Final report

Application of extracellular enzyme techniques to studying the role of bacteria in the ecology of prawn ponds and diseases of *Penaeus monodon*

Paul T. Smith Ph D

Project No. 1998/311

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1998/311: APPLICATION OF EXTRACELLULAR ENZYME TECHNIQUES TO STUDYING THE ROLE OF BACTERIA IN THE ECOLOGY OF PRAWN PONDS AND DISEASES OF *PENAEUS MONODON*.

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Objectives:

1. To apply recently developed technologies for bacterial enzyme analysis to the study of microbial processes in prawn farms.
2. To investigate the bacteriology of prawn ponds throughout the entire growouts for ponds at five Australian farms.
3. To investigate relationships between bacterial enzymes and pond productivity, with emphasis on prawn health, growth rates, feeding strategies and prawn survival.
4. To determine which are the key bacterial extracellular enzymes to study when making a rapid assessment of the status of a pond for HACCP (Hazard Analysis and Critical Control Points).
5. To investigate the associations between the various pond management practices on bacterial enzymes and pond conditions.
6. To compare the bacterial enzymes of ponds used to culture *P. monodon*.
7. To effectively communicate the results of the study to the prawn farming industry and fisheries departments through a manual for farmers on the impacts of management practices on pond bacteriology and productivity.

Non-technical Summary

Outcomes achieved

The main outcomes have been to provide new knowledge on the bacteriology of prawn farms and an innovative technique that can be used by the Australian prawn farming industry. Prior to this study there had been limited research into the bacteriology of prawn farms and this has seriously impacted on the development of the industry. To explain, it is well known that regulatory authorities need to adopt a precautionary principal when assessing development applications, particularly if there is a paucity of vital data. The discharge of effluent from prawn farms in sensitive coastal areas, such as the Great Barrier Reef, is a contentious issue. Prawn farmers reasonably explain that the effluent is relatively dilute in comparison with many other agricultural and industrial effluents. However environmentalists and regulators respond by arguing that if the effluent is benign, farmers should be capable of reusing it. In light of these views, it is clear to say that the Australian prawn farming industry has not grown according to the "Australian Prawn Farm Industry Vision" (eg Fig 10, R&D Plan 1996-2000 by Macarthur Consulting 1995).

In reality, it is the bacteria, especially the pathogenic strains of *Vibrio* that need to be managed in order to maintain healthy ponds and treat effluent. Previous reports by the author showed that a) bacteria are good bioindicators of conditions in prawn ponds and b) effluent can contain high levels of vibrios. Exciting recent developments with some farms overseas (eg in Belize) show that zero-water exchange depends on a healthy bacterial community. It is timely that this FRDC-funded project tackled an area that can provide knowledge and benefits for the industry and protection of fisheries habitats.

The project used a revolutionary technique for studying the bacteriology of prawn farms. For the first time, the rates of activity of over 20 types of extracellular enzymes were monitored in order to investigate microbial processes. Extracellular enzymes are secreted by bacteria to break down organic molecules such as proteins, lipids and carbohydrates. As a result we now have a much clearer picture of what happens during the culture cycle (ie growout) of prawns. Importantly, the project investigated the effects of a wide range of management practices and the effects they have on pond bacteriology. By carrying out this study at six farms and three hatcheries in two distinctly different areas (Far North Queensland and the Clarence River, NSW), we have new knowledge on the indicators of pond health and the factors that cause water quality to deteriorate. This is essential information for the industry to develop best management practices for ponds and improved treatment systems for effluent.

The project also used mesocosms (tank tests) to identify changes that occur to the key bacterial enzymes under a range of treatments, including addition of various supplementary feeds, use of various types of biofiltration systems and addition of probiotics. Results from mesocoms were further tested in field trials by monitoring the bacteriology of prawn ponds following additions of organic supplements. A range of hazards relating to bacteria in prawn farms were identified and a HACCP process (hazard analysis and critical control points) was developed to assist the industry in managing pond health and reducing environmental impacts to fisheries habitats.

The fundamental **aim of the project** was to study the bacteriology of prawn farms in order to provide the industry with better indicators of pond processes and prawn health. Thus providing the industry with improved methods for managing ponds and effluent treatment.

Preliminary discussions with farmers and researchers had shown that they are often at a loss to account for differences between ponds in productivity, survival and growth rates. The project focused on bacteria because they are the most abundant organism in prawn ponds, being ubiquitous in all types of sediments, water and organisms. More importantly, their processes determine: the fate of most organic matter in ponds, water quality, sediment condition and health of prawns.

Conventional methods in bacteriology (ie plate counts and identification of species) are limited because for most environmental samples, only a small proportion of bacteria can be isolated by culture. Thus a new more powerful technique for studying the actual processes carried out by bacteria was successfully developed and tested. Extracellular enzymes are important because they are secreted by microbes to breakdown organic matter in the environment. The difference between the two approaches to studying bacteriology can be summarized: the traditional methods are more concerned with how many bacteria are present and what are their names, while the extracellular enzyme method is concerned with what the bacteria are doing and how fast are they doing it. By analogy, a factory could be characterized by its number of employees and their names, or by the work the employees are doing and the rate at which they do it.

The first objective was successful in applying the new technology to studying the bacteriology of prawn farms. The technique allowed for the rapid, direct and sensitive measurement of rates of activity of over 20 bacterial enzymes in various environmental samples (i.e. water, sediment, algal blooms, detritus, prawn faeces and isolated bacteria). The main types of extracellular enzymes that were measured were those involved in breakdown of proteins, carbohydrates (sugars, starches and chitins), esterases (lipids and fats) and inorganics (phosphates and sulfates). In the first 6 months of the project the protocols for collecting samples and measuring rates of enzyme activities were successfully developed and tested.

For **the second objective**, the rates of activities of over 20 enzymes were successfully measured throughout the entire growout at 6 farms (3 in Far North Queensland and 3 on the Clarence River, NSW). Four of the farms (Tru Blu, Searanch, Melivans, Ponderosa were monitored for 3 years, while the other farms (Fortune and Pearler) were monitored for 2 years. Different sampling strategies were employed for each farm; variables included the number of ponds per farm, location and number of sample sites per pond and frequency of sampling. At the start of each crop the farmer was consulted on the work that was planned and the farmer's advice was always highly regarded. In general, sampling was carried out on a weekly basis throughout the project and each farm was visited on average every 3 to 4 weeks. The data demonstrated that the activities for each bacterial enzyme gave a strong and reliable signal that tracked the various stages of the growout for each pond.

The third objective was to investigate relationships between bacterial extracellular enzyme activity and pond productivity. The key bioindicators of the bacteriology of prawn ponds were the fluorescent substrates leucine (for protease activity ie breakdown of proteins) and n-acetyl glucosaminide (for chitinase activity ie braeakdown of prawn shell). Excessive levels of these bioindicators were associated with high mortalities and poor productivity.

The fourth objective successfully used the results of the study to develop a HACCP plan that can be used by farmers to monitor conditions and prepare corrective actions. Control points were identified from measurements of excessively levels of key extracellular enzymes associated with stress and vibriosis. The key control points were: A) Conditions for spawners during transport to hatchery and in spawner holding tanks. B) Conditions for postlarvae (PLs) during transport from hatchery to farm. C) Conditions during growout leading to mortalities, poor survival and low productivity. D) Minimisation of blooms of cyanobacteria (blue-green algae). E). Disposal of waste-water from cooking prawns. E) Treatment of effluent.

The fifth objective, investigating the association between pond management practices and bacterial enzymes was successfully completed. Pond productivity was modeled by linear regression and the key parameters were 'daily feed rate', 'depth of pond', and 'source of postlarvae'. The key enzymes for pond productivity models were proteases and chitinases.

The sixth objective of comparing results from ponds with *P monodon* and those with *P japonicus* could not be completely carried out. Unfortunately *P japonicus* was not farmed at any of the farms during the study. Tru Blu Prawn Farm had previously stocked *P japonicus* but changed to *P monodon* at the start of the investigation. Instead of monitoring *P japonicus*, the project investigated bacterial activity in hatcheries located on the Clarence River and near Cairns. This provided useful information on the vertical integration of the industry. Spawner tanks and spawner transport bags contained very high levels of bacterial enzymes responsible for the breakdown proteins (proteases) and chitin (chitinases). This type of observation was similar to that found in prawn ponds suffering from high mortalities and low survivals. High levels of proteases and chitinases were key bioindicators of poor water quality and a stressful environment.

The seventh objective, relating to communicating findings to the industry and fisheries departments, is currently underway. The main forum for communicating results to the industry is the Annual Meeting of the Australian Prawn Farmers Association and initial results were presented at the 2000 meeting. Also in July 2000 the mid-project results were presented individually to each participating farm. The draft final report was reviewed by a participating farm and FRDC prior to publishing. While some of the results from the project have been published (see Appendix) further communication of the results will be carried out through scientific publications and articles in newsletters and magazines for farmers.

In conclusions, this project was a huge study that involved 6 major Australian prawn farms and was carried out by 44 people at the University of Western Sydney who worked on some 72 sub-projects. The results provide farmers with information that can be used to develop improved techniques for pond management and improved productivity. Researchers should be able to use the technique for monitoring extracellular enzymes in future studies of effluent treatment, zero-water exchange, activated microbial communities and other exciting new developments. Regulatory authorities now have substantial base-line data on the bacteriology of coastal prawn farms so that fair assessments can be made about future developments and how farms can substantially reduce and ameliorate impacts.

KEYWORDS: ***Penaeus monodon*, shrimp, prawn, bacteria, extracellular enzyme, exoenzyme, *Vibrio*, cyanobacteria, estuarine microbiology, effluent treatment**

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CHAPTER 2. BACKGROUND

Marine prawn farming in the Asia-Pacific region produced an estimated $1.2x10^9$ kg or 90 % of the world's farmed prawns in 2001 and the culture of the giant tiger prawn, *Penaeus monodon*, accounted for over 80% of that production (pp 189-191, Rosenberry 2001). *P monodon* is generally grown in monoculture at densities of about 30-50 animals/m^2 in aerated earthen ponds with supplementary feed. The microbial ecology of ponds is believed to strongly influence farm productivity and prawn health (Horowits and Horowitz, 2001). In extensive (low density) systems, the primary producers are microalgae and cyanobacteria while in semi-intensive and intensive systems, commercial pellet feed replaces natural feeds. Bacteria and microalgae recycle organic wastes and inorganic compounds (Burford et al 2001, pp 5-27, Rosenberry, 2001), though supplementary feed is also consumed by bacteria (Moriarty, 1978). New management techniques rely on bacteria to significantly enhance pond productivity by enriching the protein content of low value carbohydrates during decomposition (Athithan and Ramadhas, 2000; Chamberlain and McIntosh, 2001). Further, brackishwater species of bacteria, such as *Vibrionaceae*, are important because they can cause major diseases, such as vibriosis (Anderson et al, 1988; Lavilla-Pitogo et al 1990; Lightner, 1996). In spite of the importance of microbial communities to prawn farming, there are relativlely few publications with data describing details of the processes they perform.

Studies of bacteria at Australian farms (Smith 1995, 1998) revealed that the level of *Vibrio* spp. was usually at least an order of magnitude higher in pond water than in nearby mangrove habitats. Also, pathogenic species, *V. harveyi and V. vulnificus,* were dominant in prawn ponds but less common in mangrove habitats. It was concluded that vibrios and sulfate-reducing bacteria were important bioindicators of pond conditions and prawn health. The bacteriology was driven by input of pelleted feed, ambient temperature and limiting levels of dissolved oxygen (Smith, 1998). However, those studies plus other bacterial studies relied on culturing bacteria with agar plates or selective media to determine levels and species of bacteria (West and Colwell, 1984, Baumann and Schubert, 1984). Unfortunately for most environmental samples, only a small proportion of bacteria are culturable (Hart et al, 1996) so traditional methods have well-recognised limitations for ecological investigations.

An alternative method, which is based on fluorescently-labelled substrates for studying bacterial processes in natural waterways, was originally developed by Hoppe (1983) and Somville and Billen (1983). It relied on the fact that microbes produce a range of extracellular enzymes (exoenzymes) to degrade dissolved and particulate organic compounds. The significant advantage of the technique is that it focuses on microbial processes rather than bacterial numbers and species. Also, methods using fluorescently-labelled substrates do not require the culture of microbial isolates and are more sensitive than chromogenic (colorimetric) methods (Morgan and Pickup 1993; Hart et al, 1996; Findlay 1997). With the invention of 96-well fluorescence plate readers in the late 1990s, it became possible for sensitive, rapid monitoring of extracellular enzyme activities in environmental studies.

The difference between the two approaches to studying bacteriology can be summarized: the traditional methods are more concerned with how many bacteria are present and what are their names, while the extracellular enzyme method is concerned

with what the bacteria are doing and how fast are they doing it. By analogy, a factory could be characterized by either its number of employees and their names or by the work the employees are doing and the rate at which they do it.

Fluorescently-labelled substrates have been used to study microbial extracellular enzymes in a range of habitats, including coastal marine ecosystems (Karner and Rassoulzadegan, 1995) and isolated bacteria (Lee et al, 1996;Oshishi et al, 1996; Liu et al, 1997; Wang et al, 1997). Since the input of natural and supplementary organic matter to prawn ponds is mainly in a complex form, it follows that bacteria in prawn ponds also need to degrade these inputs extracellularly.

This report is the first to describe a long-term ecological study of extracellular enzyme activity in earthen ponds at prawn farms. The main aim of the study was to use fluorogenic substrates to determine the types and rates of key microbial extracellular enzymes in prawn ponds and estuarine habitats. We wanted to find the key extracellular enzymes in prawn ponds and nearby estuarine habitats. Also we wanted to use statistical analysis of the data to identify significant relationships between key extracellular enzymes and variables such farm management practices, biophysical parameters, feeding regimes and prawn health. The findings should help clarify our understanding of the role played by microbes in the ecology of prawn ponds and estuarine habitats. The outputs from the project should assist the prawn farm industry in improving productivity and reducing risks.

CHAPTER 3. NEED

Preliminary discussions with farmers and researchers revealed that they are often at a loss to account for differences between ponds with respect to productivity, survival and growth rates. This study focused on bacteria because they are the most abundant organism in prawn ponds, being ubiquitous in all types of sediments, water and organisms. More importantly, their processes determine: the fate of most organic matter in ponds, water quality, sediment condition and health of prawns.

Prior to this study there has been limited research into the bacteriology of prawn farms and this has seriously impacted on the development of the industry. To explain, it is well known that regulatory authorities need to adopt a precautionary principal when assessing development applications, particularly if there is a paucity of vital data. The discharge of effluent from prawn farms in sensitive coastal areas, such as the Great Barrier Reef, is a highly contentious issue. Prawn farmers reasonably reason that the effluent is relatively dilute in comparison with many other agricultural and industrial effluents. However environmentalists and regulators respond that if the effluent is benign, farmers should be capable of totally recycling it. In light of these conflicting views, the Australian prawn farming industry has not grown according to the "Australian Prawn Farm Industry Vision" (eg Fig 10, R&D Plan 1996-2000 by Macarthur Consulting 1995). For instance, from 1993 to 2001 there has been an increase in total annual production from 1,400 tons to 3,050 tons, but this has resulted mainly from a 125% increase in pond area (from 348 ha to 740 ha) rather than from the 23% increase in the number of farms (from 35 to 43).

In reality it is the bacteria, especially the pathogenic strains of *Vibrio* that need to be managed in order to maintain healthy ponds and recycle effluent. Previous reports (Smith 1995, 1998) showed that a) bacteria are good bioindicators for conditions in prawn ponds and b) effluent can contain high levels of *Vibrio* species. CRC Aquaculture did not study *Vibrio* bacteria or the effect of treatment systems on them (Preston et al undated report; Preston et al 2001). Also, early studies which characterized the composition of the effluent, its composition, environmental load and use of settlement ponds in farms in NSW by Smith (1995) were not cited by CRC Aquaculture (Preston et al undated report; Preston et al 2001). However, exciting recent developments in some farms overseas (eg in Belize) show that zero-water exchange depends on a healthy bacterial community. It is timely that this FRDC-funded project tackled an area that admittedly is controversial, but also has the potential to provide a huge amount of knowledge which can be used for the benefit of the industry and protection of fisheries habitats.

CHAPTER 4. OBJECTIVES

The project had seven main objectives.

1. To apply recently developed technologies for bacterial enzyme analysis to the study of microbial processes in prawn farms.
2. To investigate the bacteriology of prawn ponds throughout the entire growouts for ponds at five Australian farms.
3. To investigate relationships between bacterial enzymes and pond productivity, with emphasis on prawn health, growth rates, feeding strategies and prawn survival.
4. To determine which are the key bacterial extracellular enzymes to study when making a rapid assessment of the status of a pond for HACCP (Hazard Analysis for Critical Control Points).
5. To investigate the associations between the various pond management practices on bacterial enzymes and pond conditions.
6. To compare the bacterial enzymes of ponds used to culture *P. monodon* and *P. japonicus*.*
7. To effectively communicate the results of the study to the prawn farming industry and fisheries departments through a manual for farmers on the impacts of management practices on pond bacteriology and productivity.

*Because of problems with growout, marketing and currency exchange rates, *P. japonicus* was not farmed on the Clarence River after May 1998. So it was not practical to investigate bacterial enzymes in *P. japonicus* ponds. Objective 6 was modified to include an investigation of bacterial enzymes in *P. monodon* hatcheries.

CHAPTER 5. METHODS

5.1 Experimental design

The project consisted of farm-based and laboratory-based work. In the first 6 months of the project (July to December 1998) the techniques were developed and tested, essentially to optimize methods for assaying extracellular enzymes. Tests were performed on a range of specimen types, storage temperatures, storage times and pre-treatments in order to develop protocols for sample collection, storage, and processing Also, during this period preliminary data was collected for water and sediment samples from farms in Far North Queensland and the Clarence River.

In Feb 1999 the project's two full-time research assistants, Chantelle Agha-Hamilton and Daniel Ivanoff, were employed and the focus of the work for that year was the collection and processing of field samples from 3 farms in Far North Queensland and one farm and two hatcheries on the Clarence River. This provided the project with baseline data for ponds in these two regions as well as data on prawn hatcheries. The work included studies on the extracellular enzyme profiles for water and sediment in prawn ponds with respect to: a) short-term variations (i.e. hourly, daily and weekly); b) depth in water column; c) transects across ponds and pond bottoms; and d) soluble versus particulate fractions. Replicate samples were taken at each site and the number of sites in each area/pond ranged from 2 to 12.

In 2000 the work on the farms on the Clarence River focused on four discharge systems (one had a large settlement pond, one had a tortuous stream with baffles, one had a broad canal which was lined with mangroves and one was a narrow canal with no special effluent treatment features). The sampling from Far North Queensland focused on collecting a broad range of data on productivity, farm management and environmental data for 3 ponds at each of the three farms. The data was used to look for associations between extracellular enzymes and other variables. In the laboratories at UWS, extracellular enzyme profiles were investigated with bacteria that had been isolated on Marine Agar and TCBS agar from prawn farms. Bacteria were cultured with various food sources, including prawn feeds, to determine how their extracellular enzyme activities were affected.

In 2001 the project employed three additional research assistants, Mario Godoy, Michael Cassaniti and Julianana Tasevska. Their salaries were paid for by a range of additional sources (i.e. ACIAR, UWS, P Smith's quantum funds). Three farms were studied on the Clarence River. One farm started using a new type of submerged aeration system so this was studied. Also the treatment systems were further investigated. In Far North Queensland, 75 sediment plots were set up in two ponds in two of the farms with the aim of determining what affect sediment type had on sediment bacteriology. The sediments in the plots were in duplicate and included 5 types of soils. The soils were pretreated with fertilizer, lime, lime plus fertilizer or no additive prior to filling ponds. In the third pond in Far North Queensland a major addition of ponds and a treatment system had been added, so the focus was on making comparisons between the different pond types (e.g. old vs new). In the laboratories in UWS, mesocoms experiments were carried out using small tanks (20 litre) stocked with prawns. This work investigated the bacteriology of water and sediments. Factors

that were tested in these tank tests included supplementary organic matter, types of aeration systems, biofiltration systems, probiotics, and extreme conditions. During the period from September to the end of 2001 the team concentrated on completing histology work, PCR testing of samples, and writing up reports on their contribution to the project. The last week of sampling of the farms in far North Queensland was processed in early December 2001.

Employment of research assistants and funding from FRDC ceased on 31st December 2001, however, experimental work was carried out until the end of May 2002 with funding from additional sources (P Smith's quantum funds). The work focused on one farm on the Clarence River in which supplementary organic matter was added to encourage growth of beneficial bacteria. This was a field-test which aimed to confirm findings that were obtained in mesocosm experiments during 2001. Also, final productivity data for the season of 2001-2 was received from farms in Far North Queensland in May 2002. The results for the entire project were compiled, analysed and written up in the FRDC draft report by 24th December 2002. Manuscripts for scientific journals will be written over a period of 12 months after the publicatrion of this final report.

5.2 Sample collection, field methods and chemical analysis

Samples were collected over a period of three years (1999-2001) from three farms in a sub-tropical region on the east coast of Far North Queensland, Australia (16 to 18 ^{o}S, 145 to 147 ^{o}E) and three farms on the Clarence River, NSW (30^{o}S, 144^{o}E). The farms stocked post larval *Penaeus monodon* (PL 15-18) at densities of 20 – 40 m^2 in earthen ponds. Prawns were fed commercial pellet feed and ponds were aerated at about 10 horsepower/ha (Smith, 1996). Management data, including stocking details, feeding rates, fertilization, water exchange and productivity, was routinely collected from farm records.

Samples of water and sediment were usually collected at intervals of approximately 21 days from about 50 to 100 sites at each farm. During important stages of testing, the collections occurred at intervals or 7 days and in some stages at intervals of 3-4 hr. Sites included water intake canals, culture ponds, discharge canals, bore holes and estuarine habitats. Site observations, such as water colour and Secchi disk visibility, were routinely collected at each site. The presence of a surface slick, scum, filamentous algae, cyanoacteria and foam were allocated a grade from 0 to 5 (nil to extreme). Temperature, salinity, dissolved oxygen, redox and pH were measured with a field multimeter (TPS Instruments 990-FLMV).

At each site in Far North Queensland, 13 L of water was collected in a clean vessel and 4 types of subsamples were taken as follows: 50ml (duplicate) for enzyme analysis, bacteriology and heavy metal analysis; 120mL for TSS (Total Suspended Solids) and DOC (Dissolved Organic Carbon); 1.0 L for BOD_5 and nutrients; and 10 L was poured through a hand-held 60 μm plankton net and the filtrate (50 mL) was collected for plankton identification, dry weight and organic content.

The concentrations of dissolved ammonia, phosphate, silica, iron, nitrite, nitrate and alkalinity in water samples were measured with a Palintest Photometer 5000 portable spectrophotometer. BOD_5 was measured by standard methods (APHA, 1989).

Microalgae and cyanobacteria were identified with a light microscope at 400x magnification and cell densities were determined with a haemocytometer. Filtrates from the plankton net were analysed with a dissecting microscope by identifying and counting all plankton present in a 0.5 mL aliquot.

