* species. To reanalyse the sequence data and test the conclusions of the earlier distance matrix analysis the consensus parsimony, CP, approach was used.

The **Paup** (Version 2.4.1) (Swofford 1986) parsimony program was used for primary data analysis. To serve as a hypothetical ancestor, the sequence of a Gram-positive species, *Streptococcus cremoris* (from Erdmann, Wolters, Huysmans and De Wachter, 1985), was included in the analysis. One homologous sequence from each pair of *Vibrio cholerae; Photobacterium angustum* and *P.leiognathi;* and the *Odontella* symbionts was removed. Bases were specified as coded and typed as "unordered". Missing or ambiguous loci were coded with a "?". From 142 loci, the sixty-six which remained constant were masked from analysis. OTU's were nested in parentheses to form the hierarchical structure found by single linkage cluster analysis. Fifteen trees (program maximum) were specified to be held in memory, and one hundred (program maximum) equally parsimonious trees were nominated for output. The command set is shown in A11.8.2. (A similar analysis without the above mentioned ancestral sequence and instead with the *Odontella* symbiont specified as an internal hypothetical ancestor was conducted first, but found to be less parsimonious).

The hundred equally parsimonious output trees were secondarily processed by the Strict, (Rohlf 1982), consensus tree program, **Contree** (Version 1/3/86) (Swofford, 1986) and placed into the "cladogram", Figure A10.1.

Figure A10.1: Strict Consensus Tree 5S rRNA Sequence Data for 100 Trees, 224 Steps.



A10.2. Results.

After invariant or missing sites were deleted 76 loci were available for analysis. The parsimony procedure reduced the total number of bases changed with the hypothetical ancestor from 258 to 224. If this ancestor was removed the same data as in the *Clustan* single linkage analysis was reduced from 224 "steps" to 175. Analysis without the included ancestral sequence with the same starting hierarchy (224 steps) was non-parsimoniously complete at 189 steps (i.e. only 100 trees analyzed).

The CP cladogram (Figure A10.1) placed all *Aeromonas* species into a single cladon. These were then linked to the cladon containing OTU's from *E.coli*, *Plesiomonas shigelloides* and *Proteus vulgaris* into the same hierarchy as produced by Nearhos and Fuerst (1987) and consistent with the more detailed *Enterobacteriaceae* structure shown by MacDonell and Colwell (1985).

Currently recognized *Vibrio* species were not presented as a contiguous group, most were placed as intermediates between the cladon containing *Photobacterium* spp. and the cladon with *Aeromonas* spp., *Enterobacteriaceae*, *Pseudomonas* spp. and marine symbionts. The OTU for *V.pelagius* was fused only after their combination with the *Photobacterium* group. The other species from *Listonella*, *V.anguillarum* and *V.damsela* were associated in a cladon also containing oxidase-negative OTU's.

V.cholerae was most closely associated with *V.vulnificus* and *V.mimicus* with *V.fluvialis*. The OTU's *V.logei* and *V.fischeri* respectively were the outermost OTU's of unfused *Photobacterium* and *Vibrio* clada.

A10.3 Discussion.

This analysis indicated the relatedness of *Aeromonas* species but did not support the current composition of the genus *Listonella*. Unpublished information of J.A.Fuerst (pers. comm.) and D.C.Sutton regarding the placement of *V.damsela* in the genus *Photobacterium* because it lacks a sheathed flagellum i.e. a maximum core diameter of 16 nm in ATCC 33537, is partly supported by this analysis, and other published information. Grimes *et al.*, (1984) comparatively assayed by DNA hybridization the relatedness

of an isolate (1116b) of *V.damsela* from sharks. It was found as almost 90 % homologous with the strain of *V.damsela* used in this study but only 4 % homologous with *V.anguillarum* and *E.coli*. The (1116b) strain of Grimes *et al.*, was found to be most (20 %) related to *V.fischeri* and to possess PHB granules, however this was not confirmed from either assay of UQM 2853 (ATCC 33537), (not ATCC 33539 as used for sequencing), for sudanophilic inclusions (Tables 3.19.1, 3.24.3). On the basis of higher DNA relatedness of *V.damsela* to *V.fischeri* and the similarly low G + C base ratios of *V.damsela*, 43 mole % G + C, and species of *Photobacterium*, 39-44 mole % G+C (with *V.fischeri* and *V.logei*), it would seem that this species *(V.damsela)* may be more correctly placed in the genus *Photobacterium* redefined to include species with tufts of sheathed polar flagella, in accord with 5S rRNA sequence analyses; *V.fisheri* and *V.logei*, and non-luminescent aerogenic, fish associated bacteria i.e. *P.damsela*.

The OTU *V.pelagius* because of its only peripheral association with the *Vibrio* group despite reported (Reichelt *et al.*, 1976) DNA homologies from *V.cholerae*, *V.harveyi*, *V.campbellii*, *V.parahaemolyticus*, *V.alginolyticus*, *V.natriegens*, *V.vulnificus*, *V.nereis*, both Biogroups of *V.splendidus*, and *V.logei* of between 18 and 39%, indicate placement of this species in the genus *Vibrio* is valid, and axiomatically that segregation of *V.pelagius* by parsimony analysis was artifactual. The placement of *V.cholerae* with *V.vulnificus* rather than *V.mimicus* as stated previously by Nearhos and Fuerst (1987) is also contrary to DNA homology (34-40 % *V.cholerae* X *V.vulnificus* cf. *ca* 65 % *V.mimicus* X *V.cholerae* as reported by Davis *et al.*, (1981) and also indicate susceptibility of the 5S rRNA molecule to noise and homoplasy.

The resolution of this method seems less of a fit to the traditional classification than analysis of the distance matrix by Clustan, and endorses earlier mentioned observations of Cornish-Bowden (1983) (2.9.1) regarding the non-comparison of unique features by minimum length (parsimony) procedures. Because of the small size of this molecule, together

with the proportionally high number of common loci parsimony analysis would seem less appropriate for phylogenetic determination from 5S rRNA sequences than distance (similarity) matrix analysis.

Both distance matrix sorting and parsimony analysis, failed to combine species shown by other possibly more sensitive methods to be closely related. Here the composition of specific genera, in this case of *Listonella,* may not be reliably indicated by the limited data contained in 5S rRNA sequences. While it may have been possible to delete loci known to give "spurious" results e.g. some or all non-congruent sites from *V.cholerae* and *V.mimicus*, or to weight loci according to mutation frequency such as was done by MacDonell, Ortiz-Conde, Last and Colwell (1986) the decision as to which taxonomic level to introduce generalizations for weighting or deletions is subjective, and can only be empirically evaluated.

A10.4 Recent Information.

A CP analysis as above but with new 5S rRNA sequence data for *V.aestuarianus* (Pillage *et al.,* 1987) *V.tubiashii* and *V.ordalii* (Pilladge *et al.,* 1988) reaffirmed grouping of *Aeromonas* spp. and separately nested the species *V.anguillarum, V.pelagius* and *V.tubiashii* outside of, i.e. one branch removed from a cladon latterly fusing *P.damsela* with *V.psychroerythrus* and Strain BNL 1. *V.aestuarianus* similarly appeared as an intermediate between these species and other *Vibrionaceae* and distantly related species. It was only after these fusions that *V.ordalii* was placed. These results may indicate the use of an inappropriate hypothetical ancestor but otherwise reflect the violent instability of parsimony analysis of 5S rRNA sequence data to the presence of new information from related species.

Additional References:

Pillage C.J., MacDonell M.T. and Colwell R.R. (1987) Nucleotide sequence of the 5S rRNA from *Listonella (Vibrio) aestuarianus. Nucleic Acids Research,* 15, 1879.

Pillage C.J. & Colwell R.R. (1988) Nucleotide sequence of the 5S rRNA from *Listonella (Vibrio) ordalii* ATCC 33509 and *Listonella (Vibrio) tubiashii* ATCC 19105. *Nucleic Acids Research,* 16, 3111.

APPENDIX 11.

A11.0 Computer Programs and Command Files.

Source code for FORTRAN programs is also in the directory \APNDICES\APNDX_11 of Disk_2.

A11.1 Program for Tranposition of Data Matrix, Fortran 77, (S.Heng and S.Nearhos 1987.)

```
C     THIS PROGRAM TRANSPOSES ROWS AND COLUMNS
      DIMENSION N(200,200)
      CHARACTER INFILE*12,OUTFILE*12
      WRITE (5,1)
1     FORMAT(1X,'ENTER INFILE NAME AND EXTENSION e.g. INFILE.DAT <R> ')
      READ (5,2) INFILE
2     FORMAT (A)
      WRITE (5,3)
3     FORMAT(1X,'ENTER OUTFILE NAME AND EXTENSION e.g INFILE.OUT <R> ')
      READ (5,4) OUTFILE
4     FORMAT (A)
      OPEN (20,FILE=INFILE)
      OPEN (21,FILE=OUTFILE)
      WRITE(5,10)
10    FORMAT(1X,'ENTER NO_STRAINS, NO_TESTS AS E.G. 0200,0200<R>')
      READ(5,20)NR,NT
      WRITE(*,20) NR,NT
20    FORMAT(2(I4,1X))
25    DO 40 I = 1,NT
      READ (20,35) (N(I,J),J=1,NR)
35    FORMAT(18I1)
40    CONTINUE
      DO 65 J = 1,NR
      WRITE (21,60) (N(I,J),I=1,NT)
60    FORMAT(200I1)
65    CONTINUE
      CALL EXIT
      END
```

A11.2 Program for Sorting Data Files, Fortran 77; (S.Heng and S.Nearhos, 1987).

```
C     THIS SORTS DATA ROWS INTO FILE DIRECTED SEQUENCE.
      DIMENSION DOUG(300,200)
      CHARACTER INFILE*12,SORINUM*12,SORTED*12
      WRITE (5,1)
1     FORMAT(1X,'ENTER INFILE NAME AND EXTENSION e.g. INFILE.DAT <R> ')
      READ (5,2) INFILE
2     FORMAT (A)
      WRITE (5,3)
3     FORMAT(1X,'ENTER OUTFILE NAME AND EXTENSION e.g SORINUM.DAT <R> ')
      READ (5,4) SORINUM
4     FORMAT (A)
      WRITE (5,5)
5     FORMAT(1X,'ENTER OUTFILE NAME AND EXTENSION e.g INFILE.STD <R> ')
      READ (5,6) SORTED
6     FORMAT (A)
      OPEN (20,FILE=INFILE,STATUS="OLD")
      OPEN (21,FILE=SORINUM,STATUS="OLD")
      OPEN (22,FILE=SORTED,STATUS="NEW")
      WRITE(*,7)
7     FORMAT(1X,'HOW MANY ROWS OF INPUT DATA, UP TO 300 ')
      READ(*,8)N1
8     FORMAT(I8)
      WRITE(*,16)N1
16    FORMAT(I8)
      DO 10  I=1,N1
10    READ(20,15)(DOUG(I,J),J=1,200)
15    FORMAT(200A1)
24    READ(21,25,END=999)N2
25    FORMAT(I8)
      WRITE(*,8)N2
      WRITE(22,15) (DOUG(N2,J),J=1,200)
      GOTO 24
999   STOP
      END
```

A11.3 Program for Generation of a Square or Triangular Distance Matrix using the Euclidean Similarity Criterion, Fortran 77; (S.Heng, B.Maher, and S.Nearhos 1987).

```
C     THIS PROGRAM PRODUCES A SQUARE EUCLIDEAN DISTANCE  MATRIX
      DIMENSION SR(150,150)
      INTEGER DOUG(150,200),A,D,F
      CHARACTER INFILE*12,OUTFILE*12
      WRITE (5,1)
1     FORMAT(1X,'ENTER INFILE NAME AND EXTENSION e.g. INFILE.DAT <R>')
      READ (5,2) INFILE
2     FORMAT (A)
      WRITE (5,3)
3     FORMAT(1X,'ENTER OUTFILE NAME AND EXTENSION e.g INFILE.OUT <R>')
      READ (5,4) OUTFILE
4     FORMAT (A)
      OPEN (20,FILE=INFILE)
      OPEN (22,FILE=OUTFILE)
      OPEN (UNIT=21,FILE='DUMP.FIL')
      WRITE(5,10)
10    FORMAT(1X,'ENTER NO_STRAINS, NO_TESTS AS E.G. 0300,0200<R>')
      READ(5,20)NR,NT
      WRITE(*,20) NR,NT
20    FORMAT(2(I4,1X))
      DO 500 I=1,NR
500   READ(20,30)(DOUG(I,J),J=1,NT)
30    FORMAT(250I1)
      DO 800 K=1,NR
      DO 800 J=1,NR
C     FOR TRIANGULAR MATRIX  DO 800 J=1,K
      A=0
      D=0
      F=0
      DO 600 I=1,NT
      IF (DOUG(K,I) .GE. 3 .OR.  DOUG(J,I) .GE. 3) F=F+1
      IF (DOUG(K,I) .EQ. 1 .AND. DOUG(J,I) .EQ. 1) A=A+1
      IF (DOUG(K,I) .EQ. 0 .AND. DOUG(J,I) .EQ. 0) D=D+1
600   CONTINUE
      XA=A
      XD=D
      XF=F
      S=100*(1-(XD+XA)/(NT-XF))
C     FOR SIMILARITY MATRIX  S=100*(XD+XA)/(NT-XF)
      SR(K,J)=S
      WRITE(21,40)S
40    FORMAT (F5.0)
800   CONTINUE
      DO 2000 K=1,NR
2000  WRITE(22,160)(SR(K,J),J=1,NR)
160   FORMAT(8(F10.5))
      STOP
      END
```

A11.4 Program for Generation of a Distance Matrix using Jaccard Similarity as a Distance Measure, Fortran 77; (S.Heng, B.Maher, and S.Nearhos 1987.)

```
C     THIS PROGRAM PRODUCES A SQUARE JACCARD DISTANCE MATRIX
      DIMENSION SR(300,300)
      INTEGER DOUG(300,200),A,B,C
      CHARACTER INFILE*12,OUTFILE*12
      WRITE (5,1)
1     FORMAT(1X,'ENTER INFILE NAME AND EXTENSION e.g. INFILE.DAT <R>')
      READ (5,2) INFILE
2     FORMAT (A)
      WRITE (5,3)
3     FORMAT(1X,'ENTER OUTFILE NAME AND EXTENSION e.g INFILE.OUT <R>')
      READ (5,4) OUTFILE
4     FORMAT (A)
      OPEN (20,FILE=INFILE)
      OPEN (22,FILE=OUTFILE)
      OPEN (UNIT=21,FILE='DUMP.FIL')
C     ENTER SIZE OF DATA
      WRITE(5,10)
10    FORMAT(1X,'ENTER NO_STRAINS, NO_TESTS AS E.G. 0300,0200<R>')
      READ(5,20)NR,NT
      WRITE(*,20) NR,NT
20    FORMAT(2(I4,1X))
      DO 500 I=1,NR
500   READ(20,30)(DOUG(I,J),J=1,NT)
30    FORMAT(200I1)
      DO 800 K=1,NR
      DO 800 J=1,NR
C     FOR A TRIANGULAR MATRIX SUBSTITUTE 'DO 800 J=1,K'
C     FOR AN UPPER TRIANGULAR MATRIX SUBSTITUTE  'DO 800 J=1,NR'
C                                        AND     'DO 800 K=1,J'
      A=0
      B=0
      C=0
      DO 600 I=1,NT
      IF (DOUG(K,I) .EQ. 1 .AND. DOUG(J,I) .EQ. 1) A=A+1
      IF (DOUG(K,I) .EQ. 1 .AND. DOUG(J,I) .EQ. 0) C=C+1
      IF (DOUG(K,I) .EQ. 0 .AND. DOUG(J,I) .EQ. 1) B=B+1
600   CONTINUE
      XA=A
      XB=B
      XC=C
      S=100*(1-(XA/(XA+XB+XC))
C     FOR SIMILARITY MEASURE  'S=100*XA/(XA+XB+XC)'
      SR(K,J)=S
      WRITE(21,40)S
40    FORMAT (F5.0)
800   CONTINUE
      DO 2000 K=1,NR
2000  WRITE(22,160)(SR(K,J),J=1,NR)
160   FORMAT(8(F10.5))
C     FOR "MICROCLUSTER" SUBSTITUTE '160  FORMAT(100(F8.6))'
      STOP
      END
```