TSS was determined by filtering water samples (100 mL) through pre-weighted glass fibre filters. The filtrate was acidified with 4 M HCl and kept for measuring heavy metals (by Inductiviely Coupled Plasma Analysis) and DOC (with a Shimatzu DOC Instrument). The organic content was determined by firstly rinsing the filter with 10 mL of milli-Q water, to remove salts, then determining the loss in weight after heating at 100 ^{o}C for 36 hr, and ashing at 450 ^{o}C in a muffle furnace for 5 hrs. Similarly, the dry weight of plankton was determined by filtering 20 mL of plankton filtrate through a glass fibre filter, rinsing with 10 mL milli-Q water, and determining the loss in weight after heating at 100 ^{o}C and ashing at 450 ^{o}C.

Sediment samples were collected in duplicate from sites in 50 mL plastic containers for enzyme and chemical analysis. Farm sediments were collected from the pond bottom about 2 m from the toe of the wall, while sediments from environmental sites were collected from the intertidal zone just beneath the water level. Water and sediment samples were routinely chilled on ice as they were collected and refrigerated at 4 ^{o}C upon returning from the field. Samples were processed within 48 hrs of collection. Measurement of levels of heavy metals and nutrients (ammonia, total nitrogen, available phosphate, total phosphate, sulfur and organic carbon) in sediments were determined by the methods described by Smith (1996).

Approximately 20 *P monodon* were collected from three ponds with traps or feed trays. Individual body weights were recorded and characteristics such as colouration, shell fouling, tail rot and body scaring were allocated a grade from 0 to 5 (nil to severe). Upon returning from the field, prawns were cooked according to methods used by farmers (ie cooked in fresh water and brined in 36ppt NaCl at 4 ^{0}C overnight). The next day, the prawns were taste tested and the results graded from 1 to 5 (excellent to poor).

5.3 Histopathology and disease diagnosis

When disease was evident at a farm, affected prawns (3 to 10 per pond) were collected for histological examination. Prawns were fixed in Davidson's solution for approximately 24 hours then transferred to 50% ethanol and processed and stained with Haemotoxylin and Eosin according to standard methods (Lightner 1996). Sections of major organs and tissues were examined with an Olympus microscope and observations were compared to photomicrographs of prawn histopathology (Bell and Lightner, 1988; Lightner 1996).

5.4 Assay for extracellular enzyme activity

Water and sediment samples from ponds and estuaries were assayed with fluoroescently-labelled substrates using a Spectraflur-plus microplate reader (Tecan). Various other environmental samples (i.e. algal blooms, detritus, prawn faeces and isolated bacteria) were also assayed. The excitation wavelength was 360nm, emission wavelength was 465nm and the gain on the instrument was 60. Glassware was

routinely washed in dilute acid and hot water, while plasticware was washed with hot water then deionised water.

A total of 22 methylumbelliferyl (MUB)-linked substrates were prepared at saturated concentrations (1 mM). Some 16 substrates were dissolved in 5mM $NaHCO_3$ at pH 8.2 (ie laurate, glucuronic acid, caprylate, diacetylchitobioside, phosphate, oleate, cellobioside, sulfate, α-glucoside, β-glucoside, xyloside, α-fucoside, β-fucoside, sulfo-glucopyranoside, glucosaminide and galactoside) while the remaining 6 substrates were prepared in milli-Q water (ie butyrate, propionate, acetate, guanidinobenzoate, leucine and trimethylammonium cinnimate chloride). Assays were run in black, medium-binding 96-well plates (Griener). Microplates were prepared by dispensing substrate (50 μL) and sample (200 μL) to each well then reading immediately and at intervals of up to 24 hrs. Results indicated that 210 minutes was sufficient for determining enzyme activity for all substrates, so routinely readings were made at (min) 0, 60, 150 and 210.

Extracellular enzyme activity was calculated in nmol h^{-1} mL^{-1} from:

Activity = (final FU – initial FU) / (E_m x V x T)

where FU is Fluorescence units, E_m is the molar emission coefficient, V is the volume of the reaction (0.25 mL) and T is assay interval (h). The molar emission coefficient was determined from a standard curve constructed from serial dilutions of 0.5-4.0 μM for each batch of samples. Each sample was generally assayed in duplicate and with appropriate blanks (autoclaved milli Q water, 5mM $NaHCO_3$, 2.5% NaCl, artificial sea water and non-autoclaved artificial sea water). The activity for the appropriate blank was subtracted from the sample activity for each substrate (in almost all cases the activity of the blank = 0). Also each sample was assayed with an MUB spike (50 μM) to evaluate quenching effects caused by dissolved and colloidal material.

Sediment samples usually had an activity that was at least two orders of magnitude higher than water samples, so they were diluted appropriately (usually 100x, 500x or 1000x) with autoclaved 2.5% NaCl.

In some experiments, the source of enzyme activity was examined by comparing results of assays with those obtained from filtered samples (0.45 μm) and autoclaved samples. Also commercially available enzymes were run with MUB substrates to validate results from the various substrates.

5.5 PCR tests for Gill Associated Virus (GAV) and White Spot Virus (WSSV)

5.5.1 Tissue processing and extraction of RNA and DNA

To test for viral infections, prawns were placed in a plastic bag and chilled on ice immediately after collection. At the laboratory the physical characteristics of each specimen were noted then the gills, eyes, hepatopancreas and antennule were dissected. The tissue types of the animals from each pond were pooled and placed in phosphate buffered saline with 2% bovine serum albumin (2% BSA PBS).

The pooled tissue was crushed with a sterilised, chilled mortar and pestle then mixtures were centrifuged a total of three times. The first was at 2,000rpm for 5min at 4°C. Pellets were discarded and supernatants were centrifuged at 3,000rpm for 30min at 4°C. The pellets from the second centrifugation were snap frozen and stored at -80°C and the supernatants were spun at 18,000rpm for 2 hours at 4°C. The final pellets were resuspended in 1mL 2% BSA PBS and pellets and supernatants were snap-frozen with liquid N_2 and stored at -80°C for DNA and RNA extraction.

DNA extraction was carried out using the DNeasy Tissue Kit from QIAGEN following the manufacturer's instructions. A total of 20µL homogenate was added to Buffer ATL and Proteinase K. The sample was incubated at 55°C for 1h after which time Buffer AL was added and the mixture was incubated at 70°C for 10min. Next, 96% EtOH was added and the mixture was placed on a DNeasy mini column and centrifuged. After several washing procedures, the DNA was eluted and stored at -20°C.

RNA extraction was carried out using the RNeasy Mini Kit from QIAGEN following the manufacturer's instructions. A 20µL sub-sample of homogenate was added to Buffer RTL containing 14.5M β-mercaptoethanol and incubated at room temperature for 5min. The lysate was centrifuged and the supernatant was added to 350µL of 70% EtOH. Some 700µL of this mixture was placed onto an RNeasy mini column and after several washing procedures, the RNA was eluted and stored at -20°C.

Reverse transcription was performed using the M-MLV reverse transcriptase system from Sigma and was carried out according to their instructions. The 10mm dntp, forward primer, nuclease-free water and RNA were placed in a tube to a final volume of 10µl and incubated at 70°C for 10min. After placing on ice, 0.1m DTT, 5x first strand buffer, m-mlv, RNase inhibitor (geneworks) and nuclease-free water were added to make a final volume of 20µl. This was incubated at 37°C for 50min then heated to between 80°C to 95°C to inactivate the M-MLV and stop the reaction.

5.5.2 Real Time PCR

The primers used for PCR were developed by Supershrimp USA (patent pending). Amplification was performed with a Rotorgene 2000 Real-Time Amplification Cycler and analysed with software provided by Corbett Research. The reactions were carried out in 0.2mL PCR tubes in a 25µL reaction volume containing 7.1µL 2x SYBR Green PCR Master Mix (PE Applied Biosystems), a 0.12µM concentration of each forward and reverse primer, and 5µL cDNA for GAV analysis or 5µL DNA for WSSV analysis. The thermal profile for the PCR was 95°C for 10min, followed by 40 cycles of 95°C for 10sec and 60°C for 1min and a final melt cycle ranging in temperature from 55°C to 90°C. Each sample was tested in duplicate.

5.5.3 Traditional PCR

Nested PCR on GAV was undertaken according to Cowley et al (2000a). Some 1µL cDNA was amplified in 50µL using *Taq* buffer (10mM Tris HCl pH 9.0, 50mM KCl, 0.1% Triton X-100), 1.5mM $MgCl_2$, 35pmol each primer (GAV5 and GAV6) and 200µM dNTP's. The reaction was heated at 85°C for 5min prior to adding 2.5U *Taq*

polymerase. DNA was amplified by 30 cycles of 95°C for 1min, 58°C for 1min and 72°C for 40sec, followed by a final extension of 72°C for 10min. Some 10μL of PCR product was resolved on 2% agarose gels.

If the result of the primary RT-PCR was negative or inconclusive, 5μL of the primary PCR product was amplified by nested PCR as above in a 50μL reaction-volume using primer GAV1 and GAV2. The PCR profile was 20 cycles of 95°C for 1min, 58°C for 1min and 72°C for 30sec, followed by a final extension of 72°C for 10min. Some 10μL of PCR product was resolved on 2% agarose gels.

Nested PCR on WSSV was carried out according to the OIE Diagnostic Manual for Aquatic Animal Diseases (2000). For the primary PCR reaction, 2μL template DNA was added to a 100μL reaction mixture consisting of 10mM Tris-HCl pH 8.8, 50mM KCl, 1.5mM $MgCl_2$, 0.1% Triton X-100, 200μM of each dNTP, 100pmol each primer (146F and 146R) and 2U DNA polymerase. The PCR profile was one cycle of 94°C for 4min, 55°C for 1min and 72°C for 2min followed by 39 cycles of 94°C for 1min, 55°C for 1min and 72°C for 2min and a final extension of 72°C for 5min.

For the nested PCR reaction, 10μL of the primary PCR product was added to 90μL of the same mixture as above except the primers were 146F2 and 146R2. The PCR profile is the same as above. Some 10μL of the PCR product was resolved on 1% agarose gels.

5.6 Work on isolated bacteria

Bacteria from samples of water, sediment and prawn tissue were isolated on TCBS agar and Marine Agar. TCBS agar plates were made by dissolving 88.0 g of TCBS cholera medium in 500 ml of artificial seawater and 500 ml of "milli Q" water. This was then boiled at 100 ^{o}C for 20 minutes before being poured into petri dishes and allowed to set. Similarly, Marine Agar plates were prepared by dissolving medium in "milli Q" water, autoclaving and poured into petri dishes. Once the plates were set they were stored in a cold room until used.

Samples (0.5 mL) were serially diluted (1 in 10) in a bijou bottle containing 4.5 ml autoclaved seawater. Then 0.1 mL of each dilution was spread plated onto TCBS agar plates and Marine Agar plates. Each plate was incubated at room temperature for 1-2 days before individual colony forming units were counted. These values were then converted to colony forming units per ml by multiplying by the appropriate dilution factor.

To determine the extracellular enzyme profile of isolated bacteria, a colony was picked off the agar and emulsified in 4.5 mL of autoclaved seawater. Aliquots (200 μL) were then added to 96 well-plate microplates for measuring extracellular enzyme activity (as described in Section 5.4 Assay for extracellular enzyme activity).

To test the effect of organic supplements on extracellular enzyme profiles, isolated colonies of bacteria were cultured with various food sources (i.e. prawn feed, peptone) for 24 hrs. Then the activities of extracellular enzymes were measured and compared to the results for the bacteria when it was first isolated.

5.7 Tank tests (mesocosms)

Three sets of experiments were carried out with prawns in small tanks (mesocosms of 20 litre each). In the first experiment the effect of supplementary addition of chitin on prawn growth and bacteriology was examined. Healthy post larval *P monodon* were purchased from a hatchery near Cairns then acclimatized for a period of 1 week in a stock tank (300 litre) and fed live Artemia and commercial flaked prawn food. The second and third sets of experiments used juvenile prawns (approximately 0.6 g and 10 g, respectively) that had been raised from PL in a large aquarium (5000 litre). In the second experiment the effect of organic supplements on growth and bacteriology was tested on juvenile prawns (0.6 g average weight). Prawns in all tanks were given commercial pellet feed (4% of their combined weight). Controls received no other food, while other tanks received a supplement of 2 g weekly of either "soft feed", chitin, wheat germ, corn flour, molasses, or mangrove leaf. (Soft feed was made from a mixture of fresh ingredients including, fish, oysters, cooked peas and agar.) In the third experiment the effect of biofiltration and aeration systems were tested. All mesocosms received equal quantities of commercial pellet feed.

Mesocoms were set up with conditioned estuarine gravel and 20 litre of seawater with a salinity adjusted to 28 ppt (gram/litre). Each tank was allowed to run and stabilize for a period of 2 weeks to allow stable initial biophysical and biochemical conditions (i.e. pH, temperature, salinity and nutrients) to develop before the prawns were added. Once the prawns were acclimatized, 5 of similar size were selected and placed into each tank. All tests were run in triplicate over a period of 8 to 12 weeks. The initial average weight of prawns and the final average weight were taken for each mesocosm. Water samples from all the tanks were taken twice a week for microplate enzyme assay and measurements were taken once a week of environmental conditions (i.e. the temperature, pH, and salinity). Also *Vibrio* bacterial counts were made with TCBS medium.

CHAPTER 6. RESULTS AND DISCUSSION

As an overview, this farm-based and laboratory-based study produced a number of new and important discoveries for the Australian prawn farming industry. The study was carried out from 1998-2002 on the bacteriology of Australian *Penaeus monodon* farms in which the **activities** of the bacteria and the **processes** they carry out were investigated. A new technique was employed for the first time at prawn farms to measures the rate that bacteria use their extracellular enzymes to breakdown carbohydrates, fats, proteins and phosphates.

The raw data and derived data are presented in the Supplements to this Report and the key results are discussed in this chapter. The original data is mainly contained in databases; the farm-based data is in three databases (shown in Table 6.1), and the list of variables in the "cairnsall1.sav" database is given as an example in Supplement #1. There are a number of other databases and they contain the results of experiments on: detection of disease agents by PCR/DNA tests, effect of food supplements on the extracellular enzymes produced by isolated *Vibrio* bacteria, toxicity of cyanobacteria (ie blue-green algae), use of the technique to determine the effectiveness of effluent treatment systems, use of the technique to investigate mortalities in prawn hatcheries, effect of food supplements on bacteria in tank tests and ponds, and histopathology of various bacterial diseases.

Most of the findings have not yet been communicated to the Australian industry or to the scientific community, except for the articles listed in Appendix 5 (copies of articles are in Appendices 6 to 9). Applications to present the findings to the APFA has so far been unsuccessful, hence the findings are to be presented to farmers through newsletters, aquaculture magazines and refereed journals. Chapter 6 summarises and discusses the findings of the study.

Table 6.1. Raw data collected from farms during the life of the project is primarily contained in the following 3 databases. Also, there are other smaller databases (see text above).

Name of database	*Topic of database*	*Number of variables (ie columns)*	*Number of cases (ie rows)*	*Size (Kbytes)*
cairnsall1.sav	Farm-based data from Far North Queensland	357	8,073	10,595
clarenceall1.sav	Farm-based data from the Clarence River NSW	78	2,814	1,131
ccfarmsall1.sav	Farm-based data from farmers in FNQ and Clarence River farms	149	10,851	7,477

Information Box

Q1. What are bacteria?

Bacteria are small organisms, microbes, that cannot be seen by the naked eye alone – light microscopes or electron microscopes are needed. They exist all around us; in the air, our food, water, soil and even on our skin and inside our bodies. Bacteria have a critical role in prawn farming because some cause diseases (pathogens) and most bacteria are involved in the nutrient cycle where they breakdown wastes and organic matter. Some examples of the more well-known bacteria that are found in marine and brackishwater are species of *Vibrio* and cyanobacteria (also called blue-green algae). Vibriosis is a form of a disease called septicaemia, whereby there is an invasion of tissue and blood by bacteria. It is one of the most common and serious diseases for prawn culture.

Q2. What are enzymes?

Enzymes are special proteins that all cells produce. They speed up (or catalyse) the chemical reactions of cells, usually by a factor of 1,000 to 1,000,000 times. So enzymes are the cell's tools and most enzymes are contained within the cell. However, bacteria do not have a mouth or a digestive tract, so they need to break down large organic compounds (food and wastes) by releasing some special enzymes to the environment. These are called extracellular enzymes or exoenzymes and they either remain attached to the outer surface of bacteria or are freely dissolved into the environment.

Q3. How many extracellular enzymes are there?

Just as there are many different organic compounds in the environment, there are many different extracellular enzymes. However we can describe them by their activity and this allows us to classify them into four main groups for environmental studies: proteases break down proteins, lipases and esterases break down fats or lipids, carbohydrases break down carbohydrates, and phosphatases release phosphates. Some types of extracellular enzymes are also called exotoxins because they cause tissue damage and disease.

Q4. How can extracellular enzyme activity be measured?

In this project we used a fluorescent compound called 4-methylumbelliferone (MUB), which was attached to other compounds (ie substrates). When a substrate is attached to the MUB compound, the MUB-substrate complex does not fluoresce, but when the substrate is broken off by an enzyme, the free MUB molecule fluoresces (ie it can emit light). So the rate of breakdown of a MUB-substrate complex can be determined by measuring the rate of increase in fluorescence. In total 22 substrates were used in this project: 2 were for measuring protease activity, 8 for esterase activity, 10 for carbohydrase activity (of which 3 were for chitinase activity), and 2 were for inorganics (phosphate and sulfate).

6.1 Findings from data collected at prawn farms

The focus of this study was the use of a new technique to study the bacteria in prawn farms and the reader is referred to the **Information Box** which briefly provides a summary of bacteriology, enzymology and the method for investigating extracellular enzyme activity of microbes.

6.1.1 Protocol testing

Intensive literature reviews were carried out prior to starting the study, midway through the project and continuously since early 2002. These revealed that this is the first study to use fluorescently-labelled substrates to investigate the bacteriology and ecology of prawn farms, though the technique has been used in laboratory investigations of the processes (ie chitinases) carried out by isolated bacteria and *Vibrio* species. Thorough testing was carried out by us in 1998 and 1999, to verify the suitability of the method and to develop protocols for measuring extracellular enzyme activities of environmental samples. Summaries of the results of the tests are presented here, and the findings will be reported in scientific journals and magazines for farmers.

In the laboratory, the method was found to be 100 to 1000 times more sensitive than colorimetric methods for measuring enzyme activities. Also, the enzyme activities of a range of commercially available enzymes (proteases, lipases, phosphatases, carbohydrases) with known activities were measured by the method and the results were highly satisfactory. The sensitivity and selectivity of the method was confirmed.

In the field, environmental samples were tested with a range of protocols. It was found that when samples were collected, chilled and stored at 2 to 4 ^{o}C for up to 3 days, the enzyme activities were comparable to rates that were measured immediately after samples were collected. It is likely that when bacteria are chilled for a few days, they are dormant and the enzymes generally retain the capabilities that they had when they were collected. In comparison, if samples were either kept at room temperature or frozen, the activities were not comparable. Probably because freezing causes some organisms to rupture and release their internal enzymes, while storing at room temperature allows bacterial processes to change quiet significantly.

It was possible to measure enzyme activities of sediments, however they needed to be diluted 1:100 with autoclaved saline. At higher dilutions (eg 1:1000), negative activities were measured for some substrates, probably because of interference effects. At dilutions of 1:10, rates were reduced because of quenching and the enzymes were not saturated. The effect of dilution of sediment samples on fluorescence has also been reported by other researchers.

Samples were routinely collected in duplicate from each site and very significant correlations ($P<0.001$) were obtained for activities for all substrates. Tests were carried out to examine the spatial and temporal variability in data collected from sites. Normally, it was sufficient to collect samples in duplicate from 2 sites for each pond or location. This sampling protocol was found to provide sufficiently reliable results to characterize each pond. Further, in 1999 and 2000, 2 ponds at three farms in Far

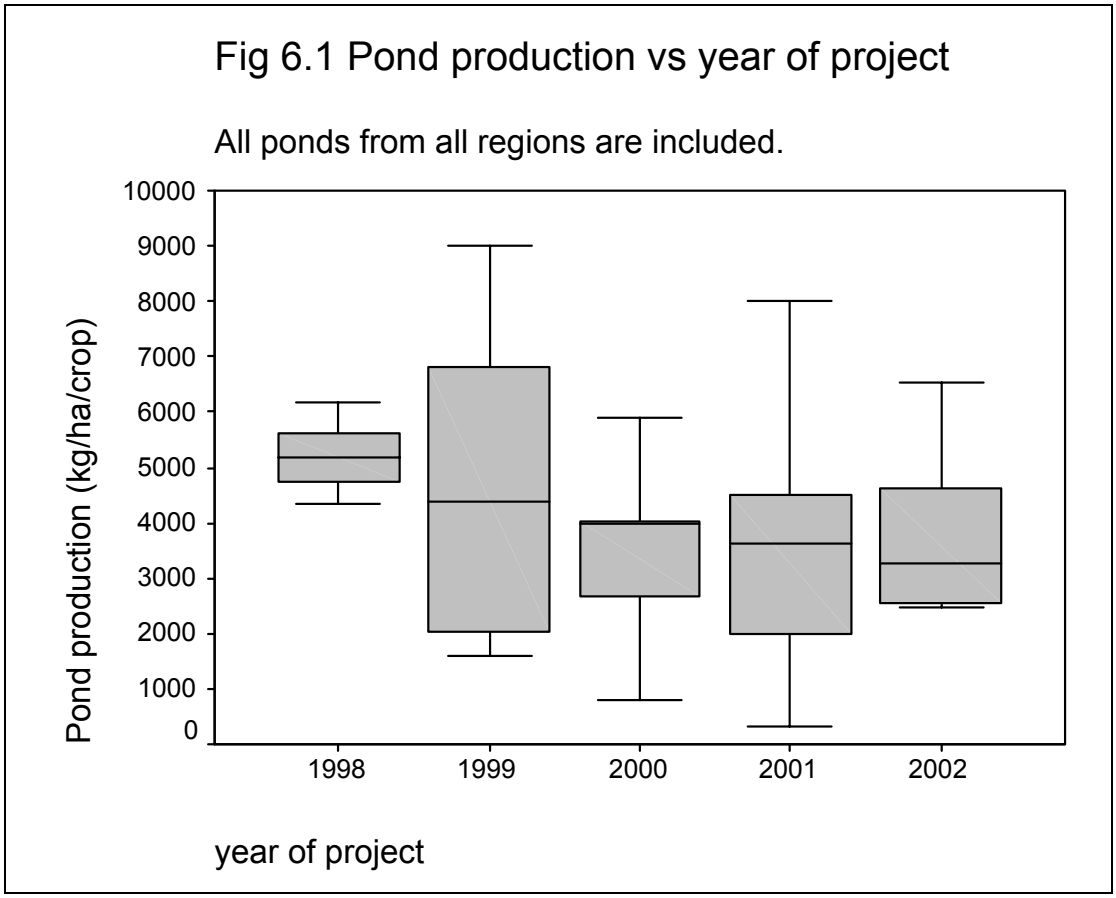
North Queensland were routinely examined for spatial variation in activities by collecting water samples in duplicate from three depths in the water column (surface, midwater and bottom) and at three locations in ponds (edge, midway and centre). Also sediments were collected in duplicate from the edge, midway and centre of ponds. In summary, the results showed that pond water was relatively well-mixed and, though enzyme activity increased slightly with depth in the water column, it was generally sufficient to characterize microbial enzyme activity by collecting water samples from the end of feeding walkways. However, sediment samples that were collected from the edge of ponds varied in activity from those collected in the centre. The results of that experiment are described in Section 6.1.4.2 Spatial and temporal variations in enzyme activity.

6.1.2 Status of industry and farms

Analysis of the data on pond production from farms that participated in the study from 1998 to 2002 indicated that there was a steady, significant ($P<0.01$) decline in pond productivity (see Fig 6.1). Average (mean) pond production decreased from 5204 kg/ha/crop in 1998 to 3721 kg/ha/crop in 2002, while the median decreased from 5160 kg/ha/crop to 3250 kg/ha/crop in the same period (Supplement #2). This finding is consistent with anecdotal evidence from the farmers. For example, one of the largest farms once produced over 155 tons/annum in the mid 1990s, but during the study could not produce more than 110 tons per annum. The trend is also consistent with the statistics provided by such bodies as QDPI and NSW Fisheries. For example, on page 102-3 WSF 1998, Ross Lobegeiger reported that over the previous three years (ie 1995-7), production per ha had continued to decline and he suggested that the causes were viral diseases, seasonal shortages of spawners, quality of spawners and PL problems.

All of the factors listed by Lobegeiger appear to be beyond the control of the average prawn farmer. But is this the end of the story? The findings of this study suggest it is not. But before we investigate the range of factors that were measured in the study, we should complete the picture of the status of the farms that participated in the study.

Supplement #3 summarises the production statistics for the participating farms. Three farms, Searanch, Ponderosa and Tru Blu, gave the project very generous access to their books and the data collected is extremely reliable. The other farms did not have extensive production data, so production data points were limited. In order to overcome this difficulty, two strategies were adopted. Firstly, the frequency of data collection was highest for farms that provided us with access to production data. Secondly, at the end of each crop, farmers were interviewed and asked to complete a questionnaire in which they provided data as well as rankings for aspects of productivity of each pond. Their responses provided data for 9 productivity variables for each pond, including a Survival Index, Production Index and FCR index, whereby 10 was the best possible result and 1 was the worst. This meant that farms such as Melivans, in which, for example, FCR was entered into the database for only 171 cases (ie 171 rows in the database), but for the FCR Index we had data for 1069 cases. Consequently, less “missing data” problems occurred when carrying out statistical analysis when using FCR index than with FCR. This was particularly important with statistical techniques such as analysis of variance (ANOVA), correlation, curve-fitting and modeling.



Figures 6.2 to 6.10 illustrate differences in the various 9 productivity variables for the farms that participated. Interpretation of these graphs and the reasons for the results is not straightforward. In order to avoid drawing strong conclusions about specific farms, it is not appropriate to compare farms in each graph in this report. The graphs are provided so that interpretation of the graphs can be undertaken by the farms themselves. It should be remembered that the purpose of the project was never to examine the performance of individual farms, rather it was to collect data from a wide range of farms and look for factors that influenced the bacteriology of those farms.

Fig 6.2 Average harvest weight of prawns at farms

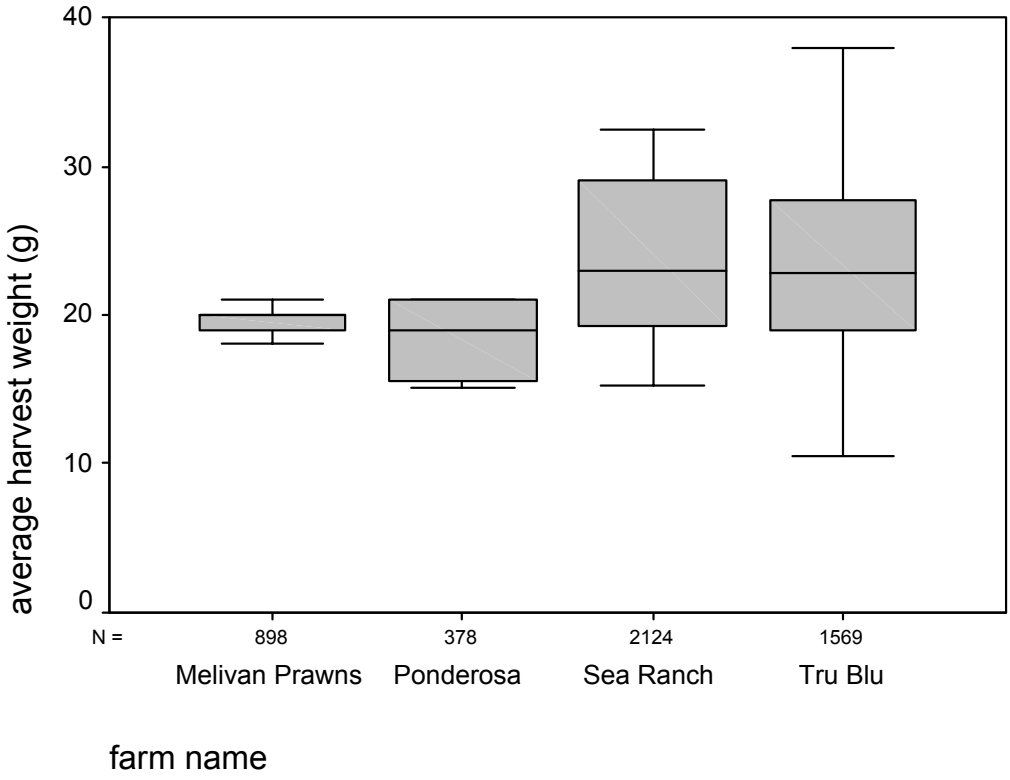


Fig 6.3 Pond FCRs for farms

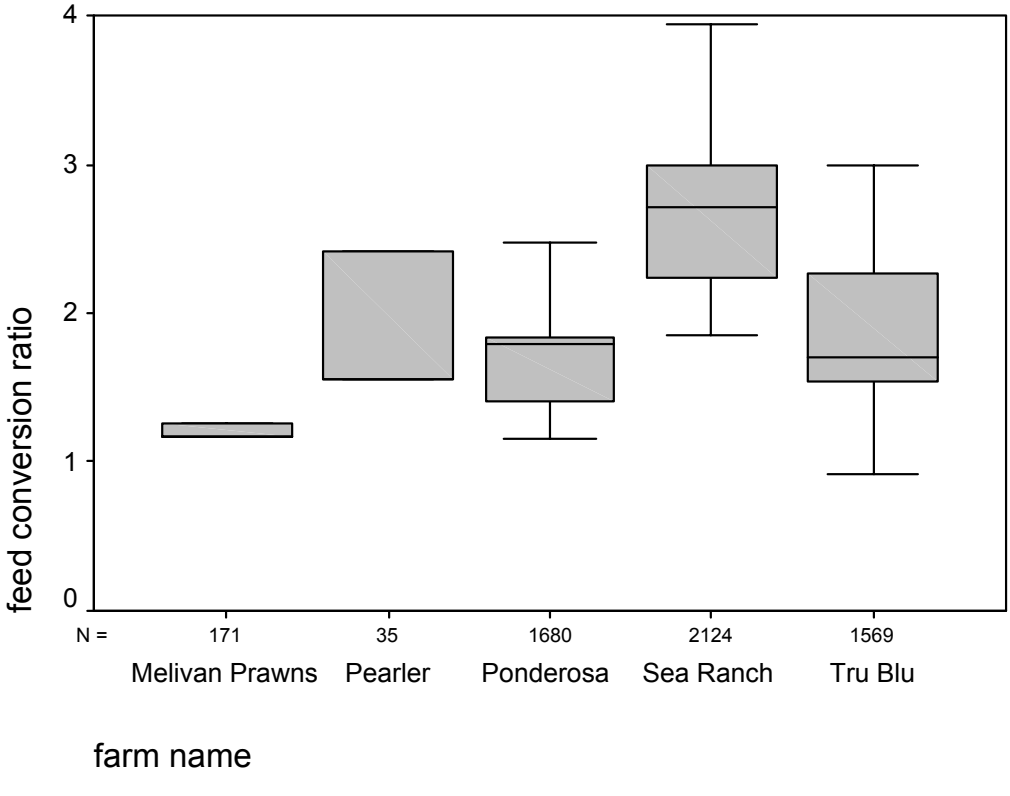
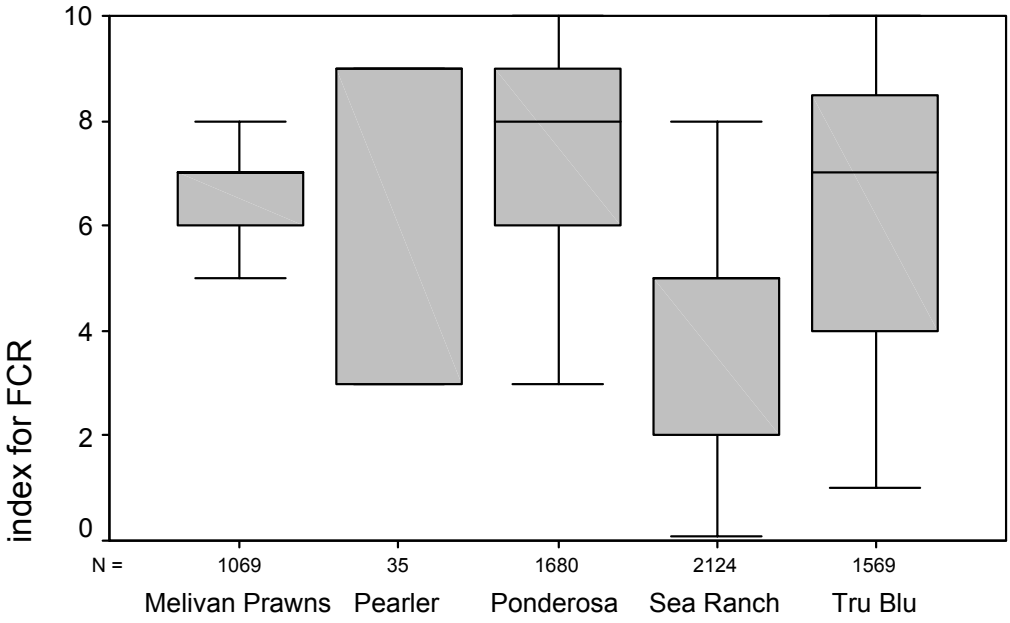


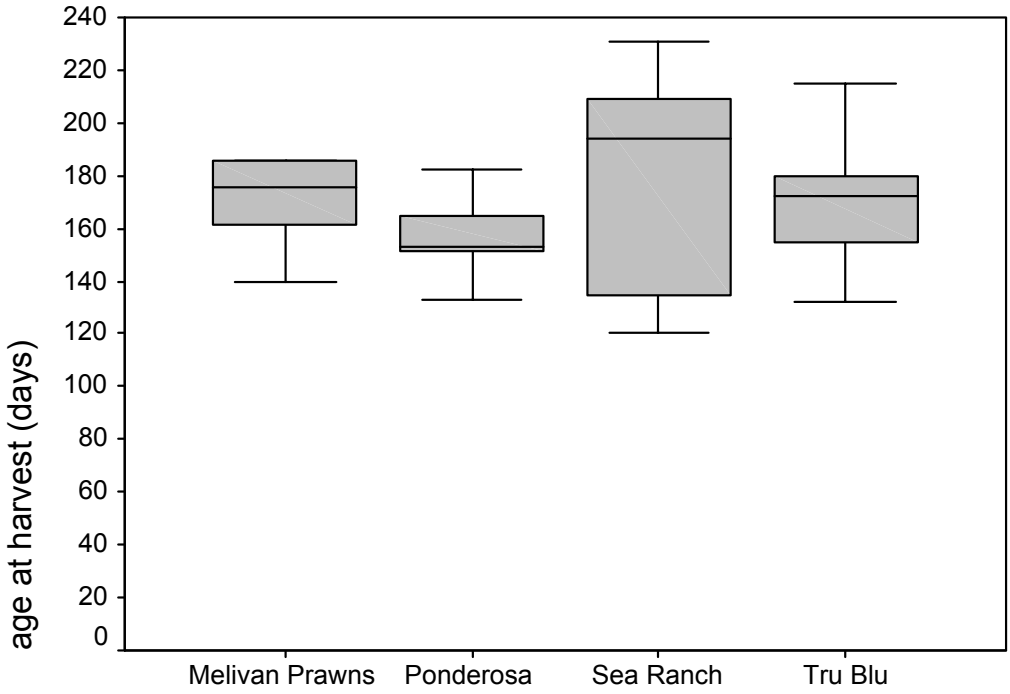
Fig 6.4 Pond FCR Index for farms

(10=best, 1 =worst)



farm name

Fig 6.5 Average age of crop at harvest for farms



farm name

Fig 6.6 Pond production for farms

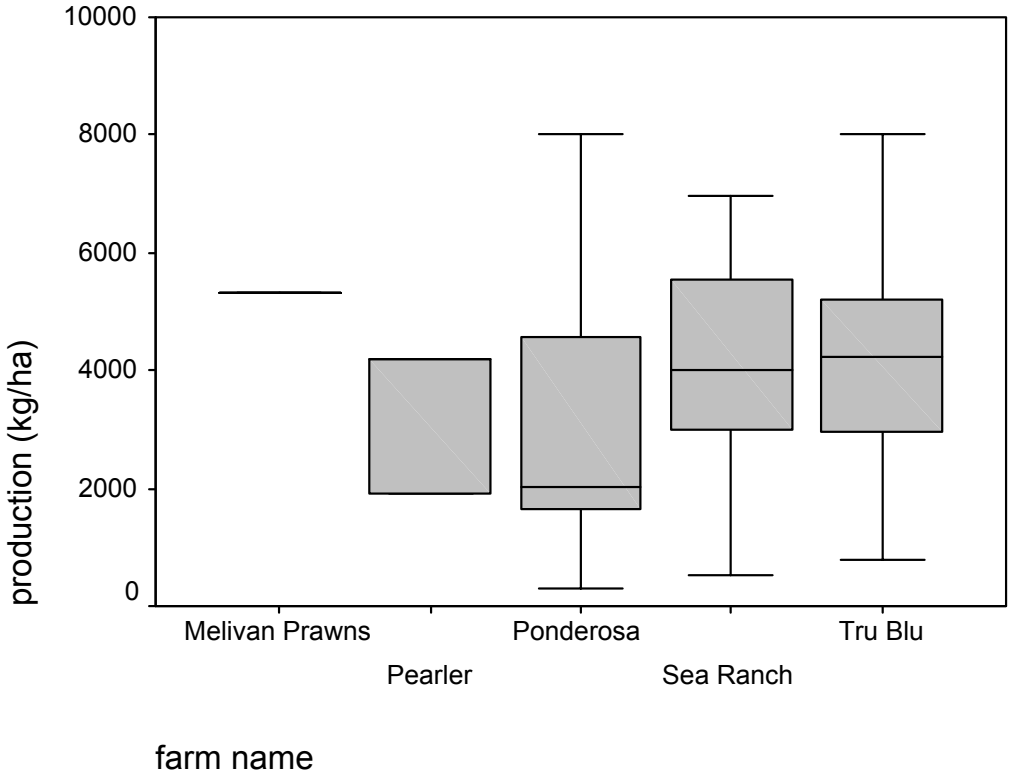


Fig 6.7 Index for production for ponds

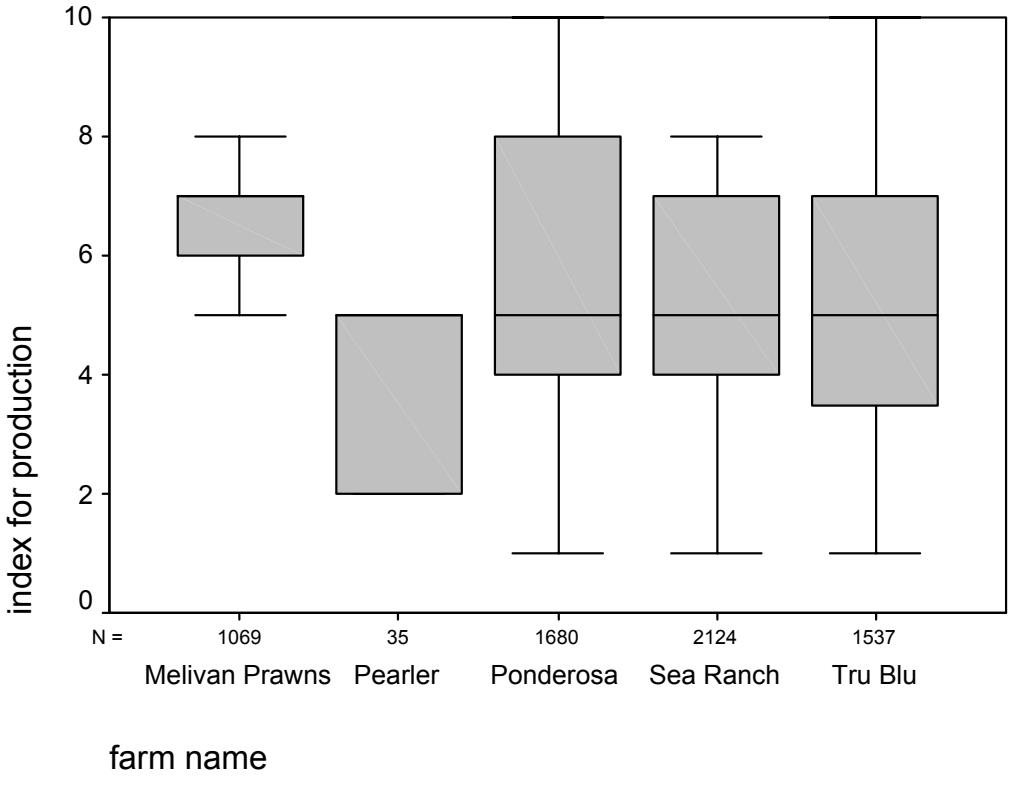


Fig 6.8 Average survival of prawns per crop

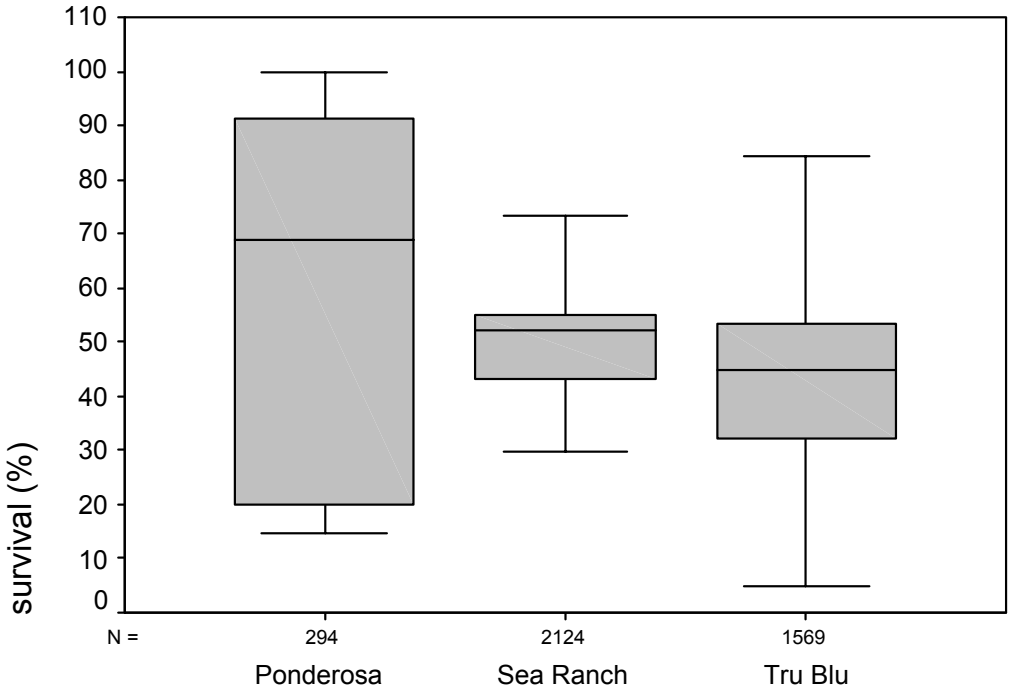
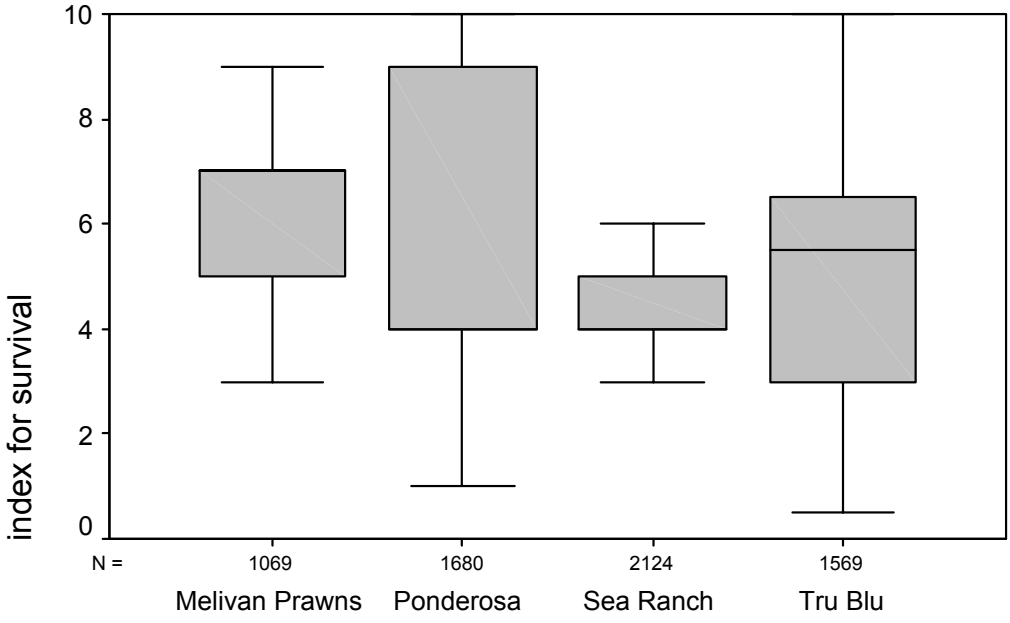
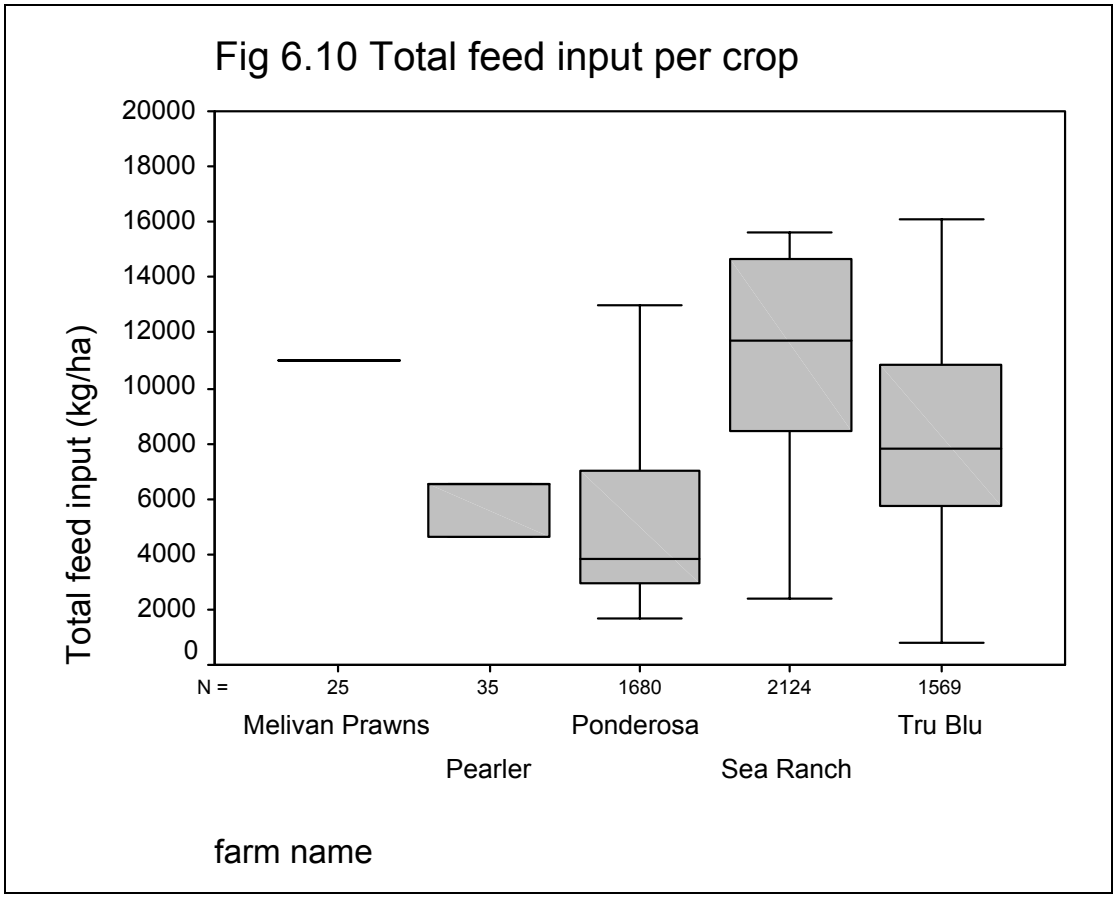


Fig 6.9 Survival index for ponds

(10=best, 1=worst)





Figs 6.11 to 6.13 show high degrees of correlation between each index and its corresponding productivity variable. For example, for the association between Survival vs Survival Index, the coefficient of determination was R^2=0.77. This indicates that 0.77 or 77% of variability in the Survival Index can be explained by the regression with Survival, and the linear model is significant at <0.0001. This illustrates the usefulness and validity of the three indices as measures of pond productivity and performance.