A11.5 Program for Determining Attribute Frequencies, Fortran 77; (S.Nearhos, 1987)

```
C     THIS PROGRAM FINDS PERCENT ATTRIBUTE FREQUENCIES.
      INTEGER DOUG(250,200),A,B,XA,XB,XN
      CHARACTER INFILE*12,OUTFILE*12
      WRITE (5,1)
1     FORMAT(1X,'ENTER INFILE NAME AND EXTENSION e.g. INFILE.DAT ')
      READ (5,2) INFILE
2     FORMAT (A)
      WRITE (5,3)
3     FORMAT(1X,'ENTER OUTFILE NAME AND EXTENSION e.g INFILE.OUT')
      READ (5,4) OUTFILE
4     FORMAT (A)
      OPEN (20,FILE=INFILE)
      OPEN (21,FILE=OUTFILE)
      WRITE(5,10)
10    FORMAT(1X,'ENTER NO_STRAINS, NO_TESTS AS E.G. 0300,0200<R>')
      READ(5,20)NR,NT
      WRITE(*,20) NR,NT
20    FORMAT(2(I4,1X))
      DO 500 I=1,NR
500   READ(20,30)(DOUG(I,J),J=1,NT)
30    FORMAT(250I1)
      DO 800  J=1,NT
      A=0
      B=0
      DO 600 I=1,NR
      IF (DOUG(I,J) .EQ. 0) B=B+1
      IF (DOUG(I,J) .EQ. 1) A=A+1
600   CONTINUE
      XA=A
      XB=B
      IF (XA+XB .LE. 0) XN=999
      IF (XA+XB .GT. 0 ) XN=XA+XB
      S=100*XA/XN
      K=K+1
      WRITE(21,40)K,S,XN
40    FORMAT (2C0(I4,1X,F5.0,1X,I4))
800   CONTINUE
      STOP
      END
```

A11.6 SAS Command Files.

A11.6.1 SAS Command File to Read Data Sets.

```
CMS FILEDEF filename DISK filename ext A;
DATA filename.ext(TYPE=DISTANCE) ;INFILE filename;
INPUT (OBS001-OBS288) (10.6) @2880 ;
;
```

A11.6.2 SAS Command File for Single Linkage Cluster Analysis with "trimming."

```
DATA; SET filename.ext(TYPE=DISTANCE);
PROC  CLUSTER DATA=filename.ext OUTTREE=A NOSQUARE METHOD=SIN  K=15
TRIM=5 NONORM;
VAR OBS001-OBS288;
PROC TREE DATA=A DIS MAXH=100 MINH=0;
```

A11.6.3 SAS Command File for Average Linkage Cluster Analysis.

```
DATA; SET filename.ext(TYPE=DISTANCE);
PROC  CLUSTER DATA=filename.ext OUTTREE=A NOSQUARE  METHOD=AVE K=15
NONORM ;
VAR OBS001-OBS288;
PROC TREE DATA=A DIS MAXH=100 MINH=0 ;
```

A11.6.4 SAS Command File for Complete Linkage Cluster Analysis.

```
DATA; SET filename.ext(TYPE=DISTANCE);
PROC CLUSTER DATA=filename.ext OUTTREE=A NOSQUARE METHOD=COM K=15
NONORM;
VAR OBS001-OBS288;
PROC TREE DATA=A DIS MAXH=100 MINH=0;
```

A11.6.5 SAS Command File Set for Principal Component Analysis.

A11.6.5.1 SAS Command File for Reading Data Sets.

```
CMS FILEDEF filename1 DISK filename1 ext A;   (datafile)
DATA set.combined;
INFILE filename1;
INPUT (OBS001-OBS00n) (10.6) @10n ;
```

A11.6.5.2 SAS Command Program for Generating Principal Component Vectors.

```
DATA;
SET Set.Combined (Type=Distance);
INFILE File2;Input OBS $1;
PROC PRINCOMP OUT=XYZ N=10;
VAR OBS001-OBS096;
```

A11.6.6 SAS Command File for Probit Analysis.

```
DATA C;
INPUT DOSE NGROUP NDEAD;
CARDS;
0 2 1
12500 5 2
125000 5 1
1250000 5 1
12500000 5 2
125000000 5 1
1250000000 5 5
;
PROC PROBIT LOG10;
VAR DOSE NGROUP NDEAD;
TITLE 'LD 50  FOR  V.ANGUILLARUM UQM 2843 TOWARDS L.ARGENTEA ';
```

A11.7 Clustan 3.2 Command File.

```
SET MODE BATCH
set width output =132
size medium
assign file SETA unit 22
assign file OUTA unit 23
assign file OUTB unit 24
READ DATA TITLE " Nearhos  SetA, 48 strains 195 characters",
variables     binary     1     - 195     cases     48     format
(40F2.0/40F2.0/40F2.0/40F2.0/40F2.0/35F2.0) infile setA MISSING -1,
cluster,
method wards,
measure seuclid,
transform ranges,
print fusions,
icicle min 2 max 47 inc 1,
save,
results outfile OUTB,
TREE OUTFILE $DENDRO
tree,
style tabular,
DENDROGRAM FULL INFILE $DENDRO
TREE,
STYLE HORIZONTAL,
DENDROGRAM FULL INFILE $DENDRO
print results,
classifications numbers,
attributes frequencies
stop
```

A11.8.1 PAUP Batch Command File For Parsimony Analysis for Strict Consensus Tree Generation from Phenotypic Data.

A11.8.1.1 Determination of the Most Parsimonious Trees.

```
PARMS NOTU=46 NCHAR=189 OUTWIDTH=132;
Symbols 1 0;
DATA (A8,A1,189A1)

[data matrix.....as Labels (1st 8chars), "hypothetical ancestor",
A.hydrophila denoted by a star in column 9; binary strings
of data 189 chars long, and 46 characters deep; missing values
denoted by "?".]

 Unordered all;
 SET HYPANC=2;
 SET TREEFILE;
 SET CONFILE;
 DELE CHAR 14 15 24 26 54 55 58 66 72 73 108 138 139 140;
 DEFINE TREEFILE OVI001;
 DEFINE CONFILE  OVI001;
 STATUS;
 SET NODESCRIBE;
 SET NOLINKS;
 GO/SWAP=ALT MULPARS BLRANG ROOT=Ancestor MAXTREE=100 HOLD=15
 TREEOUT=3;
 RELEASE TREE ALL/NOLINKS;
 END;
```

A11.8.1.2 Erection of a STRICT Consensus Tree.

```
 CONTREE  OVI001.CON OVI001.CTF <R>
```

A11.8.2 PAUP Batch Command File For Parsimony Analysis for Strict Consensus Tree Generation from 5S rRNA Sequence Data.

A11.8.2.1 PAUP Batch Command set for Parsimony Analysis of 5S rRNA Sequences.

```
!DATASET OF 5S rRNA SEQUENCES ABSTRACTED BY NEARHOS AND FUERST (1987)
!REANALYSED USING "PAUP" JUN '87.
PARMS NOTU=44 NCHAR=142 OUTWIDTH=80;
SYMBOLS A C U G;
DATA (A8,A1,1X,142A1);
[THE DATA MATRIX WAS FORMATTED AND MISSING VALUES TREATED AS IN
A9.8.1.1. ATTRIBUTE VALUES WERE PRESENTED AS INDICATED BY THE
SYMBOLS STATEMENT. THE OTU S.cremoris WAS ASSIGNED AS A
HYPOTHETICAL ANCESTOR BY PLACING A "*" IN COLUMN 9 ]
UNORDERED ALL;
DELE CHAR 14 16 18-19 26 30-34 36 39-43 48-52 54 56-61 63-65
67-68 78-80 83-89 92 95 98-100 105-107 109-110 112-113
115-116 118-125 129;
!TOTAL BRANCH LENGTH FROM CLUSTAN 2.1 (SINGLE LINKAGE)
TOPOLOGY ((((((((((((((((((((((2,3)18)(4,13))(12,15))20)5)8)((9,10)14))11)6)25)
7)19)((21(23,24))22))(17,44))(((26,27)((31((38(40(41,42)))39))43))36))(28,29))
37)((32(33,34))35))16)30)1);
RELEASE TREE 1;
SET CONFILE;
DEFINE CONFILE RNA1SEQ;
STATUS;
SET NODESCRIBE;
SET NOLINKS;
GO/SWAP=GLOB MULP BLRANG ROOT=OUTGROUP MAXTREE=100 HOLD=15 TREEOUT=3;
RELEASE TREE ALL/NOLINKS;
END;
```

A11.8.2.2 Erection of a STRICT Consensus Tree.

```
CONTREE  RNA1SEQ.CON RNA1SEQ.CTF <R>
```

APPENDIX 12.

A12.0 Phenetic Data.

Phenetic data is contained in the Disk_2 directory \APPNDICES\ APNDX_12. The file ROSETTA.TAB keys strains from Vib01-VIB08a.DAT and others with origins as specified in Tables 3.4-5 and 3.7. This sub-directory like all others can be easily accessed by typng the name of a directory change file (.BAT extension); in this case *APNDX_12* and keying "Return", (See p. 20).

APPENDIX 13.

A13.0 Principal Component Vectors.

The eigen vectors for the principal component analysis of pooled and sorted strains from Batches [1. & 3.] is contained in Disk_2 in the sub-directory \APNDICES\APNDX_13 in the file PRINCOMP.EUC and called from the file APNDX_13.BAT by typing APNDX_13 and keying "Return", (See p. 20).

APPENDIX 14.

A14.0 Cultures Deposited in The UQM Culture Collection.

Laboratory Code	UQM Accession	Designated Species.
α 72	UQM 2838	*A.hydrophila*
Stn 3310	UQM 3197	*A.hydrophila*
Stn 3230	UQM 3356	*A.hydrophila*
Stn 7700	UQM 3375	*A.hydrophila*
Stn 1800	UQM 3242	*Photobacterium* sp.
Stn 8800	UQM 3269	*Photobacterium* sp.
Stn 8200	UQM 3244	*Photobacterium* sp.
Stn 3800	UQM 3370	*Shewanella putrefaciens*
Stn 3970	UQM 3366	*Shewanella putrefaciens*
Stn 8640	UQM 3373	*Shewanella putrefaciens*
Stn 7010	UQM 3357	*V.aestuarianus*
Stn 7450	UQM 3376	*V.aestuarianus I*
Stn 8370	UQM 3359	*V.aestuarianus*
Stn 6610	UQM 3358	*V.aestuarianus II*
Stn 7300	UQM 3360	*V.aestuarianus II*
Stn 7060	UQM 3361	*V.aestuarianus II*
Σ 182	UQM 3277	*V.alginolyticus*
Stn 680	UQM 3274	*V.alginolyticus*
Stn 9099	UQM 3249	*V.alginolyticus*
Stn 5000	UQM 3270	*V.alginolyticus*
Stn 370	UQM 3254	*V.alginolyticus*
Σ 1017	UQM 2843	*V.anguillarum*
Stn 8570	UQM 3247	*V.anguillarum*
Stn 8500	UQM 3248	*V.anguillarum*
Stn 8600	UQM 3250	*V.anguillarum*
Stn 8490	UQM 3251	*V.anguillarum*
Stn 8550	UQM 3252	*V.anguillarum*
DC 276	UQM 3062	*V.campbellii*
Stn 6660	UQM 3010	*V.carchariae*
Stn 6670	UQM 3011	*V.carchariae*
Stn 6730	UQM 3362	*V.carchariae*
Stn 6580	UQM 3363	*V.carchariae*
Stn 5370	UQM 3365	*V.carchariae*
Stn 5860	UQM 3364	*V.carchariae*
Stn 3700	UQM 3261	*V.diazotrophicus*
Stn 8220	UQM 3265	*V.diazotrophicus*
Stn 8470	UQM 3266	*V.diazotrophicus*
Stn 8480	UQM 3267	*V.diazotrophicus*
Stn 7170	UQM 3264	*V.harveyi*
Y 1	UQM 2855	*V.harveyi*
Stn 1640	UQM 3033	*V.fluvialis*
Stn 1670	UQM 3031	*V.fluvialis*
Stn 8950	UQM 3239	*V.fluvialis*
Stn 2440	UQM 3240	*V.fluvialis*
Stn 2300	UQM 3241	*V.fluvialis*
Stn 4631	UQM 3273	*V.fluvialis*
Stn 5650	UQM 3275	*V.fluvialis*
Stn 7710	UQM 3262	*V.fluvialis*
Stn 5800	UQM 3275	*V.fluvialis III*
Stn 5810	UQM 3354	*V.fluvialis III*
Stn 170	UQM 2840	*V.gazogenes*
Stn 180	UQM 2842	*V.gazogenes*
Stn 130	UQM 3282	*V.hollisae*
Stn 580	UQM 3355	*V.natriegens*
Stn 4800	UQM 3278	*V.natriegens*
Stn 6780	UQM 3372	*V.mediterranei*
Σ 126	UQM 3281	*V.orientalis*
Stn 8680	UQM 3196	*V.orientalis*
Gar 180	UQM 3276	*V.orientalis*
B 1	UQM 3012	*V.orientalis*
Stn 6690	UQM 3236	*V.orientalis*
Stn 7650	UQM 3245	*V.pelagius*
Stn 1620	UQM 3032	*V.vulnificus*
Stn 3010	UQM 3061	*V.vulnificus*
Stn 750	UQM 3027	*V.zobellii*
Stn 760	UQM 3028	*V.zobellii*
Stn 770	UQM 3029	*V.zobellii*
Stn 780	UQM 3030	*V.zobellii*
Stn 860	UQM 3231	*V.zobellii*
Stn 1010	UQM 3077	*Vibrio* sp.
Gar 20	UQM 3243	*Vibrio* sp.
Stn 8200	UQM 3244	*Vibrio* sp.
Stn 2170	UQM 3371	*Vibrio* sp.
Stn 1870	UQM 3367	*Vibrio* sp.
Stn 4740	UQM 3369	*Vibrio* sp.
Stn 4710	UQM 3368	*Vibrio* sp.
Stn 9210	UQM 3263	*Vibrio* sp.
Stn 2000	UQM 3256	*Serratia*-like *Vibrionaceae*.

APPENDIX 15.

A15.0 Recommendations for *In Situ* RSD Management.

It is evident that some fish do recover from *Red-Spot* disease (2.6.2), (Table 3.6), so simple disposal of diseased fish from the river system may be a wasteful and inappropriate control measure. During epizootics it may be advisable to suspend netting activities, apart from those necessary to monitor disease incidence, as such physical (2.6.1) and physiological trauma may adversely affect fish susceptibility.

Because in this work such so many *Vibrionaceae* taxa were associated with RSD, it is apparent that simple environmental release of single-pathogen-immunized hatchery-reared fingerlings of commercially important species is inappropriate for RSD control. However multivalent vaccines may be suitable e.g. by using a genus-specific flagella-antigen such as C_2, (2.7.2.5) as an immunogen to treat such reared fish. It might also be possible to undertake a fish breeding program to further increase the populations non-specific immunity by selecting for competitively increased iron binding coefficients of the proteins transferrin and lactoferrin, such as were reported as hereditable in *S.gairdneri* (2.3.2) and as was suggested as a possible explanation for the high resistance of *L.argentea* in pathogenicity assays (3.10.3.3).