Fig 6.11 Curve Fit of FCR vs FCR Index

```
MODEL:

Dependent variable.. FCR_I              Method.. LINEAR

Listwise Deletion of Missing Data

Multiple R            .68674
R Square              .47161
Adjusted R Square     .47151
Standard Error       1.99797

            Analysis of Variance:

                 DF    Sum of Squares      Mean Square

Regression        1        19870.391        19870.391
Residuals      5577        22262.839            3.992

F =    4977.67478       Signif F =  .0000

-------------------- Variables in the Equation --------------------

Variable                    B         SE B       Beta          T  Sig T

FCR                -1.779851      .025227   -.686738   -70.553  .0000
(Constant)          9.688565      .062309              155.492  .0000
```

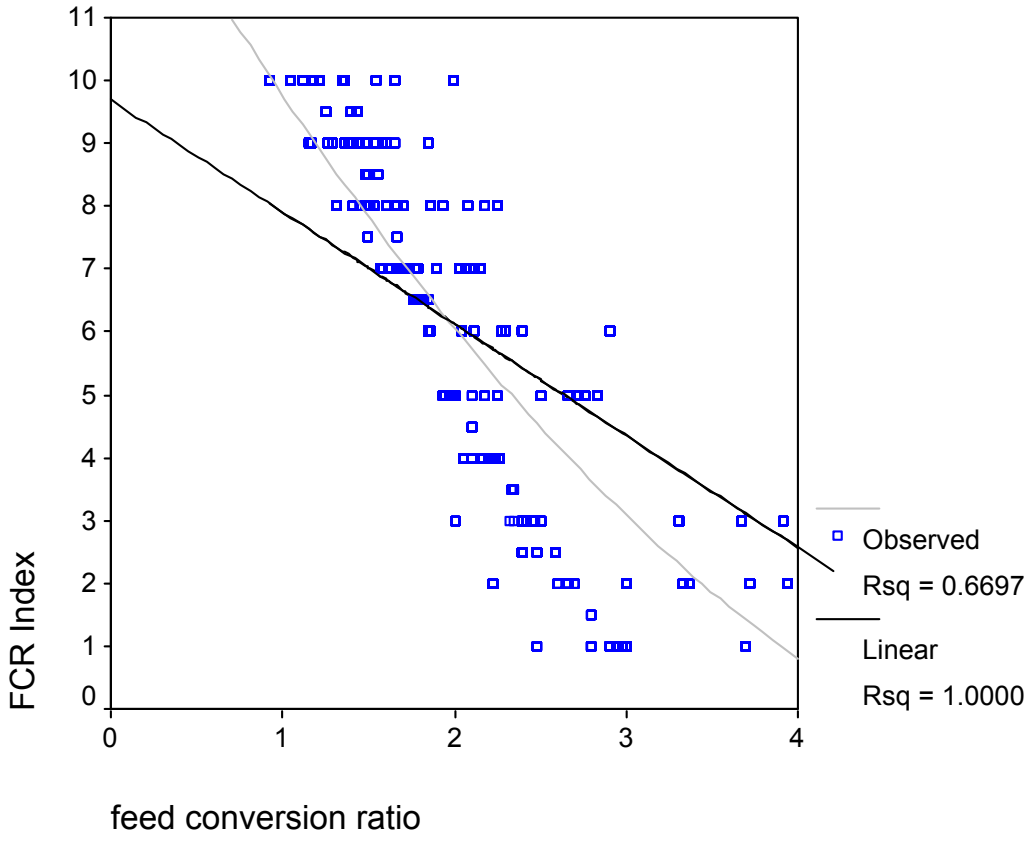


Fig 6.12 Curve Fit of Production vs Production Index

```
MODEL:


Dependent variable.. PRODN_I           Method.. LINEAR

Listwise Deletion of Missing Data

Multiple R            .80371
R Square              .64594
Adjusted R Square     .64588
Standard Error       1.15047

            Analysis of Variance:

                 DF    Sum of Squares      Mean Square

Regression        1        13389.812        13389.812
Residuals      5545         7339.315            1.324

F =    10116.27235       Signif F =  .0000

-------------------- Variables in the Equation --------------------

Variable                    B         SE B       Beta          T  Sig T

PRODUCTN             .000871  8.6581E-06    .803705    100.580  .0000
(Constant)          1.795325     .036774                48.820  .0000
```

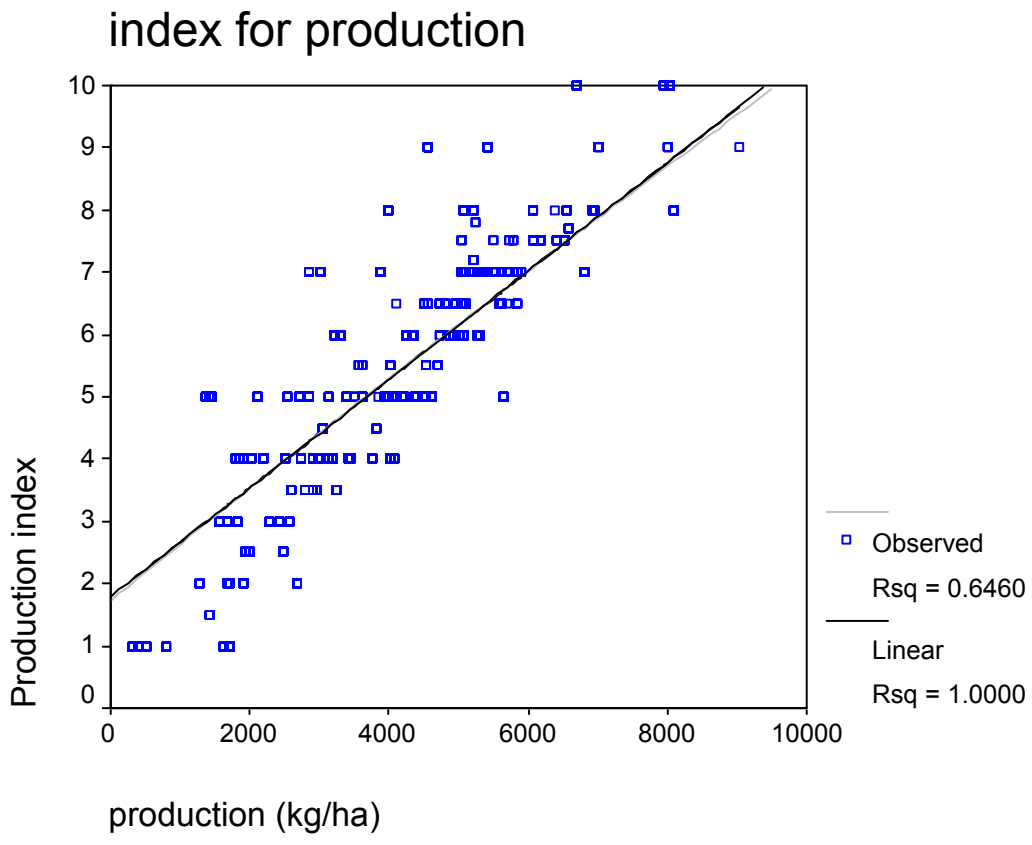
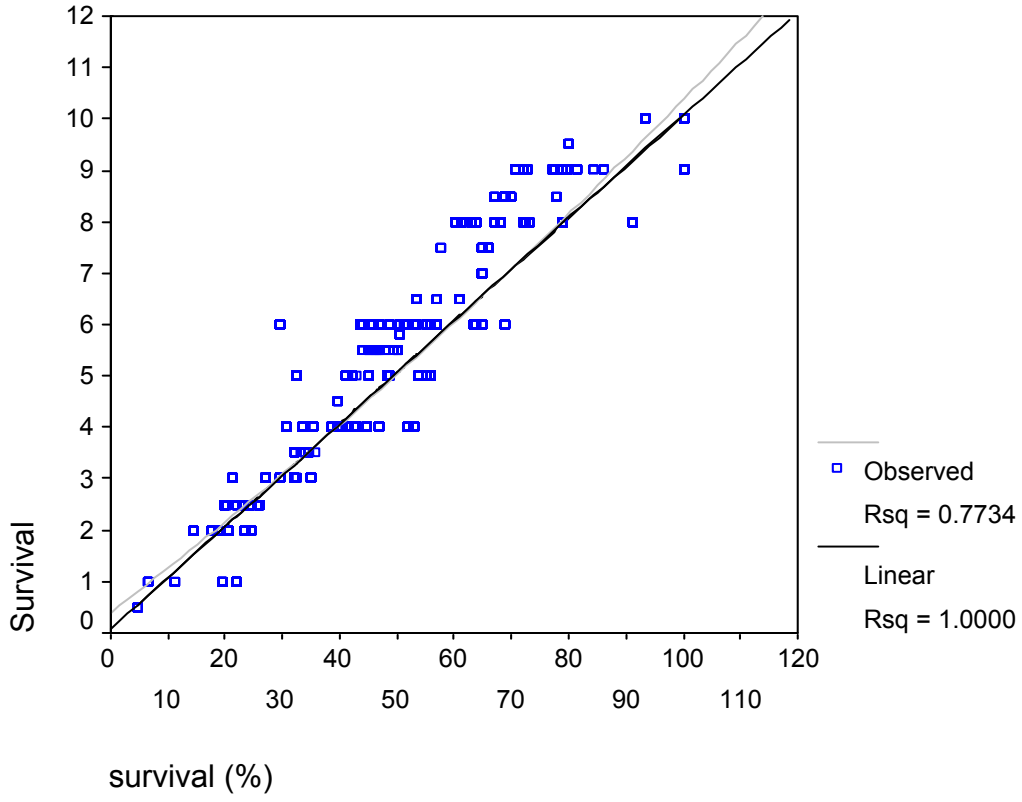


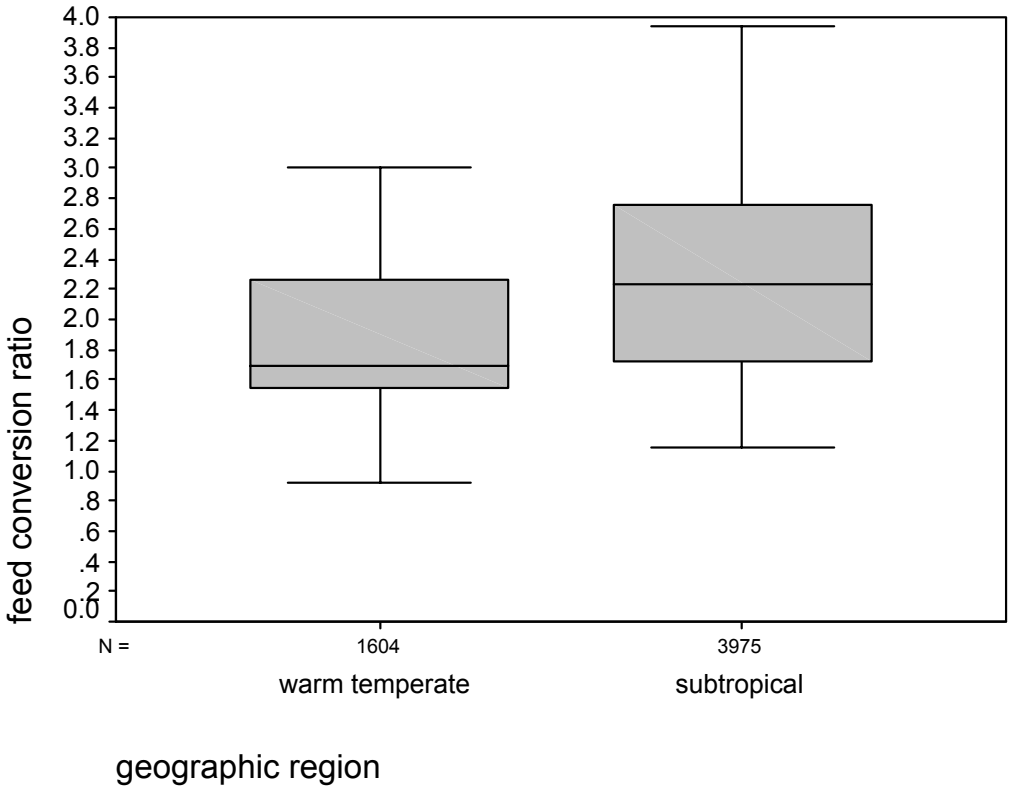
Fig 6.13 Curve Fit of Survival vs Survival Index

```
MODEL:

Dependent variable.. SURVIV_I          Method.. LINEAR

Listwise Deletion of Missing Data

Multiple R           .87878
R Square             .77226
Adjusted R Square    .77220
Standard Error       .89833

            Analysis of Variance:

                 DF    Sum of Squares      Mean Square

Regression        1        10904.824      10904.82413
Residuals      3985         3215.903           .80700

F =   13512.75865       Signif F =  .0000

-------------------- Variables in the Equation --------------------

Variable                    B        SE B       Beta          T  Sig T

SURVIVAL             .100029     .000861    .878781    116.244  .0000
(Constant)           .070465     .043121                 1.634  .1023
```



Analysis of variance (ANOVA) of the productivity indices for the two regions is shown in Supplement #4. The analysis shows that there is no significant difference between farms on the Clarence River and Far North Queensland, with respect to Age at harvest (means = 169 days and 167 days, respectively), Survival Index (means = 5.15 and 5.24), and Average harvest weight (means = 23.3g and 23.6g). However, there were significant differences between farms in the two regions (Clarence River and Far North Queensland) with respect to Survival (means = 45.5% and 48.5%, respectively), Production (means = 4051 kg/ha and 3975 kg/ha), Production Index (means = 5.0 and 5.4), Total feed input (means = 8134 kg/ha and 8960 kg/ha), FCR (means = 1.92 and 2.35) and FCR Index (means = 5.9 and 5.7). While most of these differences are small, the statistics suggest that farms in Far North Queensland have higher FCRs and use approximately 12% more feed per ha than those on the Clarence River. Figure 6.14 illustrates the difference in FCR between the two regions and the trend is consistent for comparisons between the regions for FCR Index and Total Feed input.

Fig 6.14 Comparing FCR for ponds in the two regions



Investigations of the factors associated with FCR reveal that seasonal influences may be important. Fig 6.15 illustrates that ponds on the Clarence River are only stocked in October, November and December, with FCR appearing to significantly decrease in ponds stocked in the later month (ie ponds stocked in December). On the other hand, ponds in Far North Queensland were stocked at various months throughout the year. The lowest FCRs appear to be in ponds that are stocked from July through to November. Highest FCRs occur in ponds stocked from December through to March, possibly because of the difficulties faced in growing prawns through wetter months or during colder months or when temperatures are decreasing (as shown in Fig 6.16). Interestingly, our tank tests revealed (data not shown here) that juveniles of *P. monodon* have approximately double the growth rate at 27.5°C than at 25°C. Consequently, farmers need to be aware that when PLs are stocked at 25°C or when the temperature is decreasing, there is a significant increase in FCR. At lower temperatures, *P. monodon* does not grow as fast and it is likely that significant quantities of feed are not eaten. This is also likely to cause a deterioration in the quality of water and sediment in ponds.

In Supplement #5 the statistical analysis, by ANOVA, indicates that the month that a pond is stocked is a significant factor for all 9 productivity indices. Tukey B is a statistic that was used to group the months associated with similar means. The stocking months that provided highest Indices for survival, production and FCR (ie best performance) were July to December, though there were some exceptions to this generality (see Supplement #5).

Fig 6.15 The relationship between month stocked and FCR for farms in the two regions

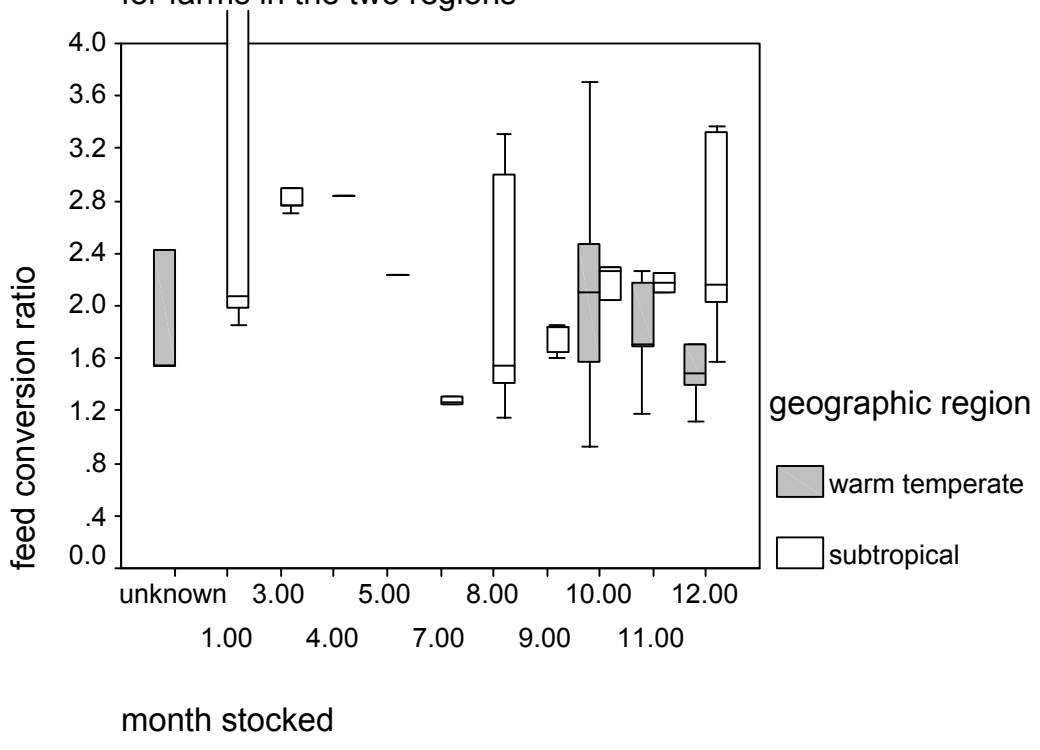
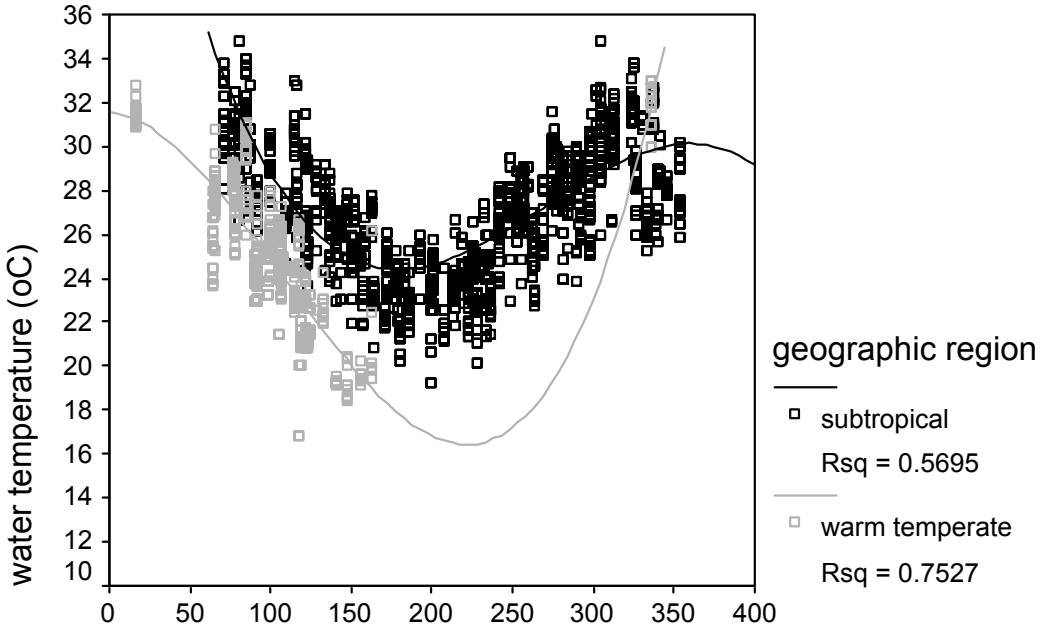


Fig 6.16 Changes in water temperature

for farms in the two regions



Another factor which strongly influenced the productivity of ponds is the source of the postlarvae (PLs), that is the hatchery. Statistical analysis by ANOVA in Supplement #6 indicates that the hatchery was a significant factor for all productivity indices. Using Tukey B analysis, the hatcheries with similar means are grouped, but the unequal number of cases from each hatchery reduces the reliability of this analysis. Hatcheries that were associated with highest productivity were Ponderosa, Great Barrier Reef and Fortune. Fig 6.17 illustrates differences in pond production for PLs sourced from the various hatcheries.

Interpretation of the statistics and graphs for hatcheries is not straightforward. As with the results for farms, we avoid drawing any conclusions about specific hatcheries in this report. The graphs are provided so that interpretation of results can be undertaken by the hatcheries themselves. Once again it should be remembered that the purpose of the project was never to examine the performance of individual farms or hatcheries, rather it was to collect data from a wide range of sources and look for factors that influenced the bacteriology. Hence, it was beyond the scope of the project to investigate the cause of the variations associated with the hatcheries. Nevertheless, the survival of PLs as shown in Fig 6.18, was consistent with influences of viral disease, probably Gill Associated Virus (GAV). If that was the case, the differences in productivity may be associated with the viral load of the batch of spawners that the hatchery used. Further, the evidence indicates that GAV is a latent virus, remaining

dormant until it is activated by one or more triggers, such as increase in stress or injury to the prawn. Our study found, and this will be described in Section 6.4, that spawners were severely stressed by bacterial pathogens and vibriosis during transport to hatcheries and PLs were often severely stressed by these same factors during the transfer from hatchery to pond. It is consequently proposed that adverse bacteriological conditions during transport may be very important factors in triggering GAV in broodstock and postlarvae, as well as in and other stages in the culture of *P. monodon*.

Supplement #7 has 32 graphs for productivity indices for Melivans, Ponderosa, Searanch and Tru Blu prawn farms. Only limited productivity data was available from Fortune and Pearler prawn farms so no graphs were produced for those farms, while some data (eg Production kg/ha) was not available from Melivans, so their productivity graphs were reduced. The graphs are provided so that interpretation of results can be undertaken by the farms themselves.

Fig 6.17 Pond production vs source of PLs.

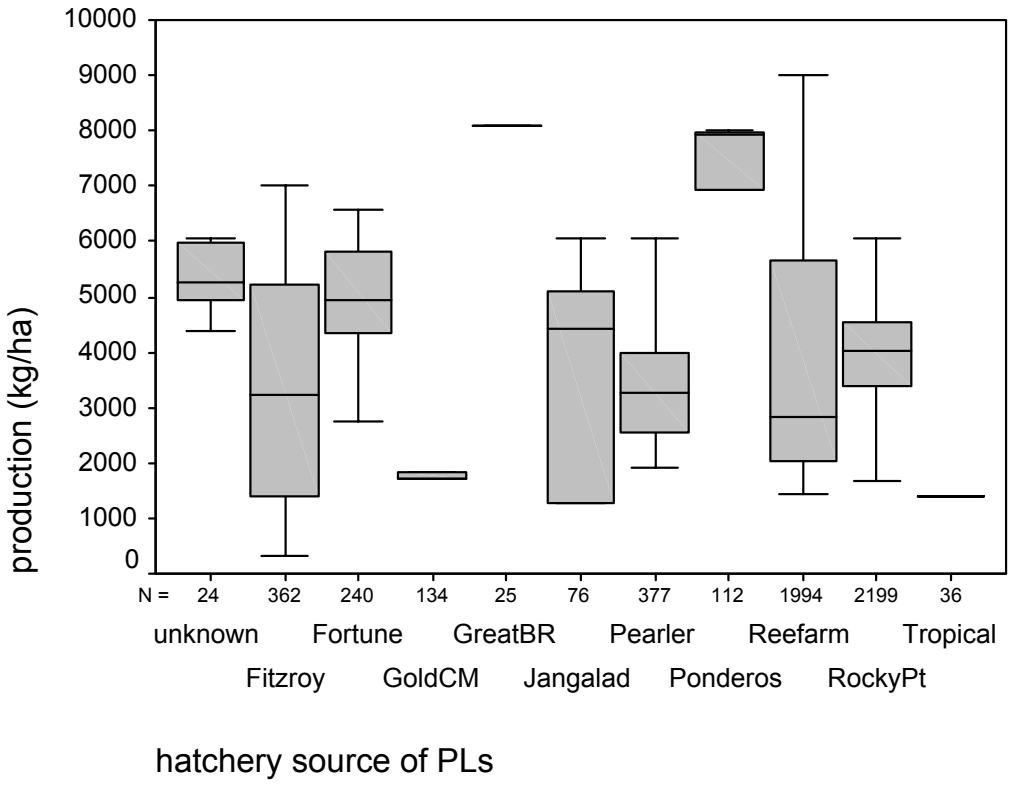
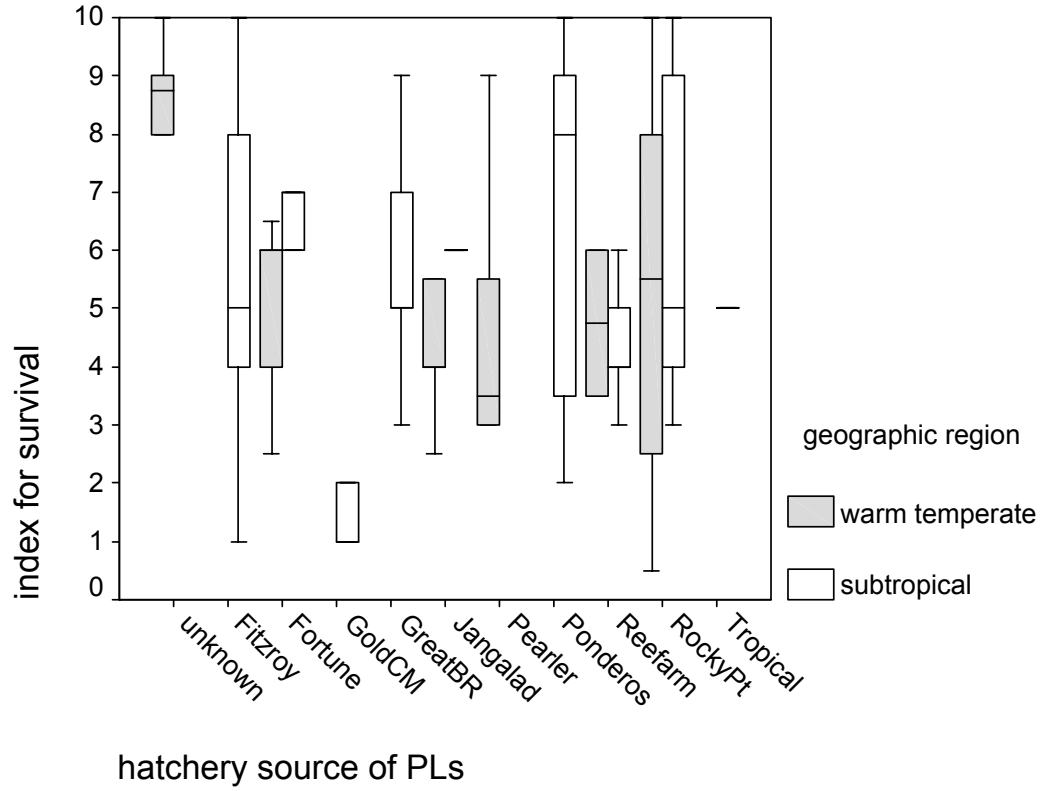


Fig 6.18 Survival index for each region vs source of PLs.



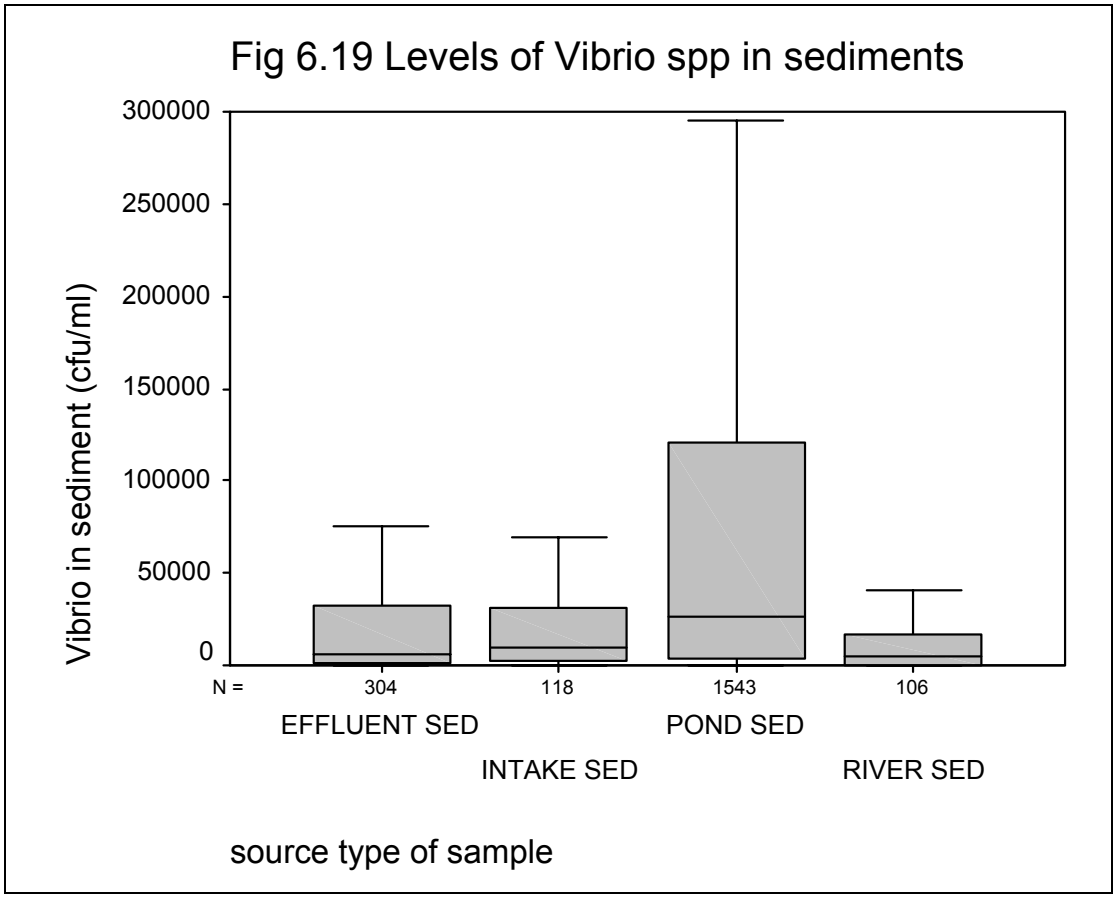
6.1.4 Extracellular Microbial Enzyme activity at the farms

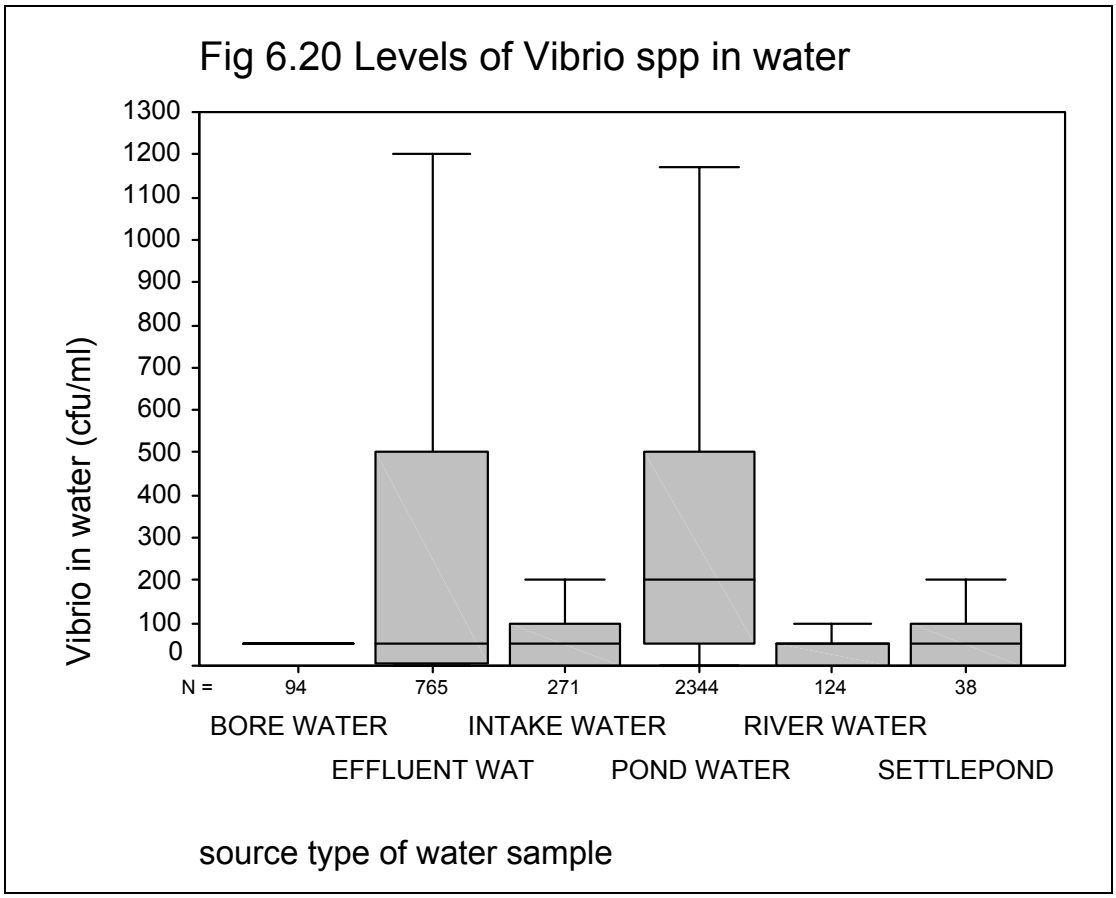
6.1.4.1 General trends in water and sediment

As discussed in the methods, autoclaved samples had either zero or very low enzyme activity. In comparison, "live" samples generally gave very strong and reproducible signals. The method produced a strong, reliable profile of the rate of extracellular enzyme activity for each sample with respect to breakdown of carbohydrates, proteins, chitins, lipids and inorganic compounds. Supplement #8 is a statistical summary of the extracellular enzyme activities of the various types of sediments (ie river, intake, effluent and pond) and waters (ie river, intake, effluent, pond and bore). In considering the relationship between the source of samples and enzyme activity, the standard parametric method of analysis was used to compare the means (ie ANOVA). This type of statistical analysis showed that for most of the 22 fluorescently-labelled compounds the means are significantly different for the various sources. However, graphs of residuals indicate that a non-parametric approach (Kruskal-Wallis) needed to be carried out and the results were found to be the same as those under the parametric approach, showing the robustness of ANOVA for large samples. The mean extracellular enzyme activities for the four main types of samples are summarized for sediments in Table 6.2 and water samples in Table 6.3.

As a preliminary statement, the data in Supplement #8,Tables 6.2.and 6.3 show that on occasions there were negative enzyme activities for some substrates. The likely causes of negative enzyme activities were discussed earlier; it was more commonly observed in sediment samples (see Table 6.2: b-glucose and l-fucoside for river sediment) but was occasionally found in samples in which bacteria levels were very low (see Table 6.3: diacetyl chitobioside for water in the intake settlement pond at Searanch). Also, because of the very high activities of sediment samples, sediments were appropriately diluted (ie usually 100x) while water samples were not. Consequently, interpretation of results from water samples was more straightforward than for sediment samples.

The results in Tables 6.2 and 6.3 illustrate some important trends. Firstly sediment samples had enzyme activities approximately 100 times (ie 2 orders of magnitude) higher than those in water samples. This is consistent with earlier reports by Smith (1994, 1998) which showed that heterotrophic bacteria and *Vibrio* spp are approximately 100 times higher in pond sediment than in pond water. The present study found that sediments from all sources had levels of *Vibrio* spp (by plate counts) that were approximately 100 times higher than those in water sources: the mean for sediments was in the range of 5,000-20,000 cfu/mL (Fig 6.19) while the mean for water sources was 50-200 cfu/mL (Fig 6.20). This finding reinforces the point that sediments in prawn farms have extremely high rates of bacterial activity. Although it is often not recognised, sediment microbiology has very profound influences on the bacteriology of prawn ponds.





It is likely that sediment bacteriology plays a more dominant role in shallow ponds (ie 1.0 m average depth) than in deeper ponds (2.0 m depth) simply because of relative bacterial activity. That is, the number and activity of bacteria in 20 mm of sediment on a pond bottom is equivalent to that for the bacteria in 2.0 m of pond water above it. Also, on those occasions when sediment is stirred up, the bacteriology of the water column is likely to change significantly. Consequently, farmers should take actions to minimize mixing of sediments in the water column. For instance, turbulents caused by wind and wave action as well as poorly oriented aerators (particularly water jets and sub-surface models) could be sources of unnecessary stirring of sediments and increases in bacteria in the water column.

Table 6.2 Summary of the mean extracellular enzyme activities (nmol/L/hr) for **sediment** samples and the level of significance of the difference in means.

Substrate	River	Intake canal	Pond	Effluent canal	Significance level for difference in means
Carbohydrates					
a-glucoside	-286	1574	4060	3734	0.000
b-glucoside	6103	5516	10293	13815	0.249
Xyloside	1920	2153	3477	2447	0.016
Galactoside	1380	1351	3508	2405	0.008
l-fucoside	-923	-206	2004	1067	0.000
b-fucoside	207	670	1026	777	0.959
Glucuronide	495	1868	2321	2189	0.846
Cellobioside	1102	1258	1588	1900	0.116
Chitins					
n-acetyl glucosaminide	10878	12162	15549	18067	0.846
di-acetyl chitobioside	434	1559	6258	3650	0.813
Sulfo glucopyranoside	5734	4400	3845	6704	0.000
Esters					
Acetate	214723	186205	147079	94832	0.000
Butyrate	297294	358193	196300	180297	0.000
Propionate	391555	358246	218870	181152	0.000
Oleate	1968	2855	1769	1884	0.002
Caprylate	90049	96181	67295	57095	0.001
Laurate	5779	6460	4741	4741	0.079
Triammonium cinnate	48969	126224	55222	24137	0.000
Proteins					
Leucine	45711	57395	76249	71573	0.272
Guanidinobenzoate	71957	55948	36103	30377	0.000
Inorganics					
Phosphate	19657	43339	18143	37125	0.000
Sulfate	6008	6980	4432	5812	0.055

Table 6.3 Summary of the mean extracellular enzyme activities (nmol/L/hr) for **water** samples and the level of significance of the difference in means.

Substrate	River	Intake canal	Intake settlement pond (at Searanch)	Pond	Effluent canal	Significance level for difference in means
Carbohydrates						
a-glucoside	3.28	5.07	0.78	25.13	15.82	0.000
b-glucoside	13.39	12.05	5.87	49.47	31.94	0.000
Xyloside	3.99	3.57	2.63	16.06	10.31	0.000
Galactoside	3.92	3.53	1.42	14.82	10.79	0.000
l-fucoside	3.42	1.30	1.19	5.47	4.96	0.672
b-fucoside	4.51	1.92	0.68	11.80	5.10	0.000
Glucuronide	5.38	1.24	0.57	6.97	10.01	0.018
Cellobioside	6.10	2.87	2.23	10.29	7.43	0.000
Chitins						
n-acetyl glucosaminide	35.14	33.95	38.69	62.02	114.64	0.000
di-acetyl chitobioside	3.27	1.50	-2.29	38.87	14.03	0.003
Sulfo glucopyranoside	19.70	10.04	4.33	26.24	52.71	0.000
Esters						
Acetate	680.66	863.90	597.55	2067.61	1147.40	0.000
Butyrate	458.24	466.87	177.70	947.80	658.14	0.000
Propionate	531.83	583.38	320.67	1162.33	777.81	0.000
Oleate	14.38	9.56	10.89	29.26	21.97	0.021
Caprylate	168.82	145.31	46.75	360.82	210.41	0.000
Laurate	21.04	21.09	12.02	30.66	38.16	0.000
Triammonium cinnate	1119.07	1235.29	826.03	1972.43	1412.86	0.000
Proteins						
Leucine	195.35	322.98	125.90	2143.18	1181.35	0.000
Guanidinobenzoate	298.37	253.52	189.87	372.92	283.66	0.000
Inorganics						
Phosphate	124.25	92.77	45.43	287.77	314.60	0.000
Sulfate	6.01	9.86	6.33	21.98	26.31	0.000

Secondly, results from river, intake canal and intake settlement pond provide useful references for interpreting results for microbial enzymes in ponds and effluent. ANOVA indicates that for the various sources of sediment, 14 of the 22 substrates had significantly different means ($P<0.05$ in Column 6, Table 6.2). While for the sources of water, the means were significantly different for 21 of the 22 substrates ($P<0.05$ in Column 7, Table 6.3). The main findings from data in Tables 6.2 and 6.3 are summarized as follows.

i) Microbial enzyme activity in sediments:

a) Carbohydrates (except chitin)

Sediments in ponds and effluent have approximately 100% higher enzyme activities for breaking down all carbohydrates than river and intake sediments. Cellulose breakdown (as measured with b-glucoside and cellobioside) is higher in sediments than for starch breakdown (as measured with a-glucoside) and other carbohydrates (xyloside, galactoside, l-fucoside, b-fucoside, glucuronide and cellobioside).

b) Chitins

Sediments in ponds and effluent have higher enzyme activity for chitin (as measured with n-acetyl glucosaminide) than for any other carbohydrate. The chitinase activity in sediments is higher in ponds and effluent than in river and intake, but because of high variability in the data the means are not significantly different.

c) Esters

In sediments the esterase activity, as measured with all substrates, is lower in ponds and effluents than in river and intake. The esters with the highest activity in sediments were butyrate, propionate followed by acetate. This may indicate that fatty acids and esters are a more important source of organic compounds in estuaries than in ponds.

d) Proteins (amino peptides)

In sediments the main indicator of protease activity (leucine) suggests there is higher activity in pond and effluent than in river and intake sediment, but the difference was not significant. For guanidinobenzoate, the enzyme activity in sediments was significantly higher in river and intake than in ponds and effluent.

e) Inorganics

Phosphatase activity in sediments was significantly higher in the intake and effluent canals than in river and ponds. Sulfatase activity in sediments was slightly higher in river and intake than in ponds and effluent.

f) Overall trends for sediments

In comparison with the reference sediments (river and intake canal), there appears to be lower enzyme activity for esterase and sulfatase in farm sediments (ie ponds and effluent). Conversely, there is higher activity in pond and effluent sediments for chitinase and most carbohydrate enzymes. Table 6.4 ranks the extracellular enzyme activity for the various groups of substrates with respect to the two general types of sediments (ie river and intake canal sediments versus pond and effluent canal sediments). It shows that changes in the pattern of enzyme activity occurred with the various carbohydrates (b-glucoside, a-glucoside, xyloside and galactoside).