A second-line, chemical strategy, in epizootic flares could involve single applications of antimicrobials such as nifurstyrenate into river-systems. Due to the inferred (Appendix 5.) complex action, of the nitrofurans on bacterial-metabolism, resistance would seem unlikely in the short term. As indicated, in Appendix 5. any such chemical control measures must first be tested for potentially deleterious environmental consequences.

The influence of specific types of pollution on RSD incidence did not fall into the province of this work however some or all of the measures suggested above may also be helpful for disease control in polluted river systems.

APPENDIX 16

A16.0 Supplier Register.

3M (Australia Pty. Ltd.) St. Mary's, Aus.
ACMM (Australian Collection Marine Microorganisms) Townsville,Aus.
Ajax Chemicals, Sydney, Aus.
American Instrument Co. Silver Spring, Md., USA.
Amersham (Australia Pty. Ltd.), Sydney, Australia.
Ansell International, Dandenong, Vic. Aus.
ATTC (American Type Culture Collection), Rockville, MD.,USA.
BAusch Lomb, Rochester, NY,, USA.
BBl - see Becton Dickinson & Co.
Becton Dickinson & Co., Rutherford, NJ., USA.
BDH (British Drug Houses) Ltd., Poole, UK.
Calbiochem, La Jolla, Cal. USA.
Canberra Packard, Mt.Waverly, Vic., Aus.
CANDU, (Canadian Government Atomic Energy Commission).
CECt (Coleccion Espanola De Cultivos Tipo), Valencia, Spain.
CNMI&E (China National Machinery Imp. and Exp. Co., Shanghai, China.
Clements Pty. Ltd., Sydney,Aus.
Clustan Ltd., Edinburgh, UK.
CIG (Commonwealth Industrial Gases), Rocklea, Aus.
CSL (Commonwealth Serum Laboratories), Parkville, Aus.
DEC (Digital Equipment Corporation), Maynard, Ma.,USA.
Disposable Products, Adelaide, Aus
DIversy (A/asia) Pty. Ltd., Brisbane, Aus.
Duran (Geschaftsbereich Chemie), Mainz, Fdr.
Edmonston B., 17 Arvine Pl. Manchester, Conn., USA.
Eflab Oy, Helsinki, Finland.
EIken Chemical Co., Tokyo, Japan.
Email-Westinghouse Pty. Ltd., Brisbane, Aus.
Faulding F.H. (Queensland), Brisbane, Aus.
Flow Laboratories (Austlalasia Pty. Ltd.), Brisbane, Aus.
Fluka, Ag Chemische, Fabrik, Switzerland.
Gelman Clemco Instrument Co. Ann Arbor, Mich., USA.
GIBCO Diagnostics, Madison, Wisc., USA.
Gilford Instrument Labs Inc., Oberlin, Ohio, USA.
Gilson France Sa-72, Villiers Le Bel, France.
Grant Instruments, Cambridge, UK.
Gurr (G.T. Gurr) Ltd. London, UK.
Hitachi Ltd., Tokyo, Japan.
IBM (International Business Machines), Kingston, NY. USA.
ICN Pharmaceuticals, Covina, Cal., USA.
IEC (International Equipment Corporation),Needham Hts.,Mass.USA.
Johns Professional Products, Melbourne, Aus.
Jones Scientific Instruments, Melbourne, Aus.
Kartell (Trading through Sieper and Co.), Strathfield, Aus.
Kimble, Toledo, Ohio, USA.
Linbro, (See Flo Laboratories).
Labline Instruments Inc., Melrose Park, Ill., USA.
Lindner and May Pty. Ltd. Brisbane, Aus.
LKB Production, A.B. Bromma, Sweden.
May and Baker, Dagenham, England.
Medical Wire and Equipment Co., Potley, UK.
Merck (E. Merck) Ag., Darmstadt, Fdr.
Meteor glass, Vinelands, NJ., USA.
Millipore Corporation, Bedford, Mass. USA.
NCTC (National Collection of Type Cultures), London, UK.
Olympus Optical Co., Tokyo, Japan.

A16.0 **Supplier Register. (cont.)**

Oxoid Ltd., London, UK.
Polaron Equipment Ltd., Watford, UK.
Robbins Scientific, Rockville, Md., USA.
SAS Institute Inc. Cary, NC., USA.
SK & F (Smith, Kline and French Labs.), Philadelphia., USA.
Sartorius (Sartorius GMBH), Gottingen, Fdr.
Selby (ANAX) Pty. Ltd., (Brisbane), Aus.
Shell Chemical Company Limited, Brisbane, Aus.
Shimadzu, Kyoto, Japan.
Sigma Chemical Company Limited, St. Louis, Mo., USA.
Silentia, Brisbane, Aus.
STSC, Rockville, Md., USA.
Swofford D.L., Champaigne, Ill., USA.
Terumo (Australia) Pty. Ltd., Melbourne, Australia.
TPS Ionode Sales, Brisbane, Aus.
UENO Fine Chemicals, Japan.
Union Carbide Corporation, Chicago, Ill. USA.
Upjohn (Australia) Pty. Ltd., Brisbane, AUS.
Waters Associates, Milford, Mass. USA.
William Freeman and Co., Barnsleigh, Yorkshire, UK.

Table 3.13.1: (Inner), Sorted Euclidean Similarity Matrix for 96 OTU's from Batch [6.].

Table 3.13.2: (Outer), Sorted Euclidean Similarity Matrix for 96 OTU's from Batch [6.].

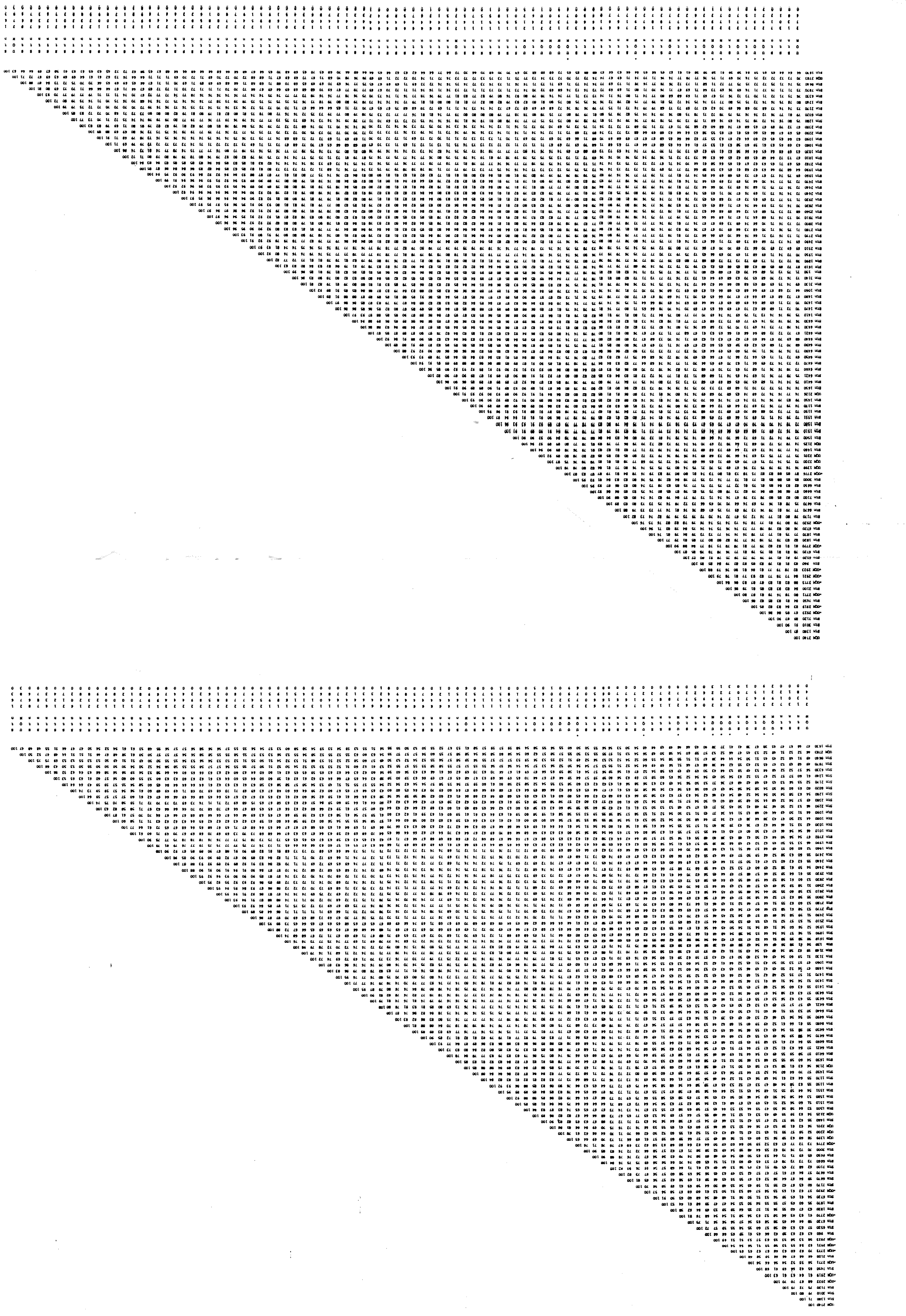
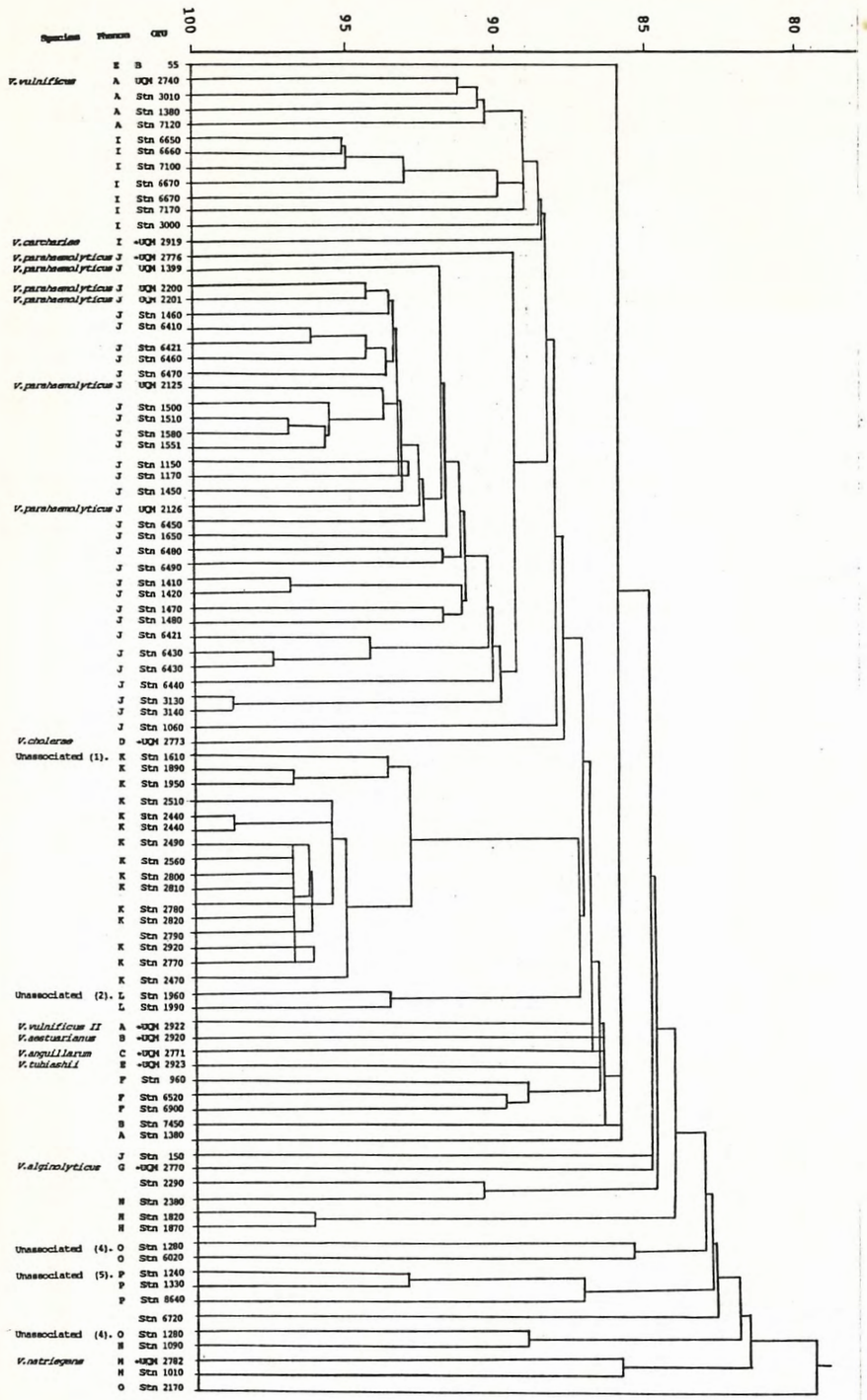


Figure 3.5.1: (Inner) UPGMS (*SAS* k = 8, Trim = 5 %), Clustering of Euclidean Matrix, (SLE); from 96 Batch [6.] OTU's.

Figure 3.5.2: (Outer) UPGMS (*SAS* k = 8, Trim = 5 %), Clustering of Jaccard Matrix, (SLJ); from 96 Batch [6.] OTU's.