The bacteria that inhabit sediments are dominated by anaerobes (ie function without oxygen) that ferment organic matter and produce gases such as hydrogen sulfide (H_2S), hydrogen (H_2), ammonia (NH_3) and carbon dioxide (CO_2). Ponds and effluent canals have significantly higher input of organic matter in the form of dying algal blooms, prawn faeces, chitin (prawn shell) and waste from pellet feed.

Table 6.4 Ranking of mean extracellular enzyme activity of sediments (highest to lowest).

Ranking of extracellular enzyme activity	Reference sediments (ie river and intake canal)	Farm sediments (ie pond and effluent canal)
HIGHEST	Esterase	Esterase
	Protease	Protease
	Phosphatase	Phosphatase
	n-acetyl glucosaminidase	n-acetyl glucosaminidase
	Sulfatase	b-glucosaminase
	b-glucosidase	Sulfatase
	Xylosidase	a-glucosidase
	Galactosidase	Xylosidase & Galactosidase
LOWEST	Other carbohydrases	Other carbohydrases

ii) Microbial enzyme activity in water:

a) Carbohydrates (except chitin)

Table 6.3 shows that pond water and effluent water have approximately 4 to 5 times higher enzyme activity for most carbohydrate substrates than water from the river and intake canal. The settlement pond for intake water at Searanch had the lowest level of activity for all carbohydrate enzymes. The trend for carbohydrate enzyme activity is similar to that for sediments; cellulose breakdown (as measured with b-glucoside and cellobioside) is higher in the water sources than for starch breakdown (as measured with a-glucoside) and other carbohydrates (xyloside, galactoside, l-fucoside, b-fucoside, glucuronide and cellobioside).

b) Chitins

Pond and effluent water have much higher enzyme activity for chitin (as measured with n-acetyl glucosaminide) than for any other carbohydrate. The chitinase activity is 2 to 10 times higher in ponds and effluent waters than in river and intake waters. This trend is more profound than that observed with chitinase in sediments.

c) Esters

The esterase activity, as measured with all substrates, is approximately 100% higher in ponds and effluent waters than in river and intake waters. This is the opposite trend to that observed for esterases in sediments. Also, the esters with the highest activity in waters were acetate, propionate followed by butyrate.

d) Proteins (amino peptides)

In pond and effluent water the protease activity (as measured with leucine) is 7 to 10 times higher than river and intake water. For guanidinobenzoate, the enzyme activity

in farm waters was slightly higher than river and intake water. The results for protease activity in the water column are clearly different to those found for sediments.

e) Inorganics
Phosphatase activity in pond and effluent waters was significantly higher by approximately 3 times than intake and river water. Similarly, sulfatase activity in farm waters was approximately 3 times higher than river and intake waters. This is also clearly different to the trend observed for sediments.

f) Overall trends
The most obvious finding is significantly higher enzyme activity for all substrates (carbohydrases, chitinases, esterases, proteases, phosphatase and sulfatase) in farm waters (ie ponds and effluent) than in reference waters (river and intake canal). The second important finding is illustrated in Table 6.5, which ranks the extracellular enzyme activity for the various groups of substrates with respect to the two general types of water (ie river and intake canal water versus pond and effluent canal water). It shows that the highest enzyme activity in pond and effluent water is protease (amino peptidase) as distinct from esterase activity. This change in the pattern of enzyme activity is highly significant and probably reflects changes in bacteriology of the farm caused by the input of protein in commercial feed.

Table 6.5 Ranking of mean extracellular enzyme activity of water (highest to lowest).

Ranking of extracellular enzyme activity	Reference water (ie river and intake canal)	Farm water (ie pond and effluent canal)
HIGHEST	Esterase	Protease
	Protease	Esterase
	Phosphatase	Phosphatase
	n-acetyl glucosaminidase	n-acetyl glucosaminidase
	b-glucosidase	b-glucosaminase
	Sulfatase	Sulfatase
	Other carbohydrases	a-glucosidase
		Xylosidase & Galactosidase
LOWEST		Other carbohydrases

6.1.4.2 Spatial and temporal variations in enzyme activity

The preceding section examined the average values that were measured for extracellular enzyme activity for the various sources of sediments and water samples. This section describes the trends in extracellular enzyme activity with respect to space and time. Spatial trends in results were investigated by comparing a) 2 regions, b) 4 farms, c) ponds within each farm and d) 12 locations within selected ponds at 4 farms. The temporal trends in results were investigated by comparing a) day of year and b) age of crop.

a) Variations between extracellular enzyme activity for two geographical regions.

The graphs in Supplement #9 illustrate that the two geographic regions appeared to have similar results for microbial enzyme activity in water sources for all substrates. A similar pattern was found for sediments in the two geographic regions. Farms in both regions use similar management techniques and brands of feed. Temperature is the major difference between the regions (see Fig 6.16). In the warm temperate region (Clarence River) stocking takes place around late spring to summer, when the temperature rises towards the annual maximum. However, in the sub-tropical farms of Queensland, stocking was carried out at most months of the year, so on occasions the water temperature in sub-tropical farms could be either higher or lower than those for farms on the Clarence River. Hence, temperature is a variable which may well influence enzyme activities but any effect would be masked in Supplement #9 because all growout periods are combined for each of the two regions.

An alternate method for investigating the effect of temperature on extracellular enzyme activity was by scatter plots. For example, Fig 6.21 reveals protease activity increased for the various types of water sources to a peak at around 28-30^{o}C then decreased. However there was considerable variability and the R^2 statistic (ie coefficient of determination) indicates that only 1.7% of the variability in pond water can be explained by the regression. The association between temperature and the other 21 enzyme activities for water and for sediment followed a similar pattern; peaks were usually obtained between 25-30^{o}C but only 0.1-2% of the variability could be accounted for by the regression. This suggests that while temperature is one factor in determining the bacteriology of prawn farms, there are more significant other factors.

A more interesting finding from scatter plots indicated that there was a highly significant association between daily feed rate and extracellular enzyme activity. Fig 6.22 shows that 12% of the variability in protease activity of pond water in sub tropics and 19% in warm temperate pond water was explained by linear regression. The extracellular enzyme activity for the other substrates also had a positive correlation with daily feed rate. Positive correlations between daily feed rate and enzyme activity in pond water are shown in Figs 6.23 for acetate esterase activity, in Fig 6.24 for b-glucosidase, Fig 6.25 for a-glucosidase activity, Fig 6.26 for chitinase activity, Fig 6.27 for phosphatase activity and Fig 6.28 for sulfatase activity. Generally the associations were stronger (ie R^2 was higher) for warm temperate ponds than for sub-tropical ponds, however this was not always true (see Fig 6.27). Also, for all of the 22 substrates, leucine enzyme activity (ie protease or aminopeptidase activity) had the strongest association with daily feed rate (Fig 6.22) and carbohydrases had the lowest. Pond sediments had associations between the daily feed rate and extracellular enzyme activities for the various substrates which were similar to those observed for pond water (data not shown here).

The traditional method for measuring bacteriology was also carried out, and results for levels of *Vibrio* bacteria, as determined by TCBS plate counts, are shown in Figs 6.29 & 6.30. For pond water, Fig 6.29 shows that the *Vibrio* level was highest for low feed rates (ie early stages of the crop or when prawns cease feeding) as well as at high feed rates (ie later stages of crop or when prawns are overfed). While for sediments, Fig 6.30 shows a similar association between daily feed rate and *Vibrio* level.

However, for both sediment and water sources, the R^2 values are in the range 0.00 to 0.048, so by themselves the data from for TCBS counts do not provide strong associations for understanding the dynamics of the bacteriology of prawn ponds.

The data from was also analyzed by considering the association between the age of the crop and extracellular enzyme activity. Fig 6.31 illustrates protease activity versus age of crop for various water sources. A linear regression for pond water explains 7% of the variability while during the same period, the other water sources had very little change in protease activity. Similar results were obtained for the other 21 types of substrates in water sources and sediment sources. The analysis revealed the relationships were not as strong as those observed with daily feed rate. This is understandable because, with respect to the biomass of prawns in ponds, the age of the crop is a cruder variable than daily feed rate because the age of the pond does not differentiate with respect to survival, FCR or productivity.

In another type of analysis, the association between the day of the year and extracellular enzyme activity was investigated. Fig 6.32 shows that there is no discernable change in protease activity in pond water with respect to the day of the year that sampling was carried out. Once again this is a cruder variable than both the age of the crop or the daily feed rate, because on any sampling day, ponds could be at any stage of their growth cycle.

To briefly summarise this section, the daily feed rate is a more significant variable than age of the crop or day of the year for determining extracellular enzyme activity. The analysis of the data with respect to daily feed rates at farms in the two geographical regions reveals that 1) protease activity is the highest extracellular enzyme activity in pond water (see Table 6.5), and 2) there is a strong positive correlation between daily feed rate and protease activity (Fig 6.22) as well as the other extracellular enzymes, and 3) the data from extracellular enzymes is more informative than tradition plate counts of *Vibrio* levels (Fig 6.29, Fig 6.30). The analysis suggests that the high protein content of prawn feed (ie 30-40% dry weight) significantly influences the bacterial processes in prawn ponds. In comparison with the proteins, the carbohydrates and lipids in pellet feed also caused increases in enzyme activity but less of the variability could be accounted for by daily feed rate. In conclusion, the composition of the feed, particularly the protein content, is a more important driver of pond bacteriology than temperature or the geographic region.