% Similarity, SLE.
100
95
90
85
80
Species
Phenon
OTU
E B 55
V.vulnificus A UQM 2740
A Stn 3010
A Stn 1380
A Stn 7120
I Stn 6650
I Stn 6660
I Stn 7100
I Stn 6670
I Stn 6670
I Stn 7170
I Stn 3000
V.carchariae I *UQM 2919
V.parahaemolyticus J *UQM 2776
V.parahaemolyticus J UQM 1399
V.parahaemolyticus J UQM 2200
V.parahaemolyticus J UQM 2201
J Stn 1460
J Stn 6410
J Stn 6421
J Stn 6460
J Stn 6470
V.parahaemolyticus J UQM 2125
J Stn 1500
J Stn 1510
J Stn 1580
J Stn 1551
J Stn 1150
J Stn 1170
J Stn 1450
V.parahaemolyticus J UQM 2126
J Stn 6450
J Stn 1650
J Stn 6480
J Stn 6490
J Stn 1410
J Stn 1420
J Stn 1470
J Stn 1480
J Stn 6421
J Stn 6430
J Stn 6430
J Stn 6440
J Stn 3130
J Stn 3140
J Stn 1060
V.cholerae D *UQM 2773
Unassociated (1). K Stn 1610
K Stn 1890
K Stn 1950
K Stn 2510
K Stn 2440
K Stn 2440
K Stn 2490
K Stn 2560
K Stn 2800
K Stn 2810
K Stn 2780
K Stn 2820
Stn 2790
K Stn 2920
K Stn 2770
K Stn 2470
Unassociated (2). L Stn 1960
L Stn 1990
V.vulnificus II A *UQM 2922
V.aestuarianus B *UQM 2920
V.anguillarum C *UQM 2771
V.tubiashii E *UQM 2923
F Stn 960
F Stn 6520
F Stn 6900
B Stn 7450
A Stn 1380
J Stn 150
V.alginolyticus G *UQM 2770
Stn 2290
N Stn 2380
H Stn 1820
H Stn 1870
Unassociated (4). O Stn 1280
O Stn 6020
Unassociated (5). P Stn 1240
P Stn 1330
P Stn 8640
Stn 6720
Unassociated (4). O Stn 1280
N Stn 1090
V.natriegens N *UQM 2782
N Stn 1010
O Stn 2170

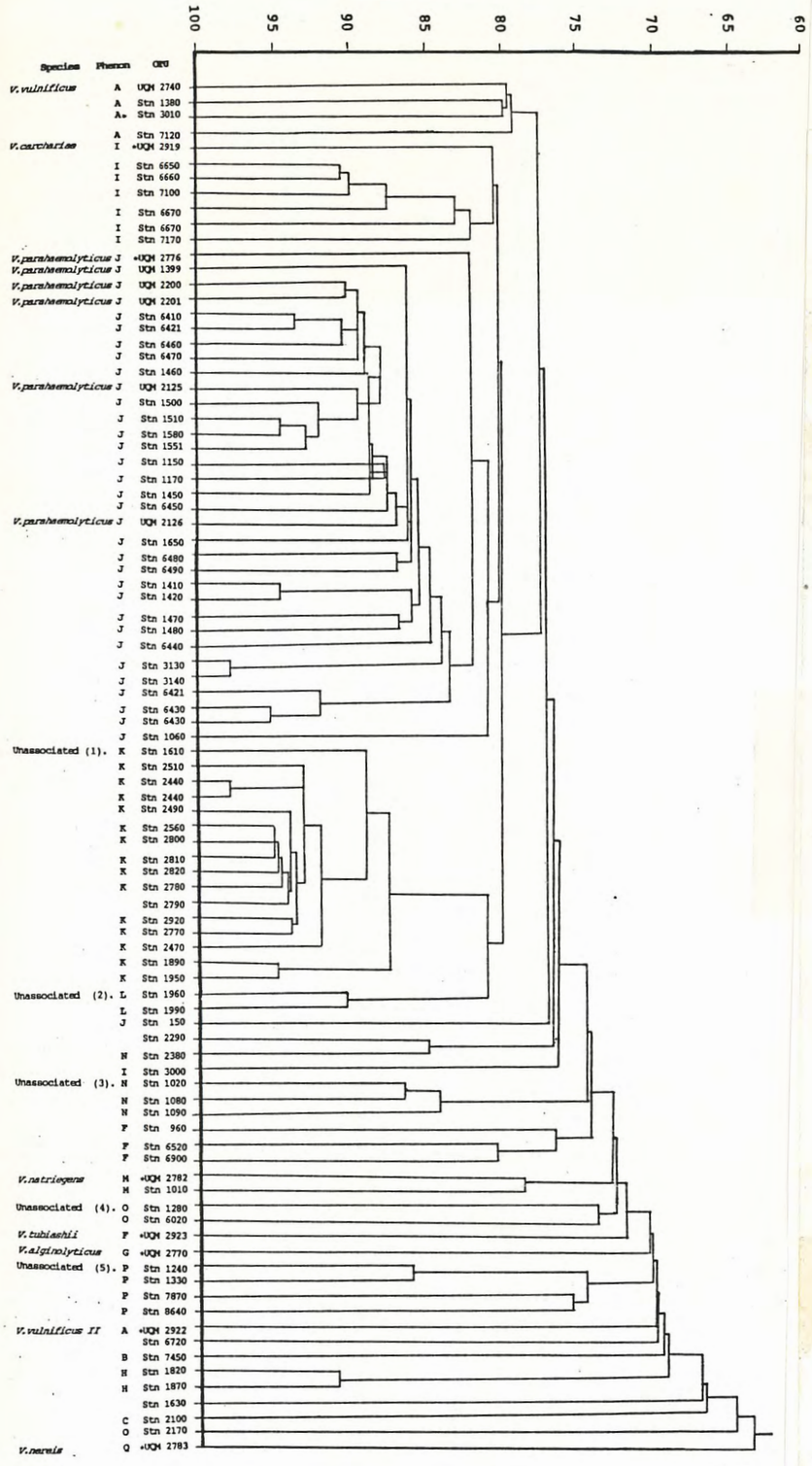
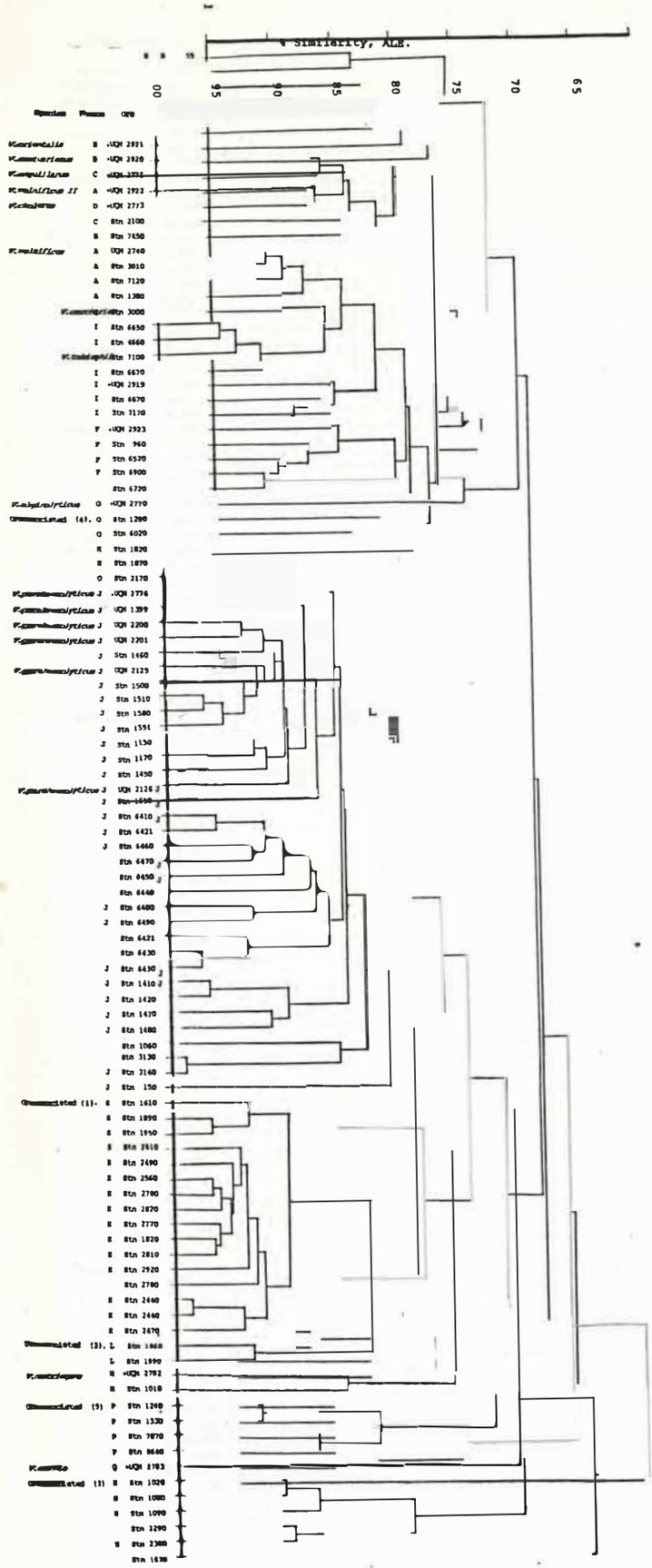

% Similarity, SLJ.
100
95
90
85
80
75
70
65
60
Species
Phenon
OTU
V.vulnificus A UQM 2740
A Stn 1380
A. Stn 3010
A Stn 7120
V.carchariae I *UQM 2919
I Stn 6650
I Stn 6660
I Stn 7100
I Stn 6670
I Stn 6670
I Stn 7170
V.parahaemolyticus J *UQM 2776
V.parahaemolyticus J UQM 1399
V.parahaemolyticus J UQM 2200
V.parahaemolyticus J UQM 2201
J Stn 6410
J Stn 6421
J Stn 6460
J Stn 6470
J Stn 1460
V.parahaemolyticus J UQM 2125
J Stn 1500
J Stn 1510
J Stn 1580
J Stn 1551
J Stn 1150
J Stn 1170
J Stn 1450
J Stn 6450
V.parahaemolyticus J UQM 2126
J Stn 1650
J Stn 6480
J Stn 6490
J Stn 1410
J Stn 1420
J Stn 1470
J Stn 1480
J Stn 6440
J Stn 3130
J Stn 3140
J Stn 6421
J Stn 6430
J Stn 6430
J Stn 1060
Unassociated (1). K Stn 1610
K Stn 2510
K Stn 2440
K Stn 2440
K Stn 2490
K Stn 2560
K Stn 2800
K Stn 2810
K Stn 2820
K Stn 2780
Stn 2790
K Stn 2920
K Stn 2770
K Stn 2470
K Stn 1890
K Stn 1950
Unassociated (2). L Stn 1960
L Stn 1990
J Stn 150
Stn 2290
N Stn 2380
I Stn 3000
Unassociated (3). N Stn 1020
N Stn 1080
N Stn 1090
F Stn 960
F Stn 6520
F Stn 6900
V.natriegens N *UQM 2782
N Stn 1010
Unassociated (4). O Stn 1280
O Stn 6020
V.tubiashii F *UQM 2923
V.alginolyticus G *UQM 2770
Unassociated (5). P Stn 1240
P Stn 1330
P Stn 7870
P Stn 8640
V.vulnificus II A *UQM 2922
Stn 6720
B Stn 7450
H Stn 1820
H Stn 1870
Stn 1630
C Stn 2100
O Stn 2170
V.nereis Q *UQM 2783

Figure 3.5.3: (Inner) UPGMA (*SAS* k = 8), Clustering of Euclidean Matrix (ALE); from 96 Batch [6.] OTU's.

Figure 3.5.4: (Outer) UPGMA (*SAS* k = 8), Clustering of Jaccard Matrix (ALJ); from 96 Batch [6.] OTU's.


% Similarity, ALK.
100
95
90
85
80
75
70
65
Species
Phase
Stn
V.orientalis
V.mediterraneus
V.anguillarum
V.vulnificus II
V.cholerae
V.vulnificus
V.carchariae
V.alginolyticus
Unassociated (4).
V.parahaemolyticus J
Unassociated (1).
Unassociated (3).
V.natriegens
Unassociated (5)
V.cincinnatiensis

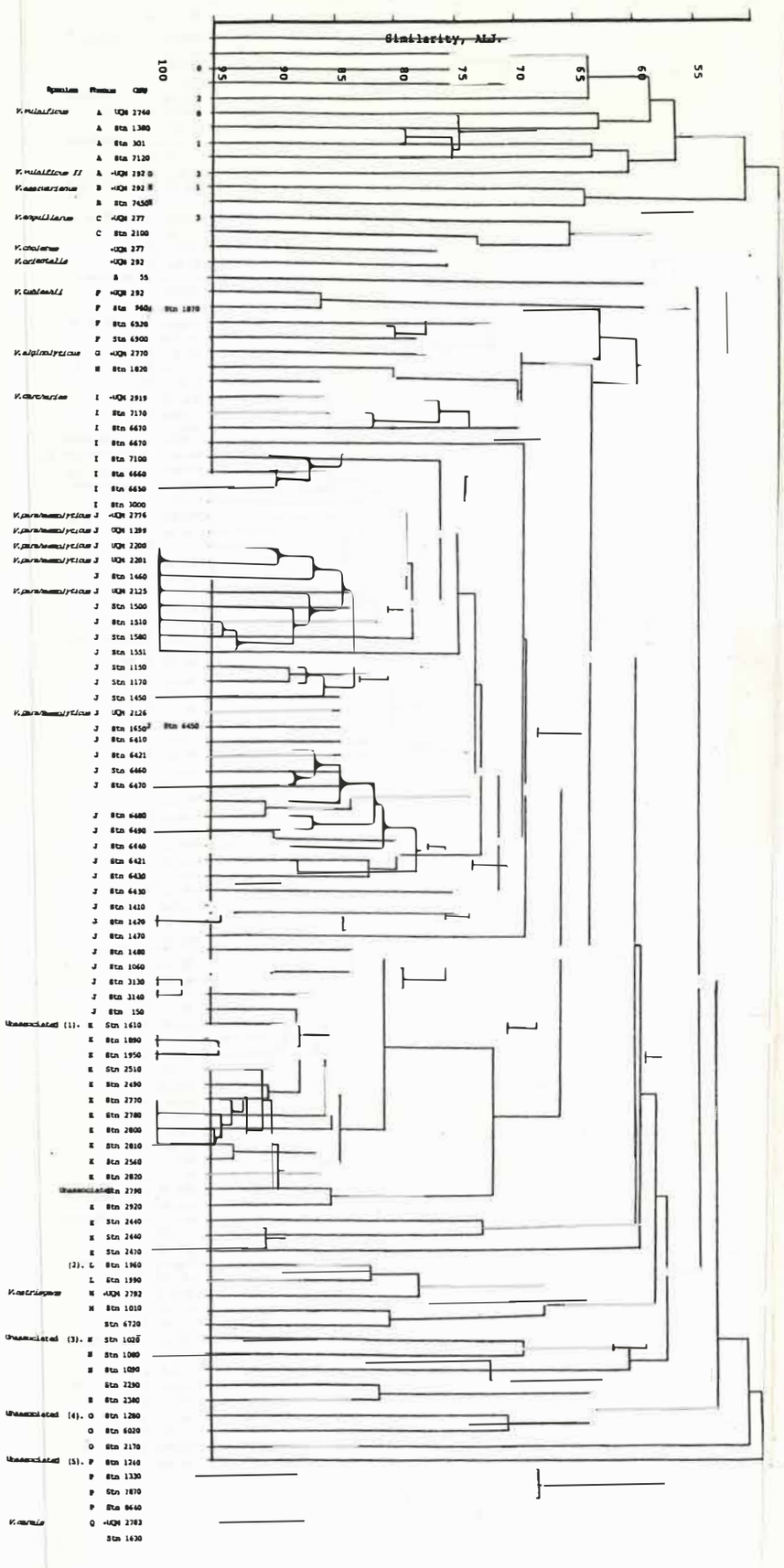
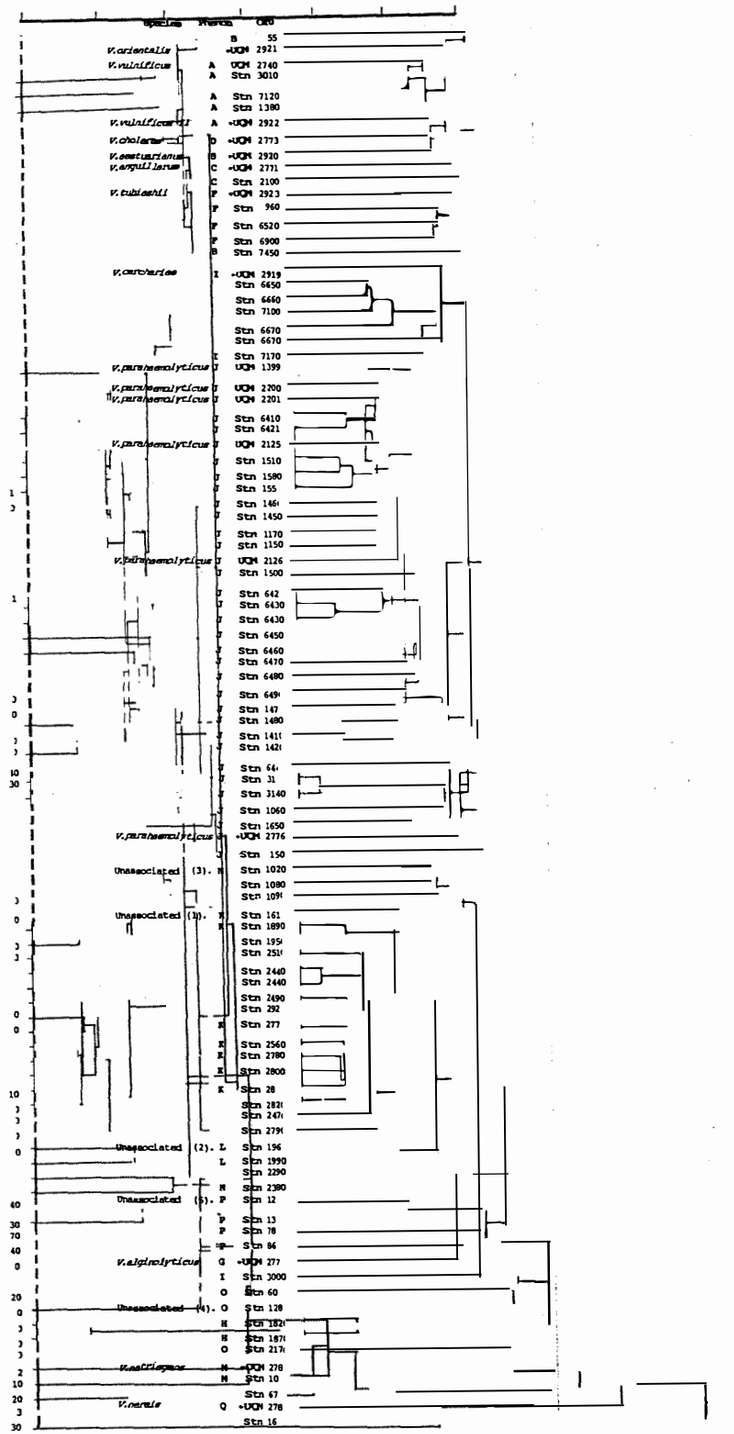