Fig 6.21 Association between temperature and protease actvity in water samples

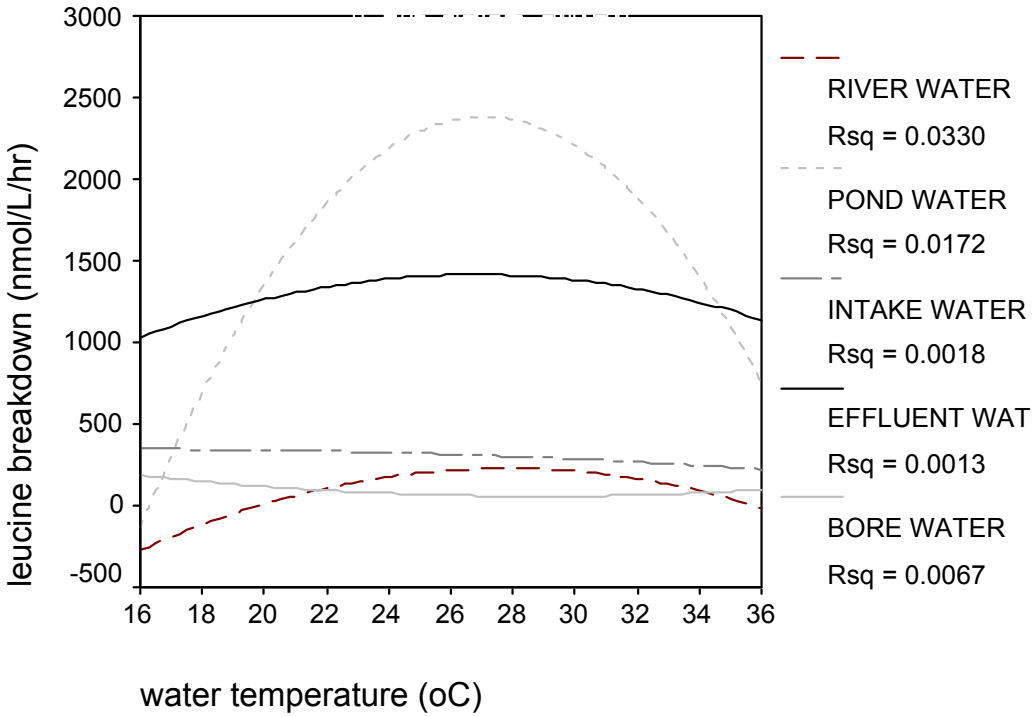


Fig 6.22 Effect of daily feed rate on protease activity in pond water

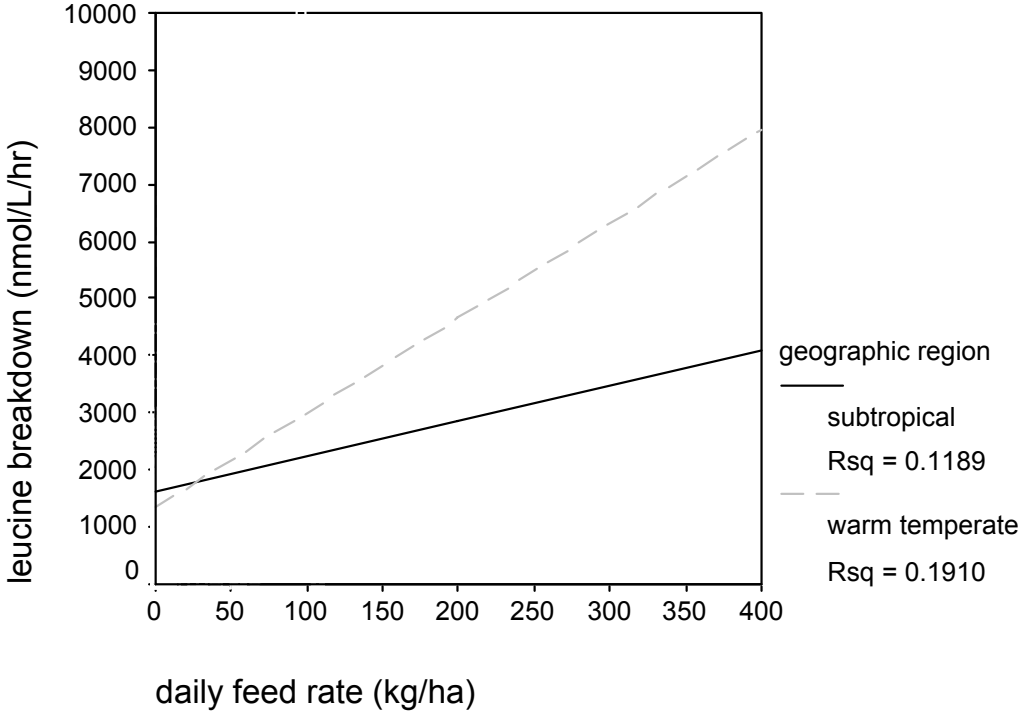


Fig 6.23 Effect of daily feed rate

on acetate enzyme activity in pond water

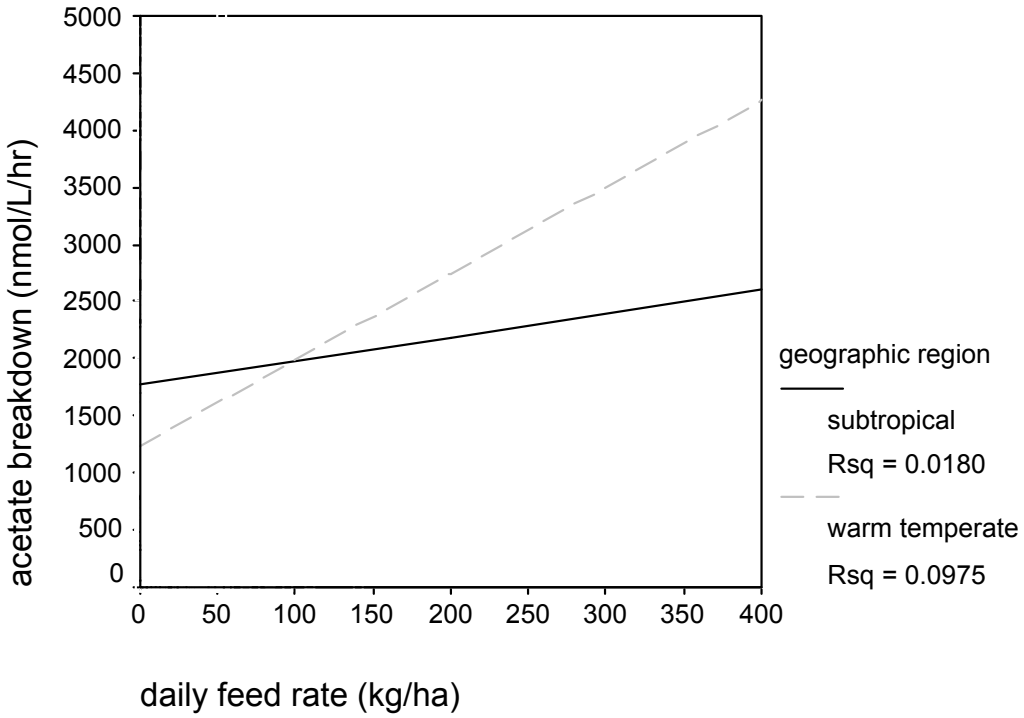


Fig 6.24 Effect of daily feed rate

on b-glucosidase activity in pond water

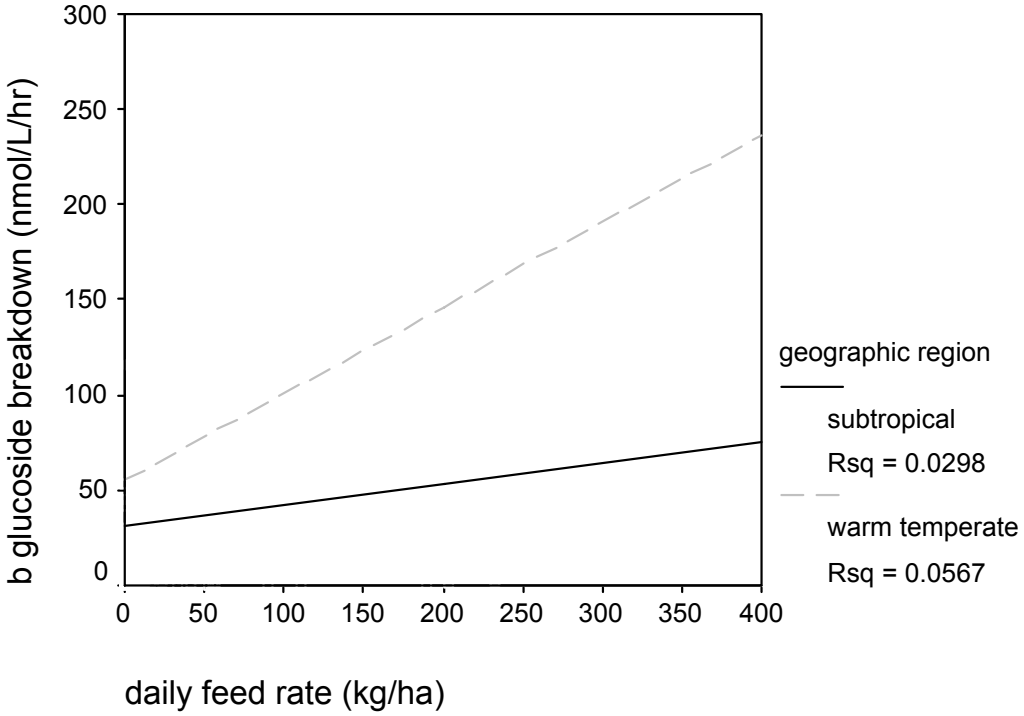


Fig 6.25 Effect of daily feed rate

on a-glucosidase actvity in pond water

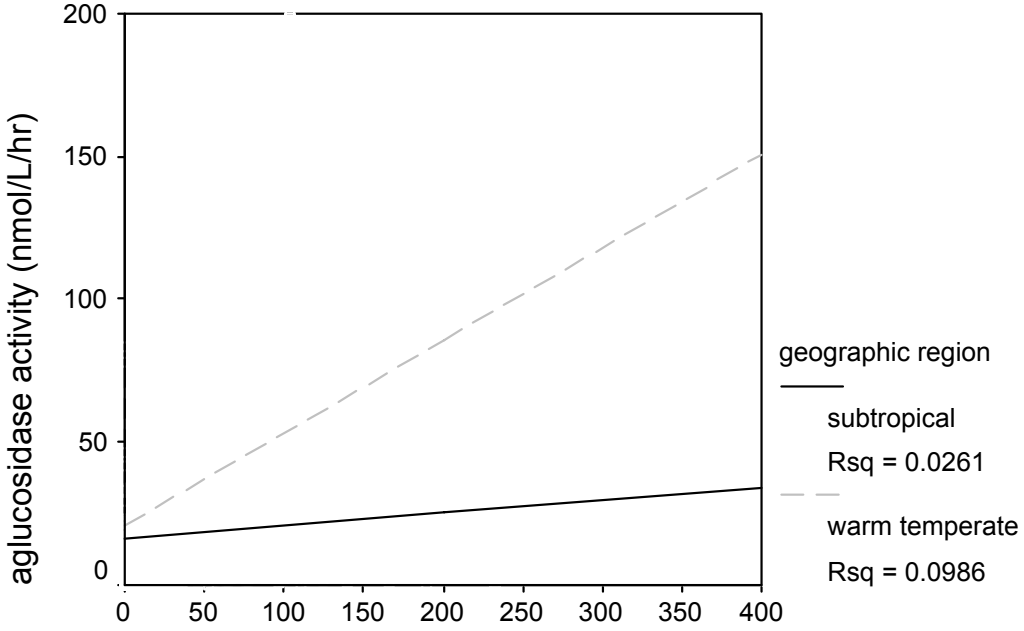


Fig 6.26 Effect of daily feed rate

on chitinase activity in pond water

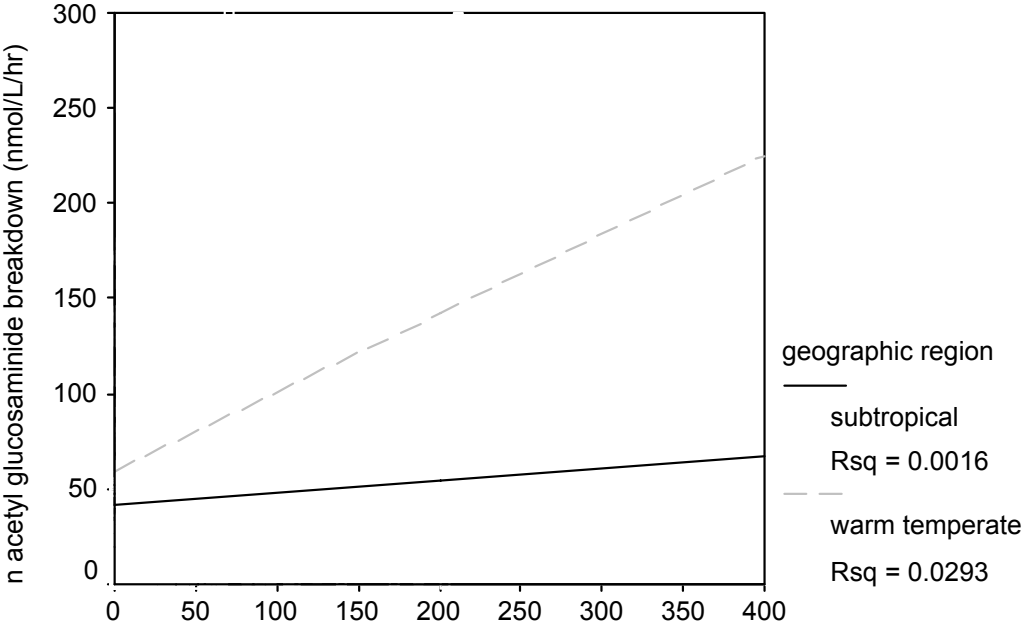


Fig 6.27 Effect of daily feed rate

on phosphatase activity in pond water

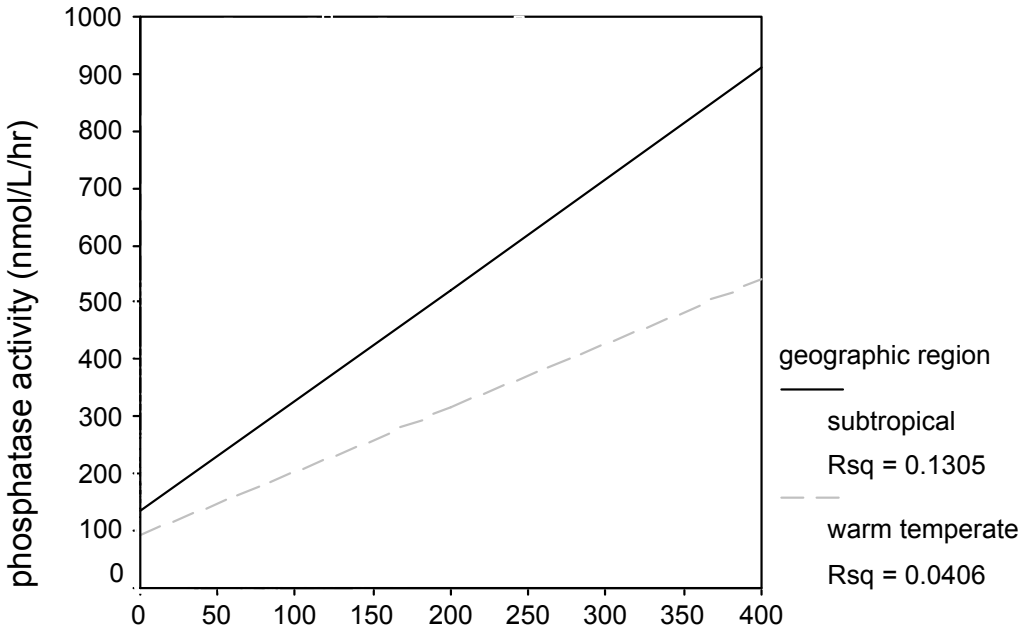


Fig 6.28 Effect of daily feed rate

on sulfatase activity in pond water

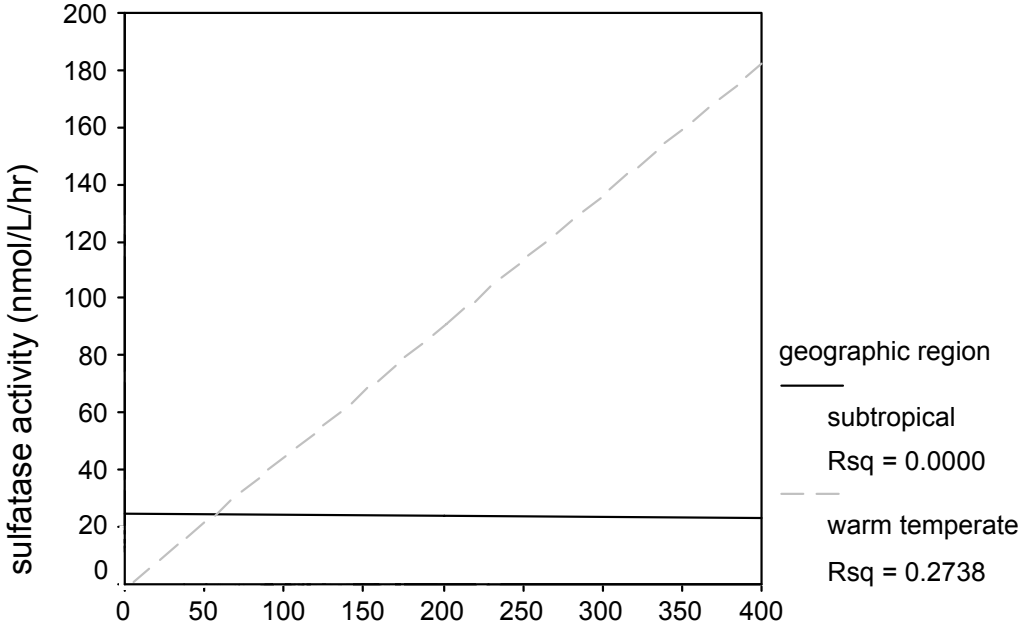


Fig 6.29 The effect of daily feed rate

on Vibrio level in pond water

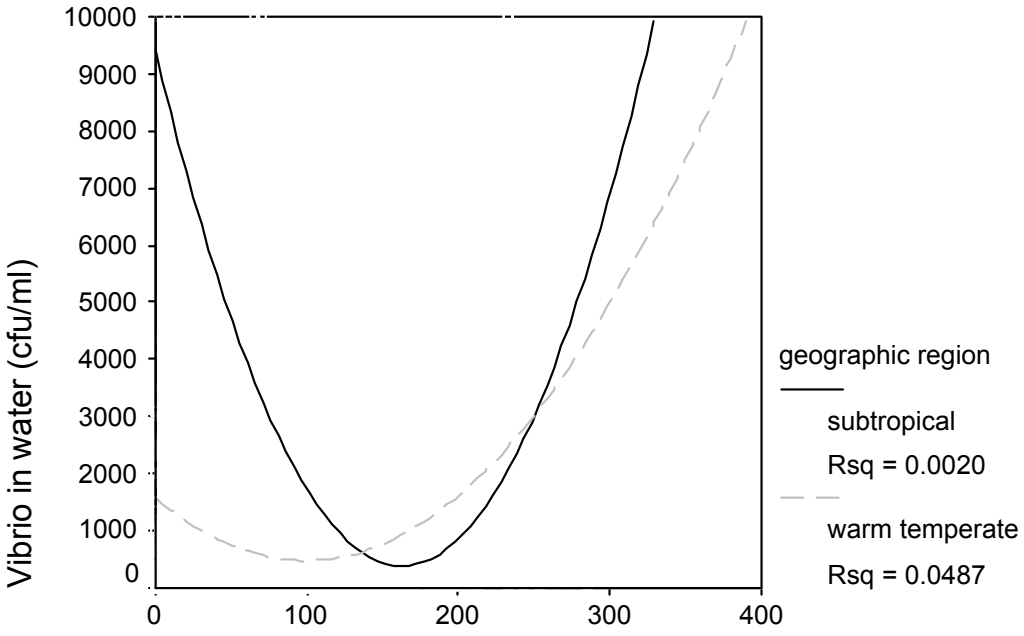


Fig 6.30 The effect of daily feed rate

on Vibrio level in pond sediment

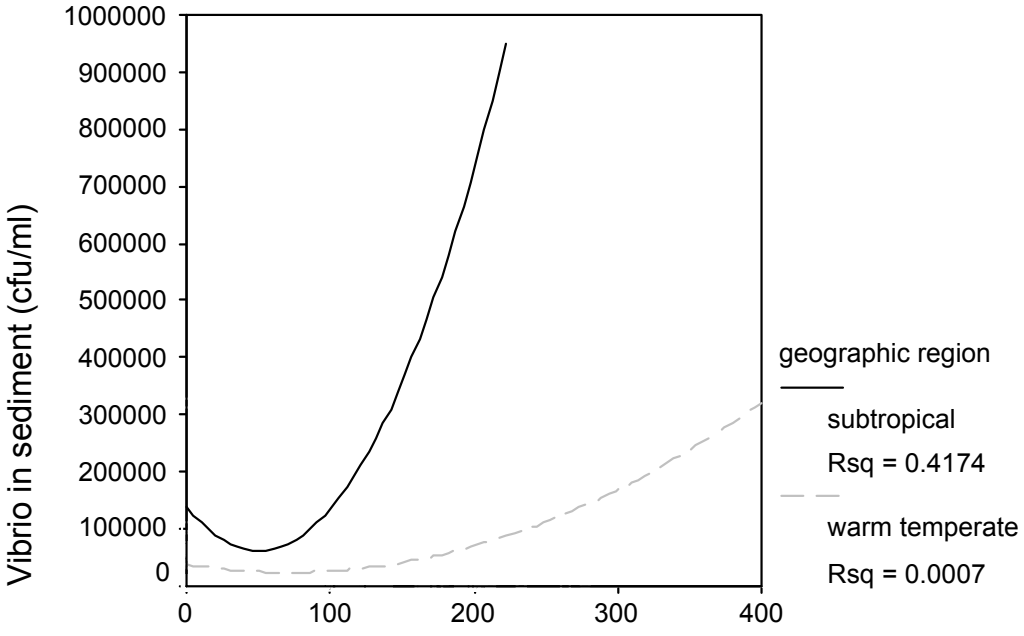


Fig 6.31 Age of crop vs protease activity for water sources

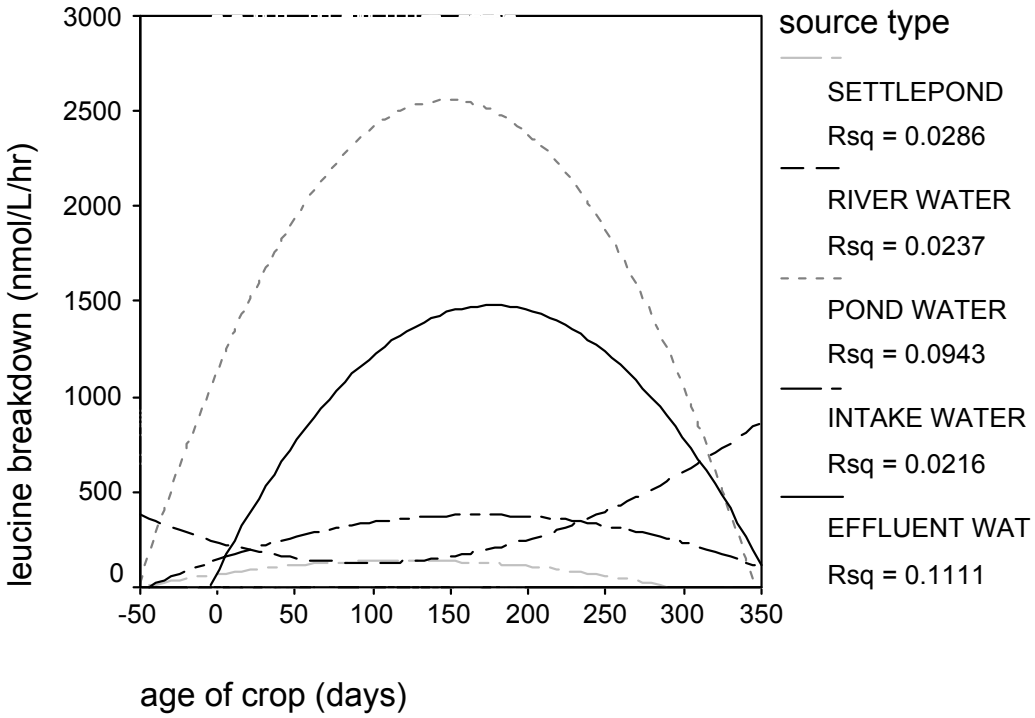
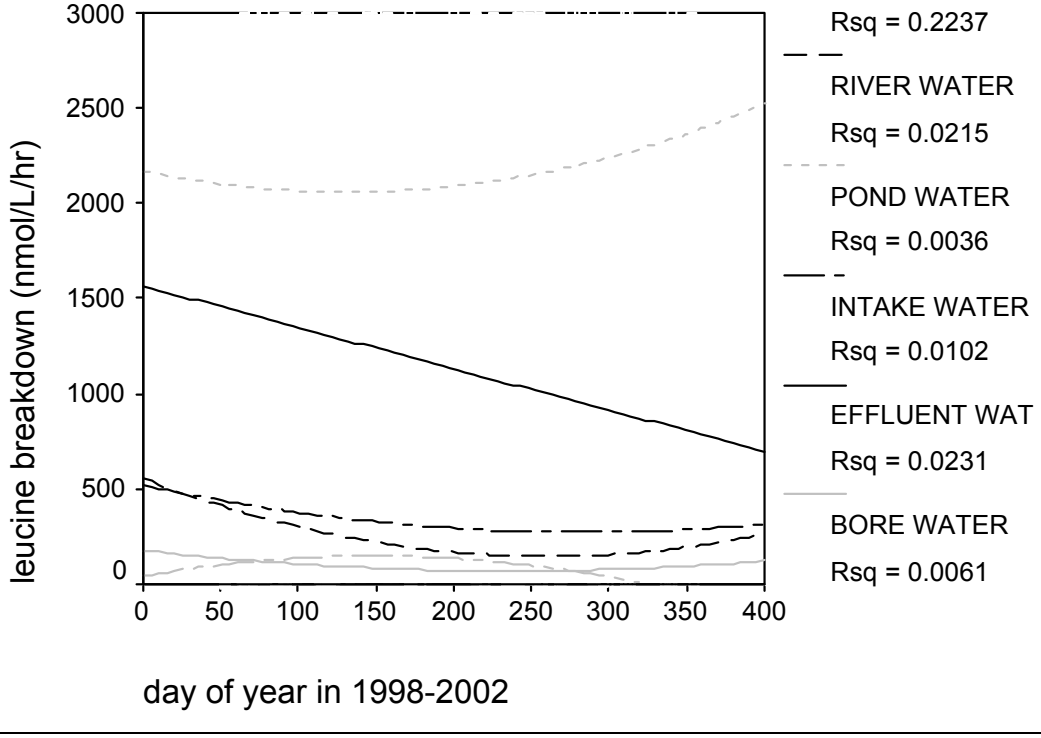
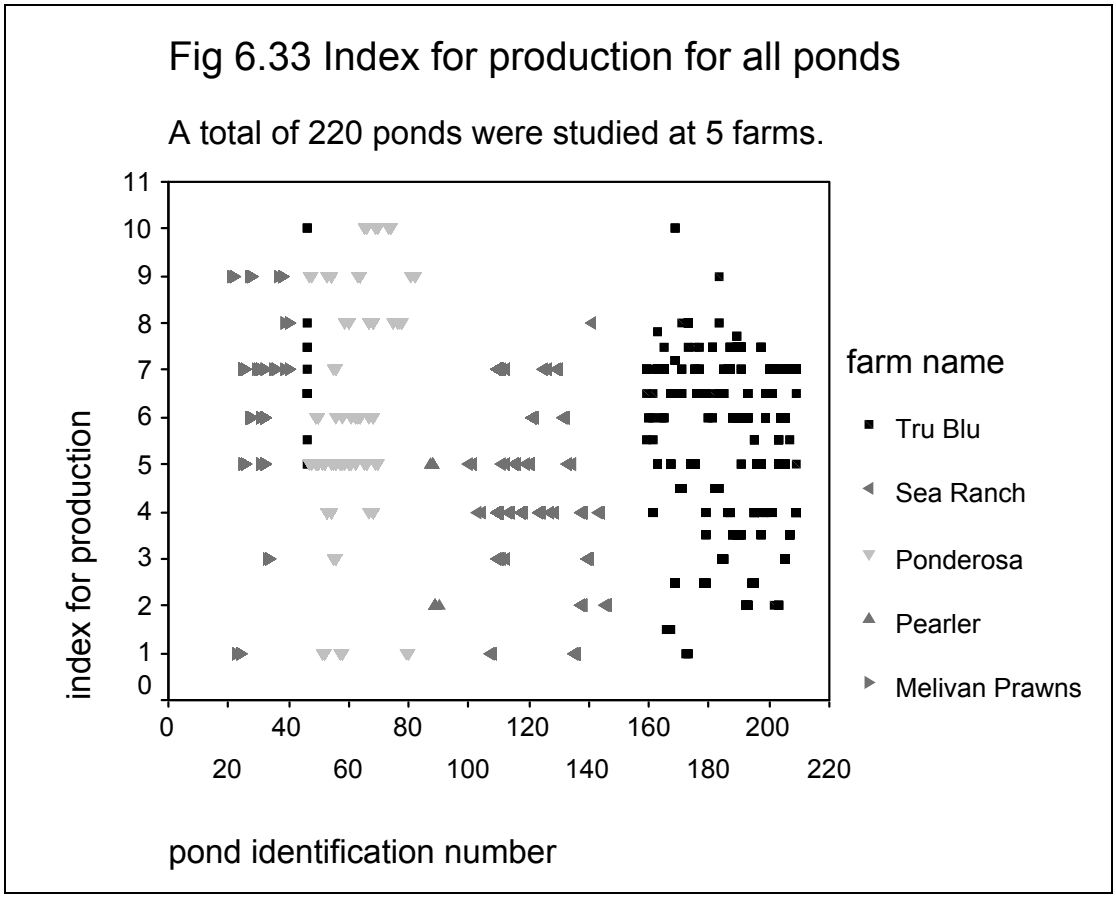


Fig 6.32 Effect of day of sampling on protease activity of water sources





b) Comparing microbial enzyme activities between ponds within farms

Fig 6.33 shows the productivity (ie Productivity Index) of all 220 ponds studied at the 5 farms during the project. The X-axis (Pond identification number) is the code for the ponds. From Fig 6.33 all ponds were classified into four groups according to the Productivity Index: low (1 to 3), below average (3.5 to 4.5), above average (5 to 6.5), and high (7 to 10). This index was used later for investigating associations, such as between extracellular enzyme activity and productivity (see later).

Supplement #11 contains the plots for four key substrates for extracellular enzyme activity: a-glucoside (glucosidease), acetate (esterase), leucine (protease) and n-acetyl glucosamininde (chitinase). The plots for pond sediment and water differentiate between productivity levels and farms. Careful examination of the plots reveals that in general the enzyme activities for each of the four representative substrates tend to reach a peak towards the end of the growing cycle. Importantly, the various farms had generally similar plots for enzyme activities, though there were exceptions to this observation. Also, the plots for each type of enzyme activity were similar for each pond productivity – for example, the plot for chitinase activity in water for a high productivity pond was similar to the plot for a below average productivity pond. This tends to indicate that microbial enzyme activity was independent of pond productivity, however there was considerable variation and scatter in the plots.

To test the null hypothesis that extracellular enzyme activity is independent of pond productivity, plots are included in Supplement #12. Once again there is considerable scatter and the plots show changes in activity over the age of the crop. However for

many substrates and the key bioindicators for chitinase and protease enzymes, there is an interesting trend. For those key indicators, there was an inverse relationship between productivity and extracellular enzyme activity. That is, high pond productivity was associated with lower extracellular enzyme activities for chitinases (N-acetyl glucosaminide, diacetyl chitobioside) and protease (leucine). This is a very important result.

c) Comparing microbial enzyme activity between sites within ponds

Supplement #10 shows the statistical results for an investigation of the differences in microbial enzyme activity for water and sediment sites within ponds at farms in FNQ. A total of 9 water samples were collected in duplicate from each of 2 ponds at three farms. Samples were from three locations (ie EDGE - 5m from the pond wall; MIDDLE – approximately 25m from the wall; CENTRE – centre of the pond or approximately 50m from the wall). At each location, water samples were collected from 3 layers in the water column: surface (T), mid-depth (M) and bottom (B). Sediment samples were collected in duplicate from the three locations described above: EDGE, MIDDLE and CENTRE.

Kruskal-Wallis analysis in Supplement #10 reveals that for water samples, there was no significant difference ($P>0.05$) between sites for most carbohydrate substrates (a-glucoside, b-glucoside, xyloside, l-fucoside, cellobioside) and inorganics (phosphate, sulfate). However, there was a significant difference between site for ester substrates (acetate, laurate, propionate, caprylate, butyrate), chitins (sulfoglucopyranoside, n-acetyl glucosaminide, diacetyl chitobioside) and proteins (leucine, guanidinaobenzoate). Examination of the mean rank activities for the substrates shows that the lowest activities are in the samples from EDGE T (ie edge of pond surface layer). However this site has a much higher number of samples than the rest and this tends to skew the results. Nevertheless, for most substrates there is a trend towards an increase in enzyme activity with depth of the water column ie activities progressively increase from top (T) to midwater (M) to bottom (B). There is also a slight increase in activity for most substrates with distance from the edge of the pond. To summarise, the findings for pond water indicate that stirring and aeration appears to be an effective tool for homogenizing and mixing the microbial communities. This management practice probably accounts for the apparent associations between site location and differences in extracellular enzymea activities.

For sediment samples, there was a significant difference between sites for all substrates except for oleate. Also, for all substrates, except for l-fucoside, the sediment in the centre of the pond had the highest extracellular enzyme activity. In general, the edge had the lowest activity for each substrate, though carbohydrate substrates where the exception (ie a-glucoside, b-glucoside, galactoside, l-fucoside). The findings for sediments are consistent with the long-held belief that it is important for successful farming to keep the bottom of the pond as “clean” as possible. Aerators are usually positioned so that organic wastes are swept away from the edge and transported to the centre of ponds. Farmers attempt to keep the edge as clean as possible so that feed can be broadcast to this area of the pond. The data from the statistical analysis reveals that the bacteriology of the edge of ponds has lower levels of extracellular enzyme activity, particularly for those enzymes our study identified as key bioindicators of pond health (proteases and chitinases).

6.1.4.3 Conclusions

1) Microbial enzyme activity in sediments is approximately 100x greater than in water. Indicating that sediments have a profound effect on the bacteriology of prawn farms.

2) Enzyme activities for farm water (ie effluent canals and ponds) are significantly higher than those of reference areas (ie nearby rivers and intake canals). The trends are not as clear with sediments.

3) Protease activity is 7x to 10x higher in pond waters than reference waters. The highest enzyme activity in reference areas is esterase, while in pond water the highest activity is protease.

4) There is a strong correlation between daily feed rate and protease activity (and other enzymes to a lesser degree). It was concluded that the high protein content of feed is a more important driver of pond bacteriology than temperature or geographic region.

5) Trends were found which suggest that there was an inverse relationship between pond productivity and extracellular enzyme activity for key enzymes, such as protease and chitinase. That is ponds with higher productivity had lower protease and chitinase activity.

6) Trends were found which suggest that within prawn ponds, enzyme activities for most substrates increases slightly with depth of water and distance from the edge of ponds.

6.2 Effluent and treatment systems

6.2.1 Introduction

The discharge of effluent from prawn farms is one of the most controversial areas for the industry. This report does not aim to discuss or analyse the "pros or cons" with respect to the impact of effluent discharge on the environment. Our starting point is simple: treatment of effluent and re-use of effluent is possible, but only when bacteria, including pathogenic types, are maintained at levels which are conducive to low risk prawn farming. Better management of bacteria will allow farmers to improve farm productivity, minimise discharges and reduce impacts on the environment. Successful prawn farmers are able to "farm" bacteria and they learn this by trial-and-error – for example, by adjusting feed rates and water exchange rates, monitoring DO and pH, assessing blooms and prawn activity. However strategies for treating effluent are not as well understood.