Similarity, ALK.
100
95
90
85
80
75
70
65
60
55
Species
Phase
Stn
V.vulnificus
V.vulnificus II
V.mediterraneus
V.anguillarum
V.cholerae
V.orientalis
V.cincinnatiensis
V.alginolyticus
V.carchariae
V.parahaemolyticus J
V.parahaemolyticus J
V.parahaemolyticus J
V.parahaemolyticus J
V.parahaemolyticus J
V.parahaemolyticus J
Unassociated (1).
V.natriegens
Unassociated (3).
Unassociated (4).
Unassociated (5).
V.cincinnatiensis

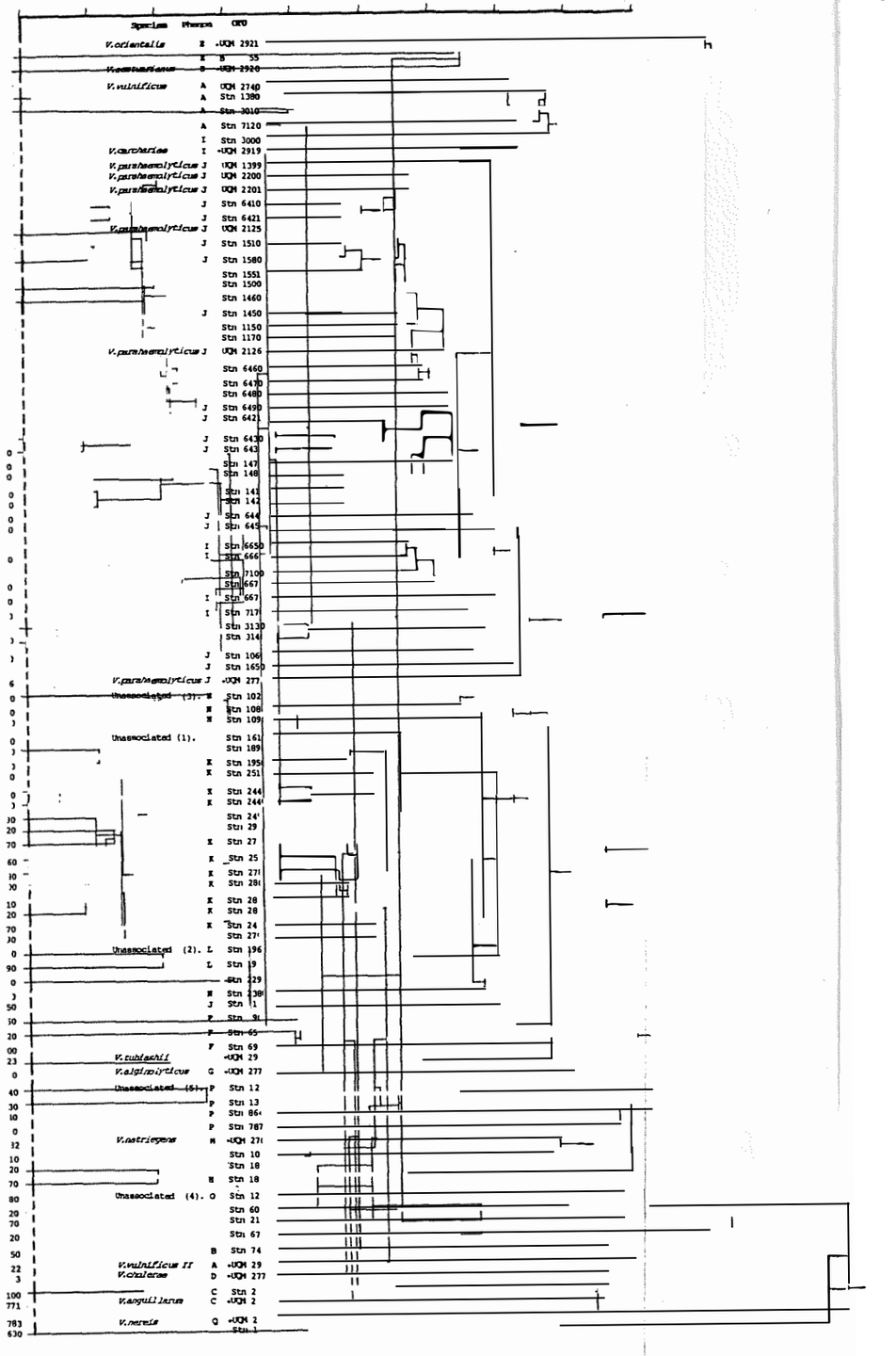
Figure 3.5.5: (Inner) UPGMM (*SAS* k = 8), Clustering of Euclidean Matrix (MLE); from 96 Batch [6.] OTU's.

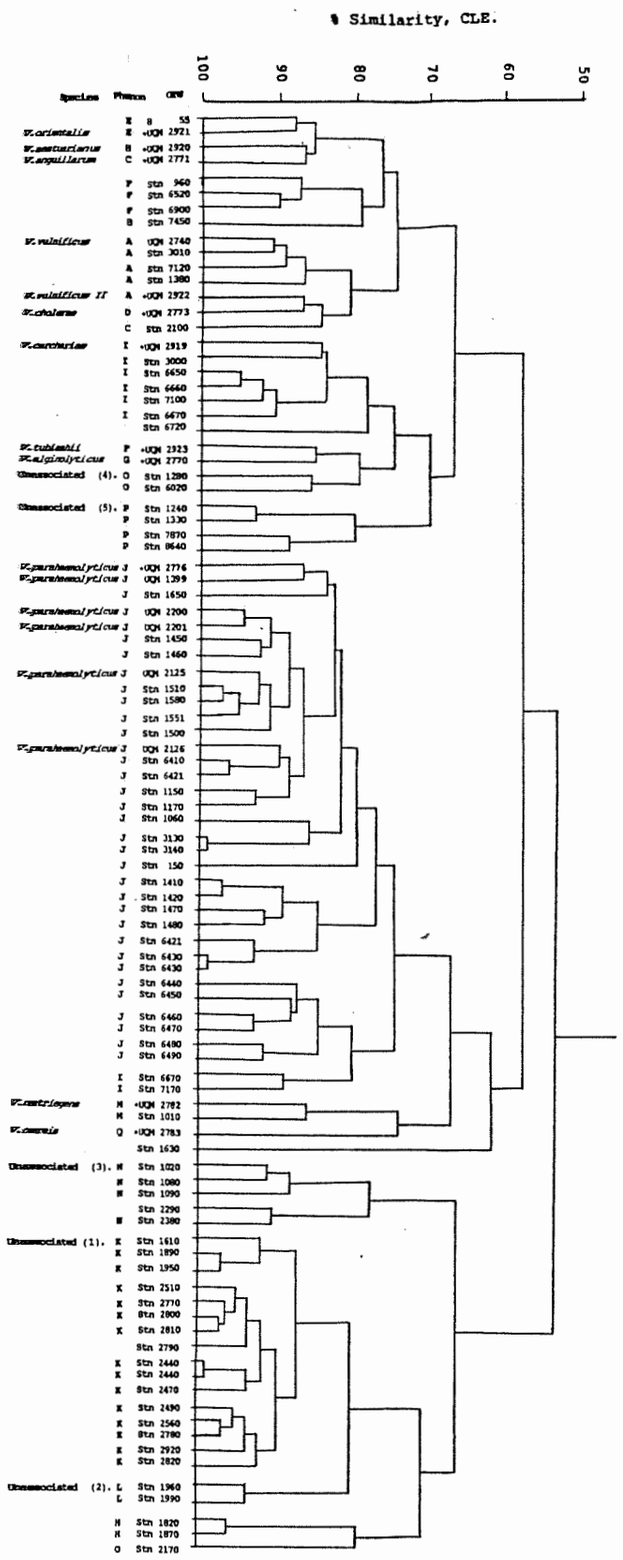
Figure 3.5.6: (Outer) UPGMM (*SAS* k = 8), Clustering of Jaccard Matrix (MLJ); from 96 Batch [6.] OTU's.

% Similarity, MLE.



% Similarity, MLJ.




% Similarity, CLE.
100
90
80
70
60
50
Species
Phenon
OTU
V.orientalis
V.cincinnatiensis
V.anguillarum
V.vulnificus
V.vulnificus II
V.cholerae
V.carchariae
V.tubiashii
V.alginolyticus
Unassociated (4).
Unassociated (5).
V.parahaemolyticus J
V.parahaemolyticus J
V.parahaemolyticus J
V.parahaemolyticus J
V.parahaemolyticus J
V.parahaemolyticus J
V.mediterranei
V.nereis
Unassociated (3).
Unassociated (1).
Unassociated (2).
V.natriegens

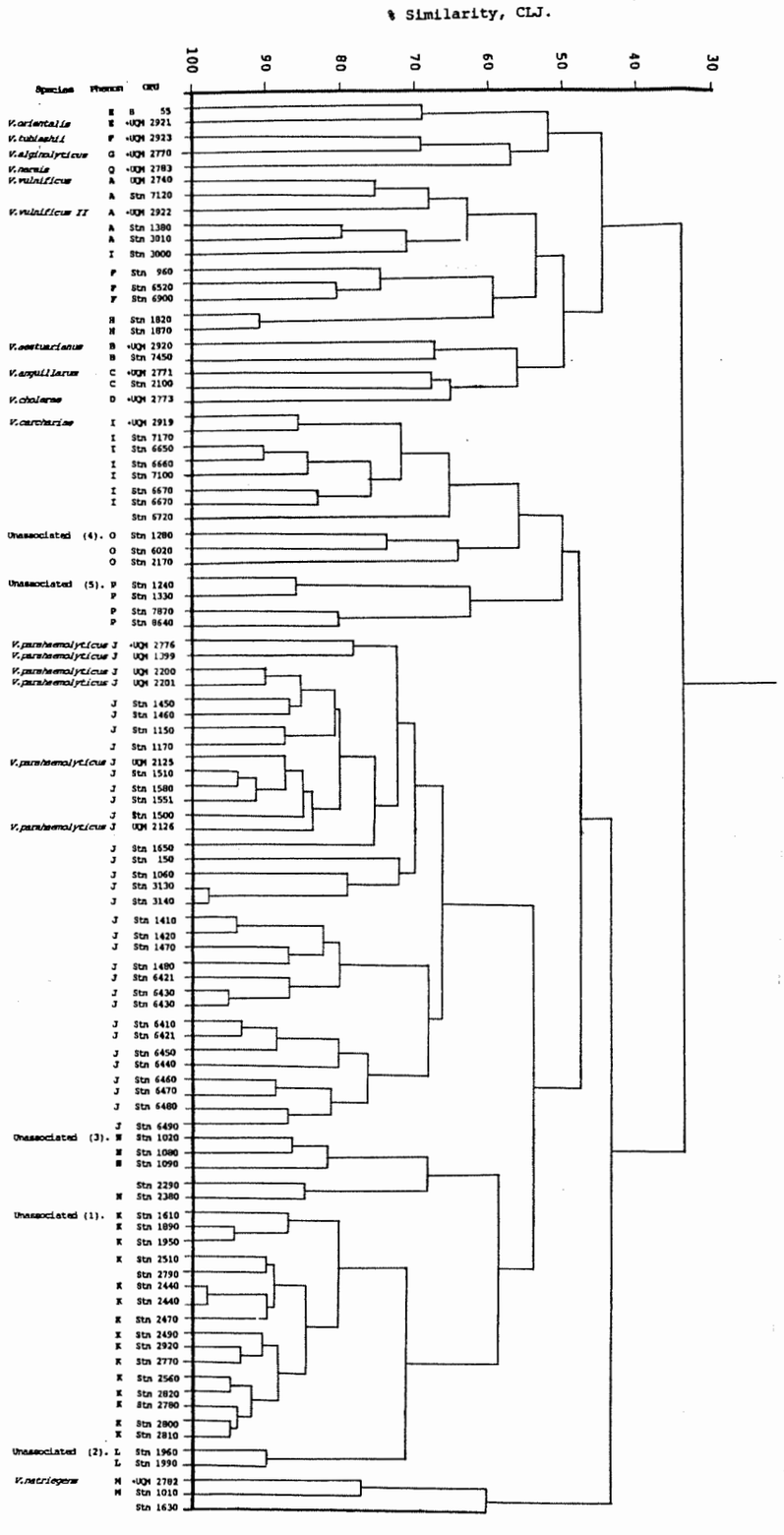
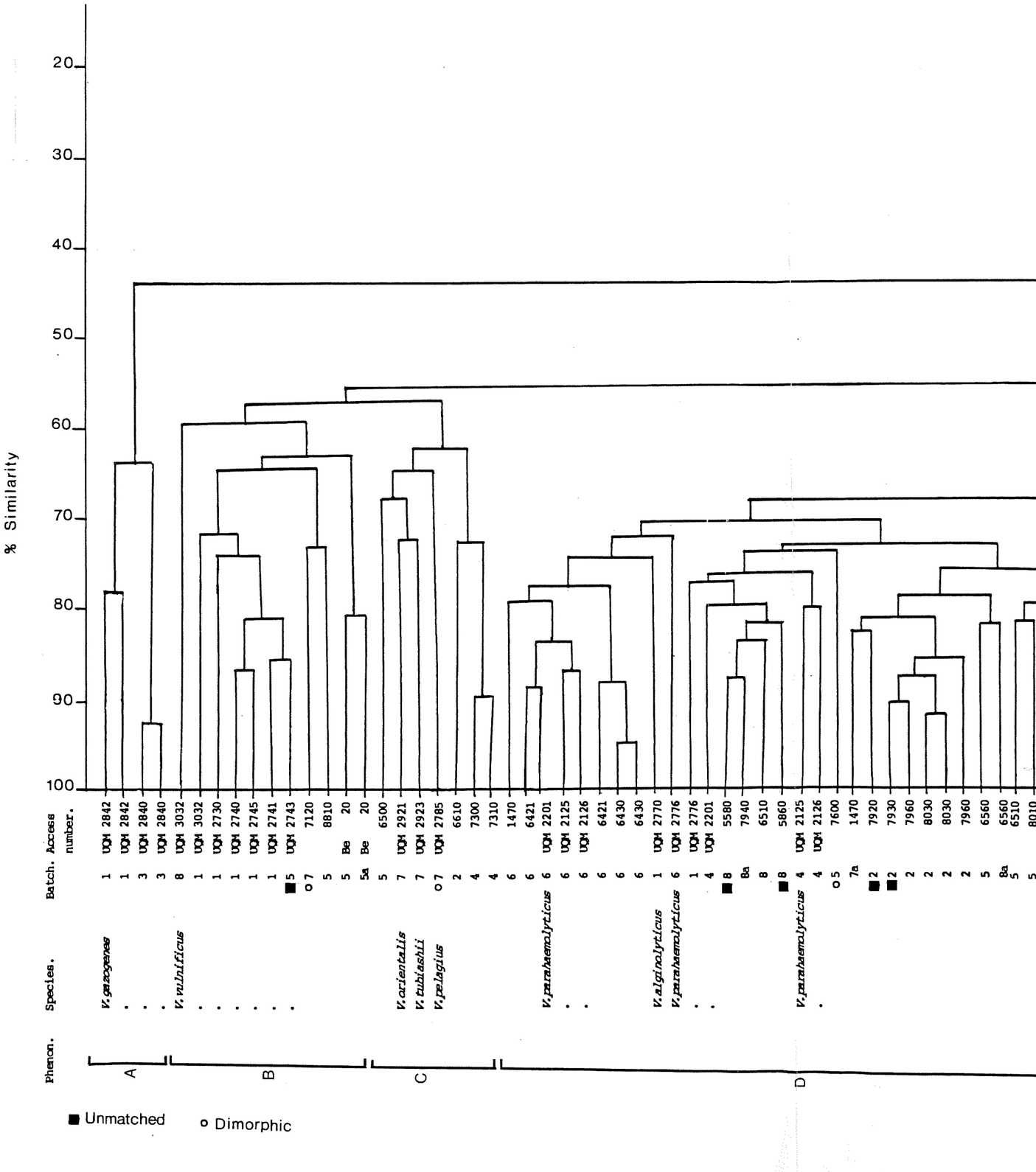
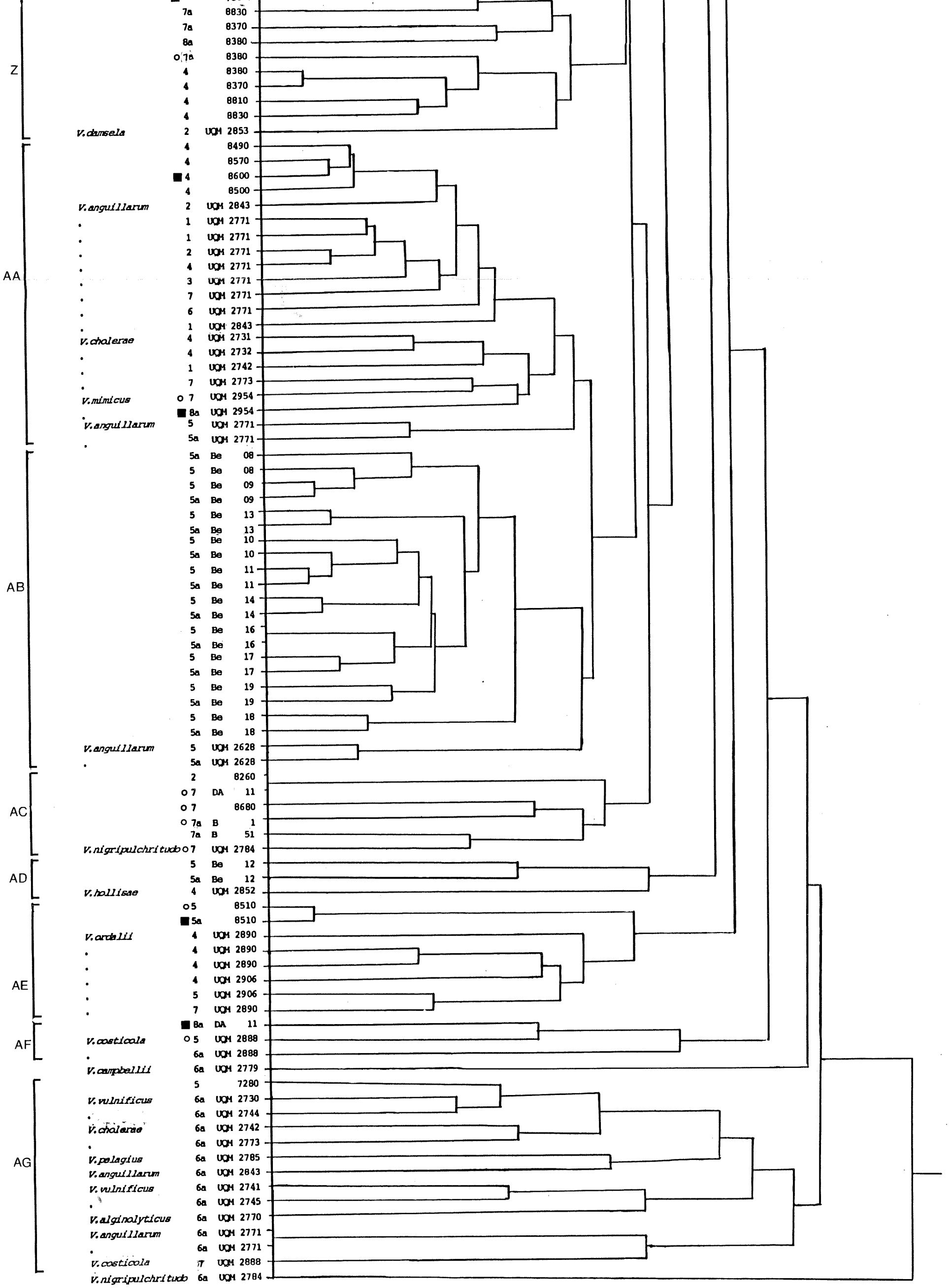

% Similarity, CLJ.
100
90
80
70
60
50
40
30
Species
Phenon
OTU
V.orientalis
V.tubiashii
V.alginolyticus
V.nereis
V.vulnificus
V.vulnificus II
V.cincinnatiensis
V.anguillarum
V.cholerae
V.carchariae
Unassociated (4).
Unassociated (5).
V.parahaemolyticus J
V.parahaemolyticus J
V.parahaemolyticus J
V.parahaemolyticus J
V.parahaemolyticus J
V.parahaemolyticus J
Unassociated (3).
Unassociated (1).
Unassociated (2).
V.natriegens

Figure 3.5.7: (Inner) UPGMC (*SAS* k = 8), Clustering of Euclidean Matrix (CLE); from 96 Batch [6.] OTU's.

Figure 3.5.8: (Outer) UPGMC (*SAS* k = 8), Clustering of Jaccard Matrix (CLJ); from 96 Batch [6.] OTU's.

Figure 3.7: UPGMA (*SAS* k = 8), Clustering of Jaccard Matrix from 277 Completely or Partly Replicated OTU's from all Batches; (unflagged OTU's are referred to in the text with an *Stn* prefix).




Z
AA
AB
AC
AD
AE
AF
AG
V.damsela
V.anguillarum
V.cholerae
V.mimicus
V.anguillarum
V.anguillarum
V.nigripulchritudo
V.hollisae
V.ordalii
V.costicola
V.campbellii
V.vulnificus
V.cholerae
V.pelagius
V.anguillarum
V.vulnificus
V.alginolyticus
V.anguillarum
V.costicola
V.nigripulchritudo

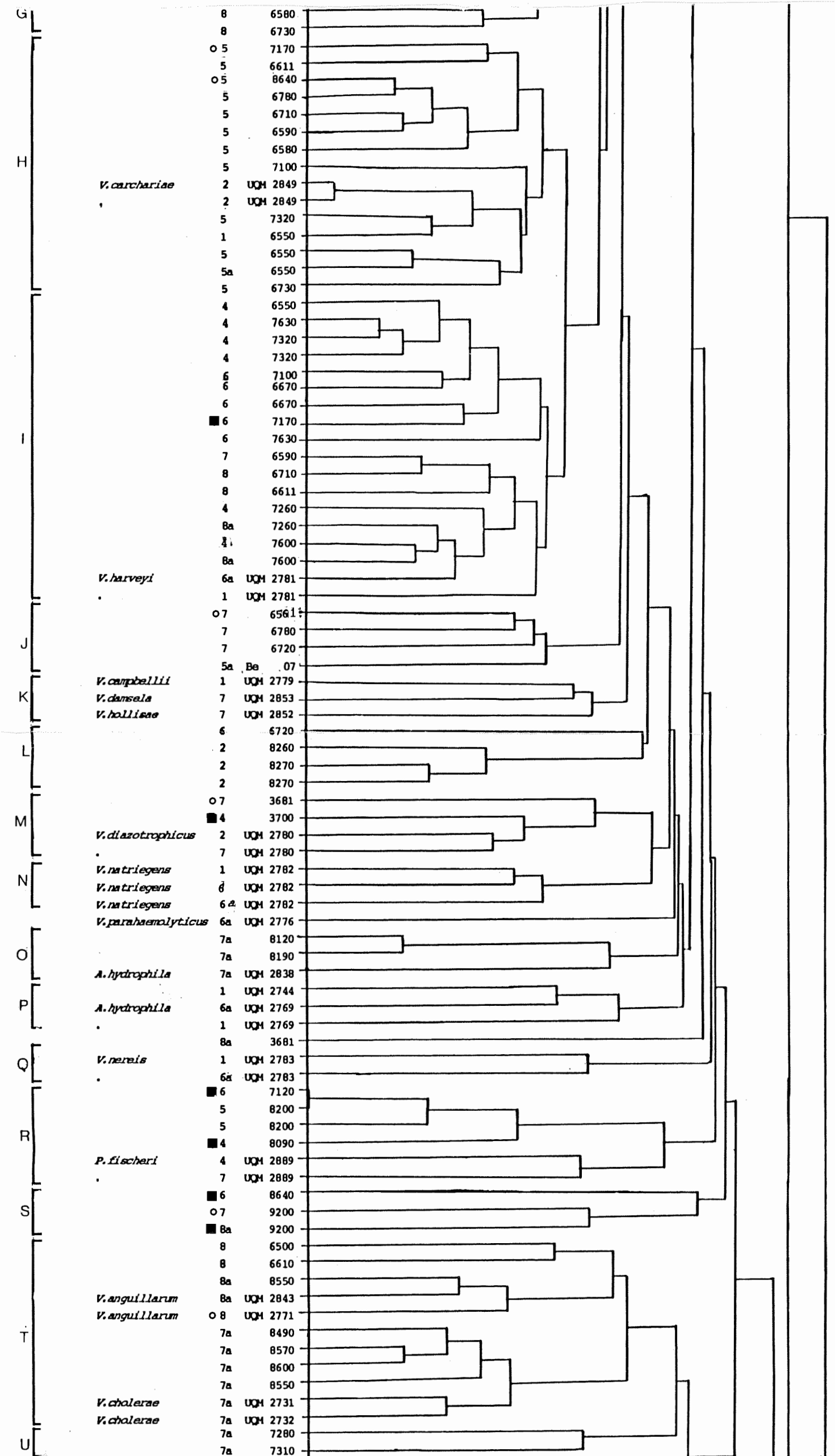
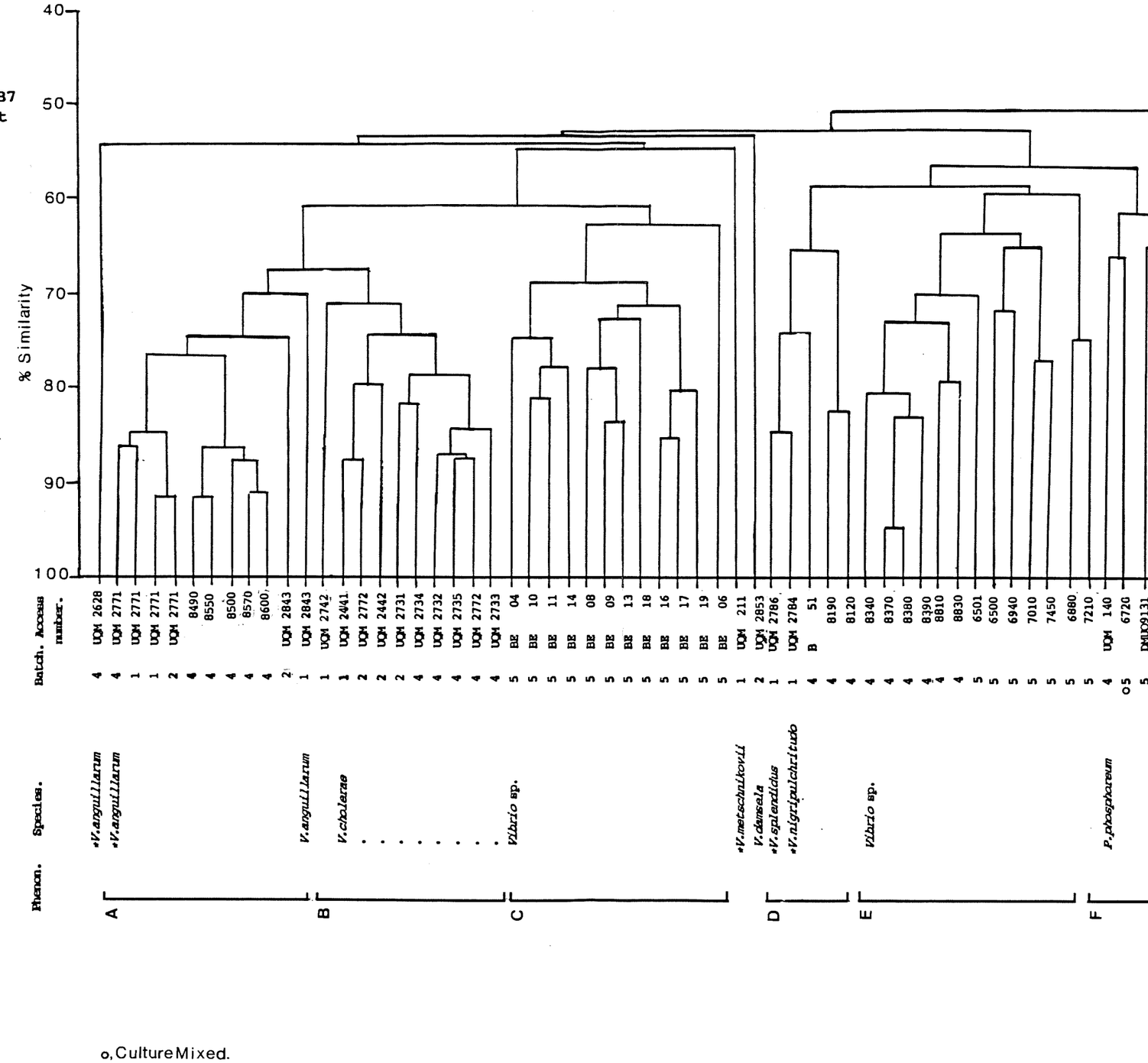
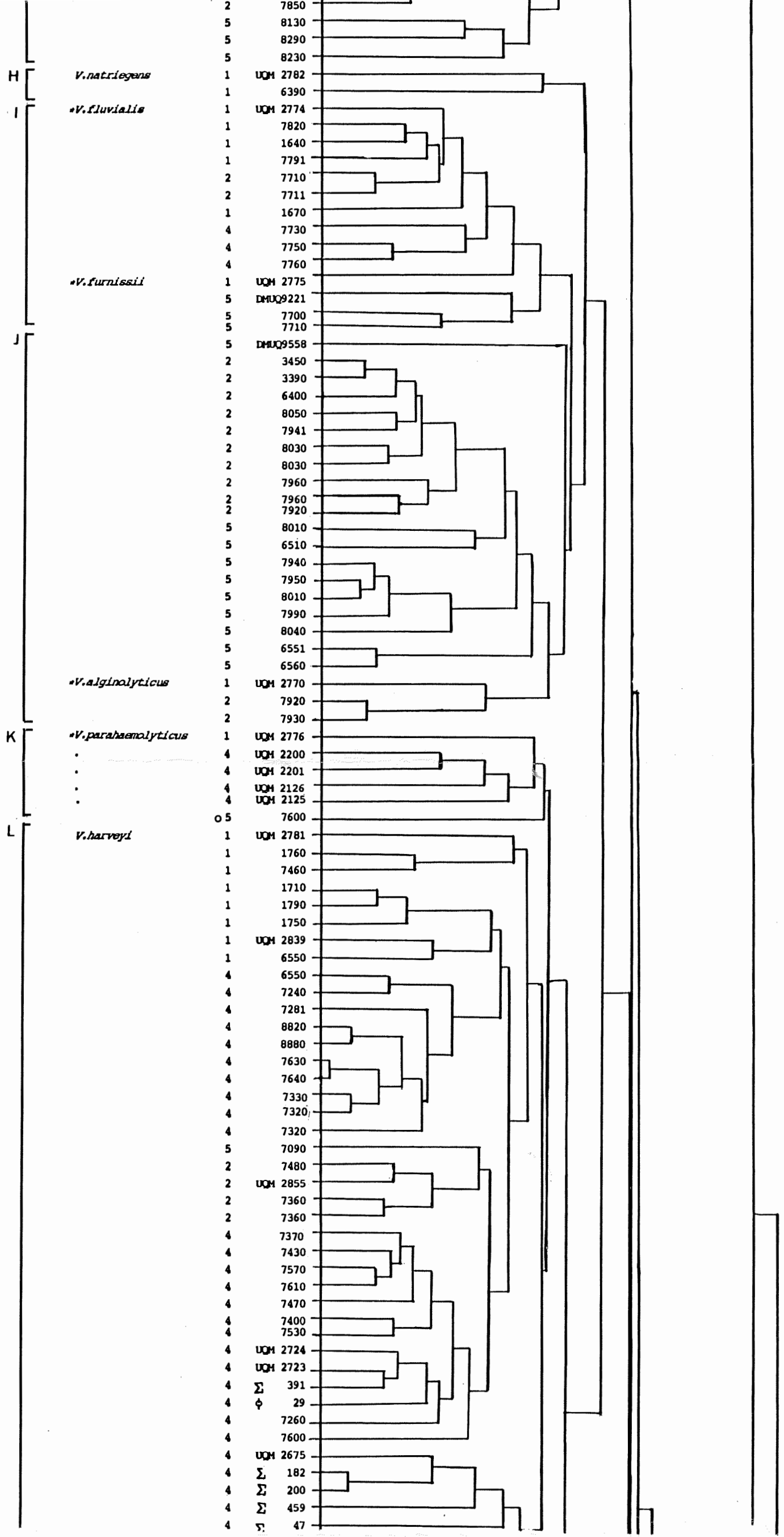

G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V. carchariae
V. harveyi
V. campbellii
V. damsela
V. hollisae
V. diazotrophicus
V. natriegens
V. natriegens
V. natriegens
V. parahaemolyticus
A. hydrophila
A. hydrophila
V. nereis
P. fischeri
V. anguillarum
V. anguillarum
V. cholerae
V. cholerae
8 6580
8 6730
o 5 7170
5 6611
o 5 8640
5 6780
5 6710
5 6590
5 6580
5 7100
2 UQM 2849
2 UQM 2849
5 7320
1 6550
5 6550
5a 6550
5 6730
4 6550
4 7630
4 7320
4 7320
6 7100
6 6670
6 6670
■ 6 7170
6 7630
7 6590
8 6710
8 6611
4 7260
8a 7260
8a 7600
6a UQM 2781
1 UQM 2781
o 7 6561
7 6780
7 6720
5a Be 07
1 UQM 2779
7 UQM 2853
7 UQM 2852
6 6720
2 8260
2 8270
2 8270
o 7 3681
■ 4 3700
2 UQM 2780
7 UQM 2780
1 UQM 2782
6 UQM 2782
6a UQM 2782
6a UQM 2776
7a 8120
7a 8190
7a UQM 2838
1 UQM 2744
6a UQM 2769
1 UQM 2769
8a 3681
1 UQM 2783
6a UQM 2783
■ 6 7120
5 8200
5 8200
■ 4 8090
4 UQM 2889
7 UQM 2889
■ 6 8640
o 7 9200
■ 8a 9200
8 6500
8 6610
8a 8550
8a UQM 2843
o 8 UQM 2771
7a 8490
7a 8570
7a 8600
7a 8550
7a UQM 2731
7a UQM 2732
7a 7280
7a 7310

Figure 3.8: UPGMA (*SAS* k = 8), Clustering of Jaccard Matrix from 287 OTU's from Batches 1,2,4 & 5; (unflagged OTU's are referred to in the text with an *Stn* prefix).



o, Culture Mixed.


H
V.natriegens
1 UQM 2782
1 6390
I
*V.fluvialis
1 UQM 2774
1 7820
1 1640
1 7791
2 7710
2 7711
1 1670
4 7730
4 7750
4 7760
*V.furnissii
1 UQM 2775
5 DMUQ9221
5 7700
5 7710
J
5 DMUQ9558
2 3450
2 3390
2 6400
2 8050
2 7941
2 8030
2 8030
2 7960
2 7960
2 7920
5 8010
5 6510
5 7940
5 7950
5 8010
5 7990
5 8040
5 6551
5 6560
*V.alginolyticus
1 UQM 2770
2 7920
2 7930
K
*V.parahaemolyticus
1 UQM 2776
4 UQM 2200
4 UQM 2201
4 UQM 2126
4 UQM 2125
o 5 7600
L
V.harveyi
1 UQM 2781
1 1760
1 7460
1 1710
1 1790
1 1750
1 UQM 2839
1 6550
4 6550
4 7240
4 7281
4 8820
4 8880
4 7630
4 7640
4 7330
4 7320
4 7320
5 7090
2 7480
2 UQM 2855
2 7360
2 7360
4 7370
4 7430
4 7570
4 7610
4 7470
4 7400
4 7530
4 UQM 2724
4 UQM 2723
4 Σ 391
4 ϕ 29
4 7260
4 7600
4 UQM 2675
4 Σ 182
4 Σ 200
4 Σ 459
4 Σ 47

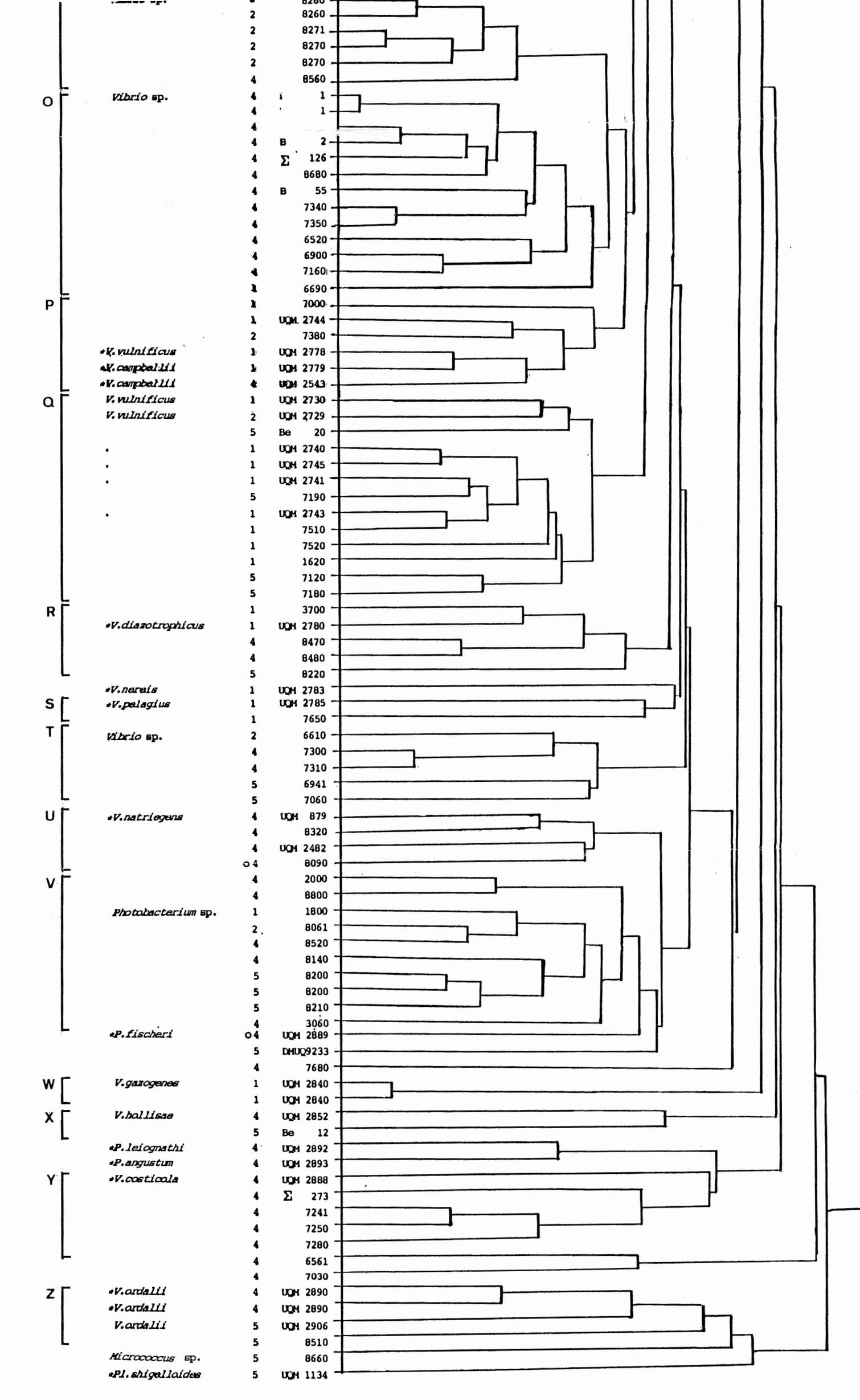
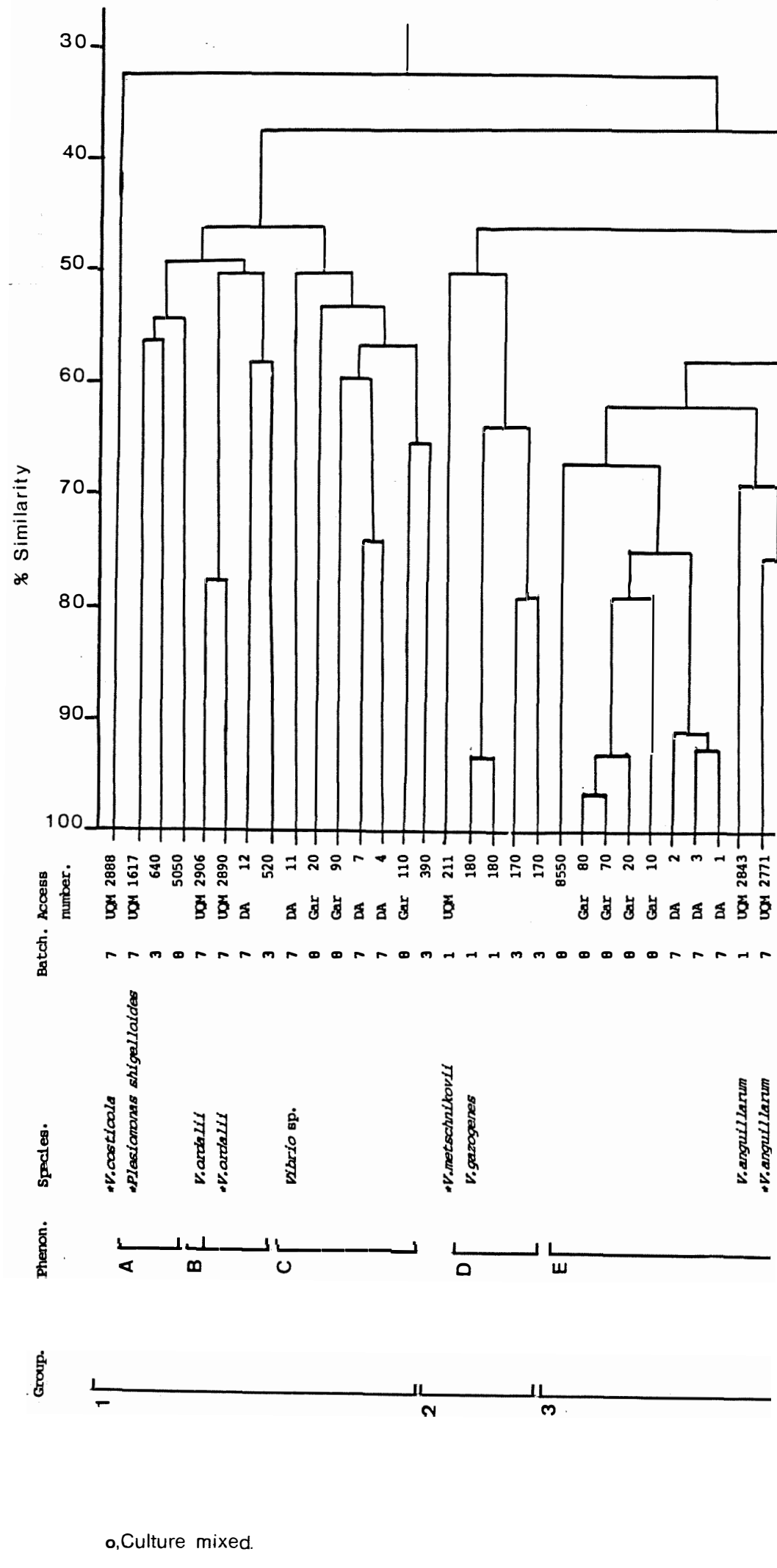
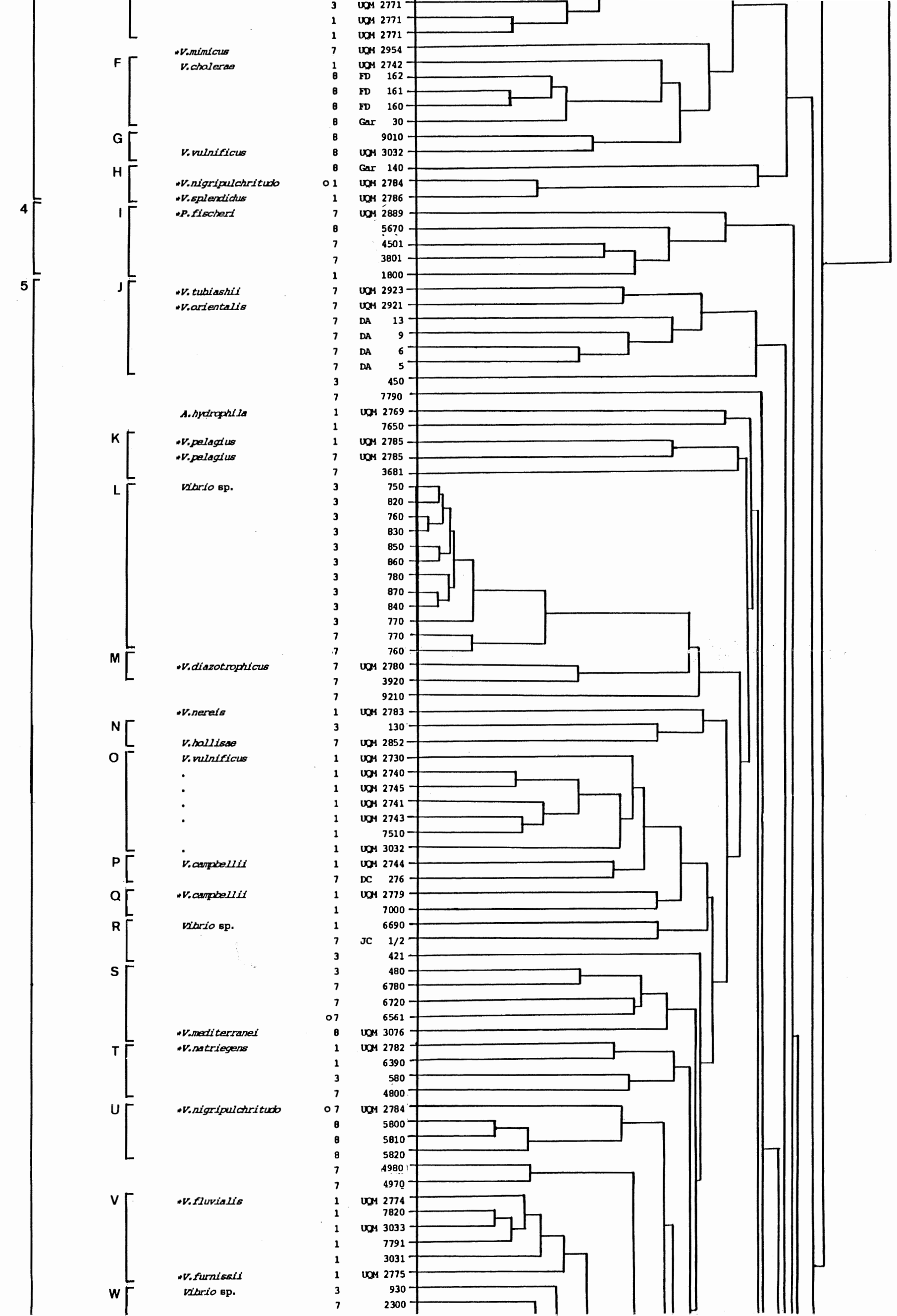
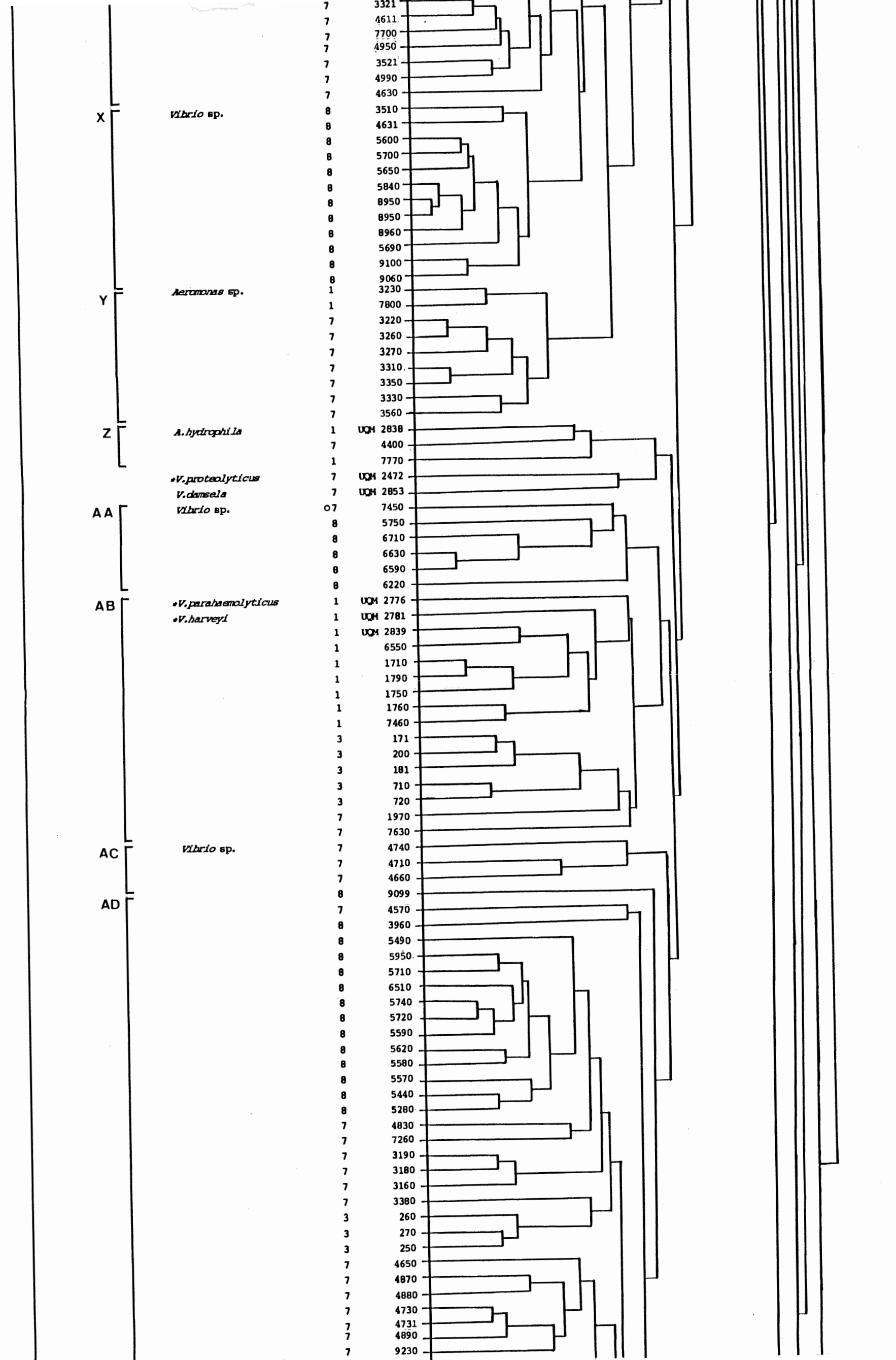

O
P
Q
R
S
T
U
V
W
X
Y
Z
Vibrio sp.
*V.vulnificus
*V.campbellii
*V.campbellii
V.vulnificus
V.vulnificus
*V.diazotrophicus
*V.nereis
*V.pelagius
Vibrio sp.
*V.natriegens
Photobacterium sp.
*P.fischeri
V.gazogenes
V.hollisae
*P.leiognathi
*P.angustum
*V.costicola
*V.ordalii
*V.ordalii
V.ordalii
Micrococcus sp.
*Pl.shigelloides
UQM 2744
UQM 2778
UQM 2779
UQM 2543
UQM 2730
UQM 2729
UQM 2740
UQM 2745
UQM 2741
UQM 2743
UQM 2780
UQM 2783
UQM 2785
UQM 879
UQM 2482
UQM 2889
DMUQ9233
UQM 2840
UQM 2840
UQM 2852
UQM 2892
UQM 2893
UQM 2888
UQM 2890
UQM 2890
UQM 2906
UQM 1134

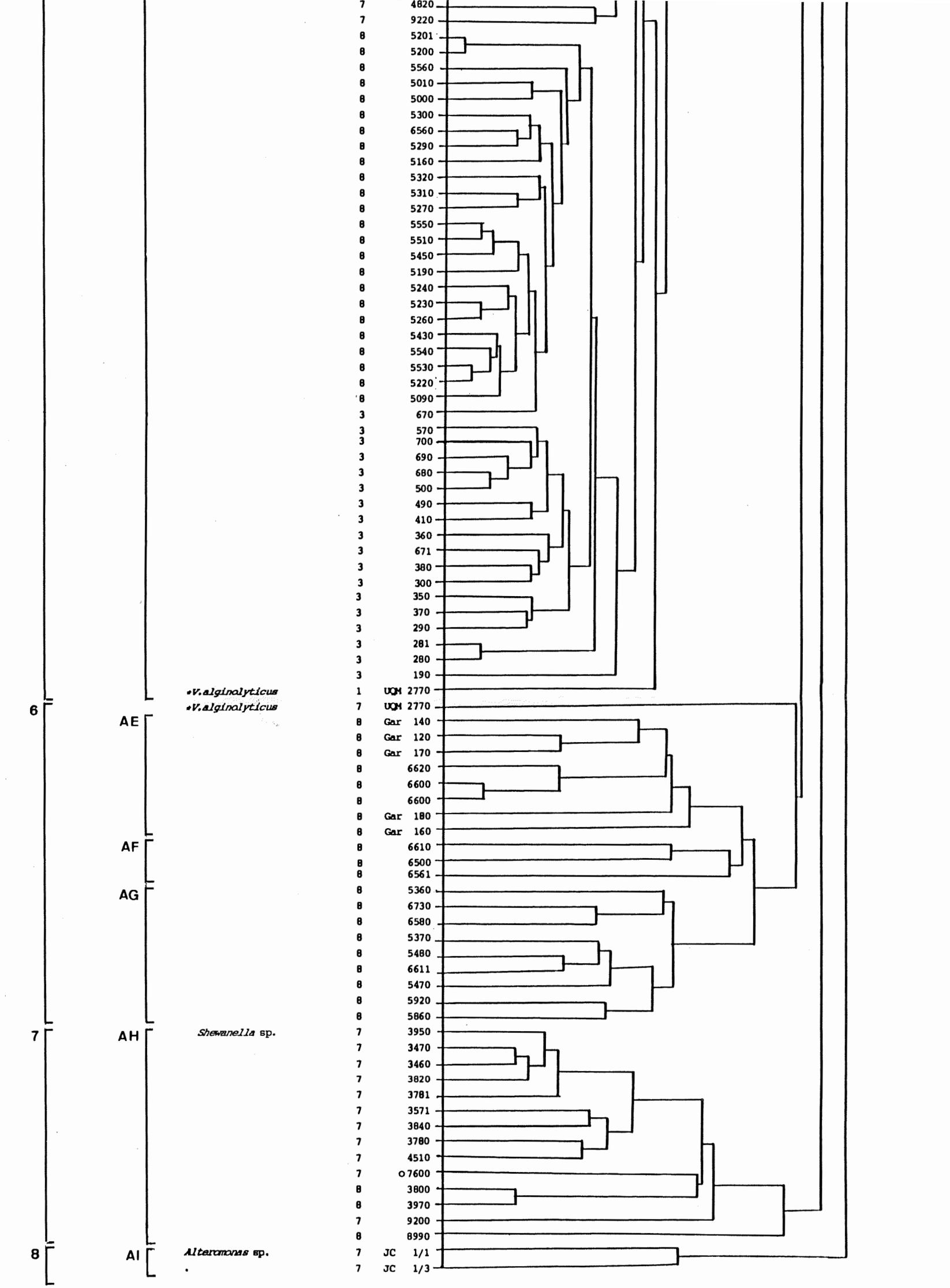
Figure 3.9: UPGMA (*Microcluster*), Clustering of Jaccard Matrix from 289 OTU's from Batches 1,3,7 & 8; (unflagged OTU's are referred to in the text with an *Stn* prefix).



o,Culture mixed.


4
5
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
*V.mimicus
V.cholerae
V.vulnificus
*V.nigripulchritudo
*V.splendidus
*P.fischeri
*V.tubiashii
*V.orientalis
A.hydrophila
*V.pelagius
*V.pelagius
Vibrio sp.
*V.diazotrophicus
*V.nereis
V.hollisae
V.vulnificus
V.campbellii
*V.campbellii
Vibrio sp.
*V.mediterranei
*V.natriegens
*V.nigripulchritudo
*V.fluvialis
*V.furnissii
Vibrio sp.
3 UQM 2771
1 UQM 2771
1 UQM 2771
7 UQM 2954
1 UQM 2742
8 FD 162
8 FD 161
8 FD 160
8 Gar 30
8 9010
8 UQM 3032
8 Gar 140
o 1 UQM 2784
1 UQM 2786
7 UQM 2889
8 5670
7 4501
7 3801
1 1800
7 UQM 2923
7 UQM 2921
7 DA 13
7 DA 9
7 DA 6
7 DA 5
3 450
7 7790
1 UQM 2769
1 7650
1 UQM 2785
7 UQM 2785
7 3681
3 750
3 820
3 760
3 830
3 850
3 860
3 780
3 870
3 840
3 770
7 770
7 760
7 UQM 2780
7 3920
7 9210
1 UQM 2783
3 130
7 UQM 2852
1 UQM 2730
1 UQM 2740
1 UQM 2745
1 UQM 2741
1 UQM 2743
1 7510
1 UQM 3032
1 UQM 2744
7 DC 276
1 UQM 2779
1 7000
1 6690
7 JC 1/2
3 421
3 480
7 6780
7 6720
o 7 6561
8 UQM 3076
1 UQM 2782
1 6390
3 580
7 4800
o 7 UQM 2784
8 5800
8 5810
8 5820
7 4980
7 4970
1 UQM 2774
1 7820
1 UQM 3033
1 7791
1 3031
1 UQM 2775
3 930
7 2300


X
Vibrio sp.
Y
Aeromonas sp.
Z
A.hydrophila
*V.proteolyticus
V.damsela
Vibrio sp.
AA
AB
*V.parahaemolyticus
*V.harveyi
Vibrio sp.
AC
AD
7 3321
7 4611
7 7700
7 4950
7 3521
7 4990
7 4630
8 3510
8 4631
8 5600
8 5700
8 5650
8 5840
8 8950
8 8950
8 8960
8 5690
8 9100
8 9060
1 3230
1 7800
7 3220
7 3260
7 3270
7 3310
7 3350
7 3330
7 3560
1 UQM 2838
7 4400
1 7770
7 UQM 2472
7 UQM 2853
07 7450
8 5750
8 6710
8 6630
8 6590
8 6220
1 UQM 2776
1 UQM 2781
1 UQM 2839
1 6550
1 1710
1 1790
1 1750
1 1760
1 7460
3 171
3 200
3 181
3 710
3 720
7 1970
7 7630
7 4740
7 4710
7 4660
8 9099
7 4570
8 3960
8 5490
8 5950
8 5710
8 6510
8 5740
8 5720
8 5590
8 5620
8 5580
8 5570
8 5440
8 5280
7 4830
7 7260
7 3190
7 3180
7 3160
7 3380
3 260
3 270
3 250
7 4650
7 4870
7 4880
7 4730
7 4731
7 4890
7 9230


7 4820
7 9220
8 5201
8 5200
8 5560
8 5010
8 5000
8 5300
8 6560
8 5290
8 5160
8 5320
8 5310
8 5270
8 5550
8 5510
8 5450
8 5190
8 5240
8 5230
8 5260
8 5430
8 5540
8 5530
8 5220
8 5090
3 670
3 570
3 700
3 690
3 680
3 500
3 490
3 410
3 360
3 671
3 380
3 300
3 350
3 370
3 290
3 281
3 280
3 190
1 UQM 2770
*V.alginolyticus
7 UQM 2770
*V.alginolyticus
6
AE
8 Gar 140
8 Gar 120
8 Gar 170
8 6620
8 6600
8 6600
8 Gar 180
8 Gar 160
AF
8 6610
8 6500
8 6561
AG
8 5360
8 6730
8 6580
8 5370
8 5480
8 6611
8 5470
8 5920
8 5860
7
AH
Shewanella sp.
7 3950
7 3470
7 3460
7 3820
7 3781
7 3571
7 3840
7 3780
7 4510
7 o7600
8 3800
8 3970
7 9200
8 8990
8
AI
Alteromonas sp.
7 JC 1/1
7 JC 1/3