There have been some studies that have investigated prawn effluent and treatment systems in Australia and overseas. Smith (1995) characterised the effluent from Australian prawn farms in terms of a range of biophysical parameters, including *Vibrio* levels and bacterial load. Also, the settlement velocity of particles in effluent was measured and calculations were given for determining appropriate sizes of settlement ponds. Partially as a result of that work, 3 treatment systems were installed at farms in NSW. More recent work by CRC Aquaculture carried out comprehensive, studies on the composition, origin and treatment of prawn farm effluent. Also, that research developed models to describe pond dynamics. The main parameters that were measured by CRC Aquaculture were biophysical parameters, nutrient levels (ie nitrogen, phosphorus) and total suspended solids (TSS). The bacteriology of effluent and treatment systems apparently was not investigated.

Our goal was to develop an improved method for measuring the bacteriology of effluent and to use the method to assess different types of effluent treatment systems. Some of the findings were presented to the Australian Prawn Farmers Association in July 2000 (see Appendix 5) and further findings are summarised here. Full details of the findings will be presented to journals for publication and magazines for prawn farmers.

6.2.2 Materials and methods

Four types of effluent systems were monitored at three farms. Samples were collected from between 4 and 12 sites in each effluent system and extracellular enzyme profiles were recorded as well as flow rates, nutrient levels and biophysical parameters. The maps for the four treatment systems are given in Supplement #15.

6.2.3 Extracellular enzyme activities of effluent and treatment systems

The mean extracellular enzyme activities for the four effluent systems in April 2000 are shown in Supplement #15. The profiles were recorded towards the end of the 1999-2000 growout and they reveal some very important findings.

1) **Sugars**. The enzyme activities for sugar substrates (ie a-glucoside, b-glucoside, xyloside, galactoside, b-fucoside, glucuronic acid) have a general trend of decreasing activity from site 1 to site 4 at each farm, though this was not always so. That is, enzyme activities decreased as the effluent travelled throught the effluent discharge system. River levels had the lowest activities while ponds and td1.1 generally had the highest activites. A significant finding was the high level of glucuronidase activity in effluent system, td1. This substrate is used as an indicator of faecal contamination (ie *E.coli* bacteria.) so this finding was startling. Our work with isolated bacteria revealed that stains of *Vibrio* were capable of high rates of glucuronic acid breakdown (see Sections 6.3, 6.4).

2) **Esters & lipids.** The enzyme activities for esters and lipid substrates (ie acetate, propionate, butyrate, capryate, oleate, laurate) generally showed a slight reduction in activity as the water travelled through the effluent systems. As with sugar enzyme activities, the river had the lowest activities while ponds and td1.1 had the highest activities. Laurate (or dodeconoic acid) is a fatty acid which is an indicator of animal tissue. The highest enzyme activities for this substrate were in td1.

3) **Proteins.** The enzyme activities for protein substrates (ie leucine, guanidinaobenzoate) showed similar trends to those observed for sugars and esters.

4) **Chitins.** The enzyme activities for chitin substrates (ie sulfoglucopyranoside, n-acetyl glucosamininde, diacetyl chitobioside) had a remarkably consistent pattern. There was low to negligible activities for most sites in comparison to td1.1. The results for sites within td1 show that chitinase activity was high at the top of the system and decreased significantly as the effluent flowed towards the river.

5) **Phosphate and sulfate**. The enzyme activities for phosphate substrate were low for most sites, except for td1 and ponds. There was a significant reduction in phosphatase activity as effluent flowed through td1. Sulfatase activity was quite low at all sites (ie <40nmol/L/hr) and there was no significant trend.

The remarkably high levels of enzyme activity in the effluent system, td1, was investigated further and it was found that the cooking waste from boiling prawns was disposed of in the headwaters of the canal. This discovery explained the unusually high levels of enzyme activities for various substrates (glucuronic acid, laurate, chitins, leucine, phosphate). These are clearly key bioindicators of the bacteriology and quality of the effluent from prawn farms. Importantly, routine environmental monitoring of the discharge into the river has been carried out for many years and levels of BOD, nitrogen and phosphorus have not indicated that there was an environmental hazard. Hence the results from this enzyme study verify the sensitivity of the new method for measuring bacterial activity.

Supplement #16 shows results of a further study of the effluent system, td1, in April and May 2002. The plots compare the extracellular enzyme activities of cooking water (ie sampled directly from the cooker) and effluent water approximately 100 metres downstream from the point of discharge of cooking water into the effluent canal (ie td1.1). The plots in Supplement #16 show that for all substrates, the enzyme activity is significantly higher in the effluent canal than the cooking water. This is consistent with the explanation that cooking water is relatively sterile and has very

low levels of microbial activity. However, cooking water contains nutrients, organic matter and particles, which are sources of food for microbes. Hence once the cooking water enters the canal, it causes growth of bacteria and increases in the enzyme profile as shown. High levels of enzyme activity were recorded in the effluent canal during the harvest period for the key bioindicators identified in Supplements #15 and #16 (ie glucuronic acid, laurate, chitins, leucine, phosphate).

Supplement #15 shows that the effluent systems had a capacity to reduce the levels of microbial enzyme activities. This was most noticeable in td1 which had high activities in the headwater. While some of the reduced activities may be explained by dilution effects, a careful study of 12 sites along the effluent system, td1, in 2001 and 2002 showed that there was a gradual reduction in extracellular enzyme profiles that was independent of dilution effects. This indicates that the effluent systems were relatively effective in improving the bacteriology of the effluent prior to its discharge.

As to the effectiveness of the effluent systems, it appeared that the settlement ponds were less effective than narrow long canals at reducing extracellular enzyme activity. Settlement ponds developed stratified and stagnant conditions, while narrow, tortuous canals had faster flow, greater input of dissolved oxygen and greater contact with sediments. Since it is generally accepted that high levels of oxygen are required to efficiently decompose organic matter, it follows that an effective effluent treatment system requires a high oxygen concentration. It is also likely that because sediment has 100x greater bacterial activity than water. Therefore the findings of the study support the conclusion that effluent is more efficiently treated in a shallow, narrow canal than in a broad body of water. This finding is contrary to the general practise of treating effluent in large settlement ponds.

6.2.4 Conclusions

The investigation of the effluent systems provided information about the bacteriology that was more advanced than previous research. The main conclusions were as follows.

1) Disposal of cooking waste has important implications for the industry. From the results in Supplements #15 and #16 it was concluded that the disposal of prawn cooking wastes into waters near the farm encourages the development of bacteria that feed on prawn shell and tissue (ie pathogenic bacteria). This may have serious effects on the health of prawns and other marine organisms within the farm as well as nearby environment. It is recommended that farms disposing of cooking waste on-site, should have a simple sullage pit to digest the wastes before they seep into the effluent system.

2) Comparisons between enzyme levels in ponds, sites along the effluent systems and the discharge sites, revealed that the four effluent systems were generally capable of reducing the enzyme activities of most substrates, particularly the key bioindicator substrates for proteases and chitinases. That is, the lowest levels of enzyme activity in each system were usually at the final stages of the system.

3) The effluent systems were not capable of treating effluent to the extent that its bacteriology was comparable to that of river water. Since none of the farms in this

project re-used their effluent, it was not possible to determine whether the treated effluent was satisfactory for recycling.

4) Improvements in the design of effluent treatment systems can be developed from this baseline information. For instance, it is recommended that settlement ponds be small enough to simply reduce suspended solids according the equations of Smith (1995). A long tortuous, narrow canal system provided better treatment of effluent than a single large body of water, such as a settlement pond. We could speculated about ways of improving the efficiency of treatment systems, however further research is needed using the tools that we have developed.

6.3 Experiments on isolated bacteria, mesocosms and pond trials

The project undertook a major study of factors that affect the extracellular enzyme profile of isolated bacteria, probiotic bacteria and mesocosms. Also, in order to apply the knowledge that had been gained, a final set of experiments was carried out at one prawn farm in Northern NSW. The findings are presented in this section.

6.3.1 Extracellular enzymes of isolated bacteria

Bacteria were cultured from freshly collected samples of water, sediment, prawn tissue and other materials. Bacteria were isolated on marine agar and TCBS agar by traditional microbiological techniques, such as spread-plating, then purified and cleaned up by streak-plating. Fig 6.3.1.1 shows examples of the isolated bacteria as viewed with the Scanning Electron Microscope.

Extracellular enzyme profiles of isolated bacteria were measured after the initial isolation. The bacteria were then subcultured and stored. In one type of experiment isolates were fed a range of food sources, including peptone broth and emulsified prawn feeds. Extracellular enzyme profiles were re-measured 24hrs after incubation. (In order to maintain equivalent bacterial densities, samples were diluted with sterile saline prior to measurement.) Supplement #13 shows a typical set of results for 24 strains of *Vibrio* bacteria that were isolated from prawns suffering from shell vibriosis (tail rot) and septicemia. Similar results were obtained from other sets of bacterial isolates – the food test was replicated on 4 occasions, with variations in the types of foods. Peptone was often included as a reference material in these tests because it is a commonly used general food additive for culturing bacterial. (Peptone is prepared by partially digesting it with proteolytic enzymes, such as pepsin.)

The results in Supplement #13 are summarized as follows.

a) **Without supplementary food** (ie Nil food) all *Vibrio* isolates had negligible enzymes for sugar breakdown (ie a glucoside, b glucoside, xyloside, glactoside, fucoside, glucuronic acid). The main types of extracellular enzyme activity were for esterases (butyrate, caprylate, propionate, acetate, laurate). Protease activity (ie leucine) was significant in some isolates (#9, 10, 15, 22, 24). Chitinase activity (ie n-acetyl glucosaminide, di-acetyl chitobioside, glucopyranoside) was negligible for all isolates. Phosphatase activity was low but significant for most isolates.
b) **Peptone as a food source** encouraged increased production of extracellular enzyme activity in all *Vibrio* isolates. In general, isolates produced low or negligible enzymes for breaking down sugar substrates – a-glucoside and b-glucoside were exceptions. Esterase activity was highest for acetate followed by propionate and butyrate. Importantly, protease activity (ie leucine) was now very significant for most isolates, in fact for some isolates it was now the dominant enzyme activity. Also, chitinase activity (ie n-acetyl glucosaminide, di-acetyl chitobioside, glucopyranoside) was now dominant for most isolates. Phosphatase activity was substantially higher with peptone as a food source.
c) **Star Brand monodon prawn feed** produced enzyme profiles that were similar to the "Nil food" enzyme profile apart from protease activity. For 7 of the isolates, protease activity (ie leucine) was very significant and protease

activity was the highest extracellular enzyme activity for at least 5 isolates (see isolates 4, 9, 10, 11, 15, 22 23).

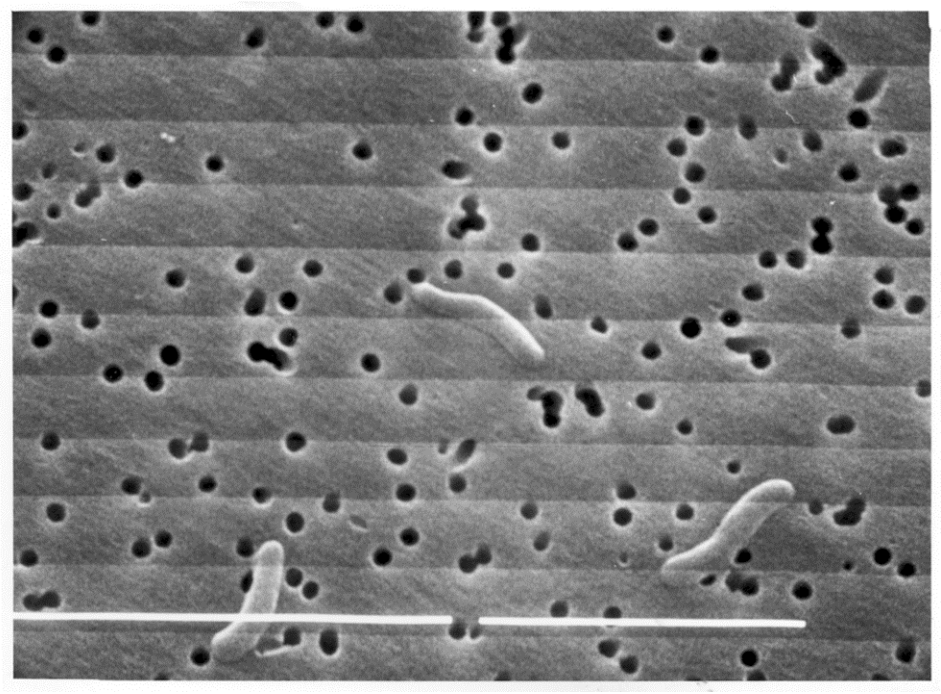
d) **Higarshimaru japonicus feed** produced enzyme profiles that were similar to those for Star Brand monodon prawn feed. The same *Vibrio* isolates (ie isolates 4, 9, 10, 11, 15, 22 23) had significant protease activity (ie leucine substrate).

Conclusions: As a result of food experiments with bacterial isolates from diseased prawns, it was concluded that *Vibrio* bacteria have an extracellular enzyme profile that is very responsive to the type of food that is available. In other words, enzyme activity is inducible. For example, general purpose food supplements, such as peptone, encourage a broad range of exracellular enzymes. On the other hand, the major impact of prawn food is to encourage production of protease enzymes in a significant proportion of *Vibrio* isolates. In fact, prawn feed induced many pathogenic bacteria to modify their profile so that protease activity was the dominant class of extracellular enzymes. Interestingly, most pathogenic vibrios did not have a wide range of enzymes for sugars, though they were induced to have high chitinase actvity. The encouragement of high protease activity and high chitinase activity is a potentially lethal mixture for prawn culture. This issue is developed further in the remainder of Section 6.3 and Section 6.4.

Fig 6.3.1.1 Views of pure cultures of bacteria using Scanning Electron Microscopy

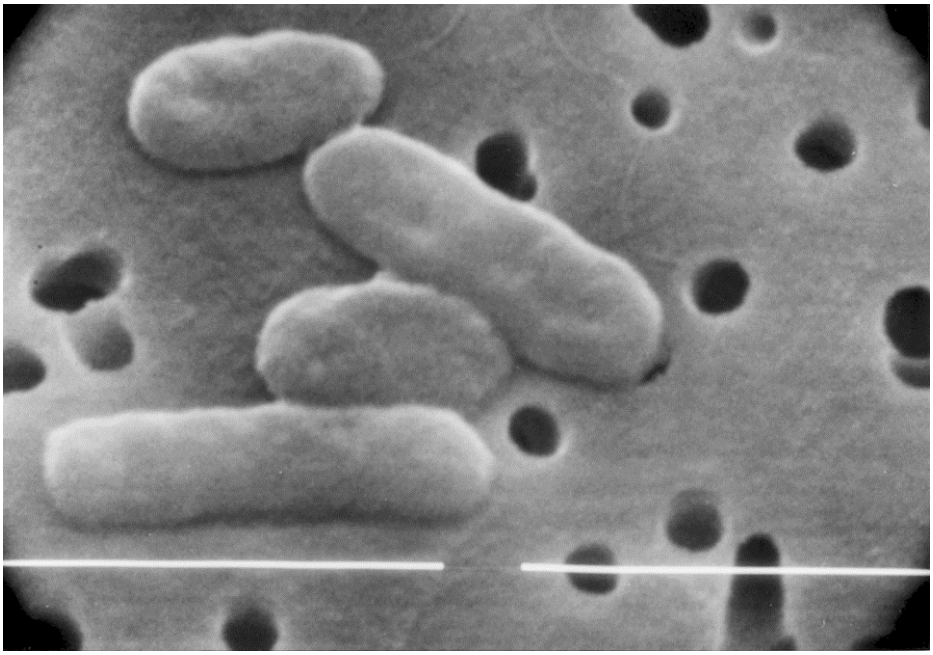
***a)Vibrio* bacteria often have a curved shape (ie vibroid) as seen here, however some are straight rods.**

The round holes are 0.3µm diameter and they are properties of the filter paper (SEM x3,000).



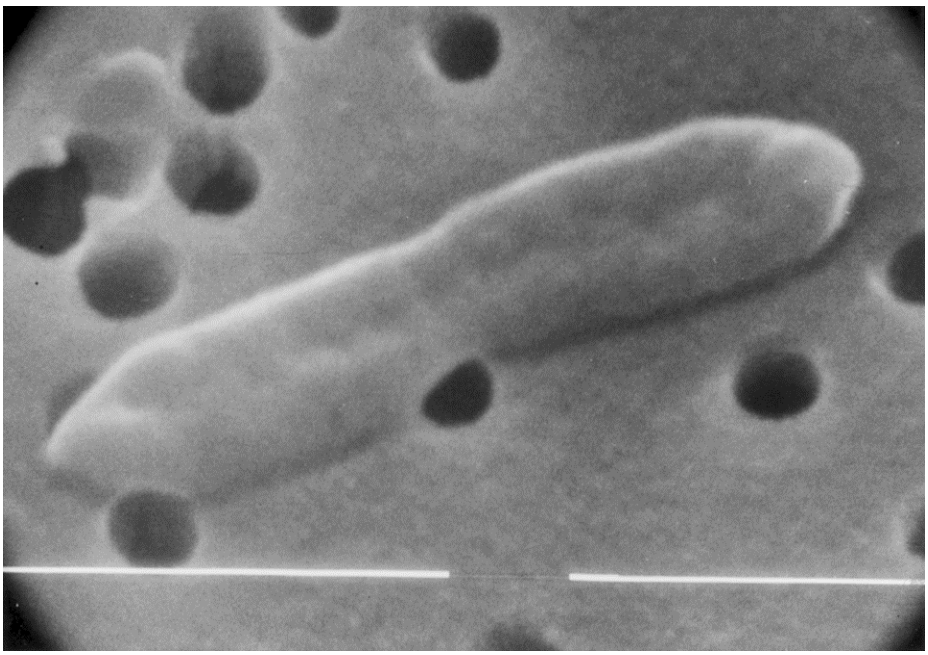
b) Bacteria in a pure culture.

These bacteria are 0.6µm wide and up to 3µm long. Flagella can be seen at the top of the photomicrograph - often flagella are lost in the preparation of bacteria for SEM. The round holes are 0.3µm diameter and they are properties of the filter paper (x17,000).



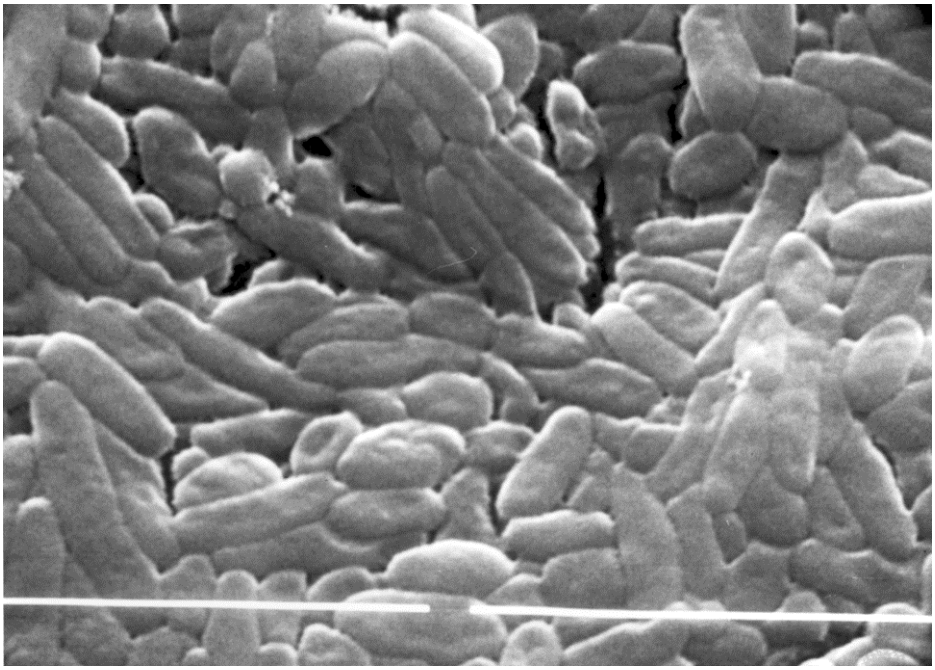
c) *Vibrio* bacterium in the process of cell division (binary fission).

The holes are 0.3µm and they are properties of the filter paper (SEM x26,000).



***d) Vibrio* bacteria in a pure culture have a variety of sizes and shapes depending on their stage in the growth cycle.**

The round holes are 0.3μm and are properties of the filter paper (SEM x8,000).



6.3.2 Study of extracellular enzymes in mesocosms

6.3.2.1 Introduction

Current practices in prawn farming rely on feeds that have a protein content of approximately 30-50% dry weight. Prawn wastes and uneaten feed cause organic enrichment and elevated levels of nitrogen and phosphorus in the water column and sediment. Our study found that these current practices tend to cause blooms of bacteria that have high levels of extracellular protease enzymes and we concluded that the level of protease activity is a key bioindicator of the health of a pond.

New research carried out in this project, as well as work overseas (eg Chamberlain et al 2001: "Advantages of aerated microbial reuse systems with balanced C:N". Advocate 4(3): 22-24), suggests that there may be a better way of providing protein and essential organic compounds to prawns. In aquatic environments, organic material is colonised by bacterial biofilms that: a) decompose organic matter by secreting extracellular enzymes, b) absorb the breakdown products and c) multiply until the food has been completely digested. Although the original organic matter may be high in carbohydrates and low in proteins, bacteria convert the carbohydrates to proteins and essential organic compounds (ie vitamins), making it a more suitable food source for marine organisms, such as prawns. That is, carbohydrates with a high carbon to nitrogen ratio (i.e. poor food value for prawns), can be readily digested by bacteria and converted into an enriched food source. It is highly likely that the bacteria use dissolved compounds (eg ammonium ions and phosphates) as substrates for making essential fatty acids and proteins. The flocs of bacteria and detritus, which have improved levels of organic compounds, can be eaten by prawns as they browse.

In this part of the project, experiments were performed which had two aims. Firstly, various low value food sources were tested in order to find a type of supplement that would be broken down by bacteria and provide a high value food to prawns. Secondly, culture methods and other management practices were investigated in order to find ways of reducing levels of protease activity and producing a better extracellular enzyme profile for the culture of prawns. The results of this study may be used by farmers to reduce feed costs, improve feed conversion efficiencies and reduce environmental impacts.

6.3.2.2 Materials and methods

The food and management experiments were carried out in 20L aquarium tanks (ie meoscosms). Each test was carried out in duplicate in the first set of experiments (run from June to August 2001) and in triplicate in the second set (from September to December 2001). Tanks were located in a laboratory with constant temperature and each tank had 1L of conditioned marine sediment and an airstone to aerate the water.

A test was carried out to determine the effect of the filtration system on bacteriology: tanks either had a biofilter, undergravel filters or no filter (ie control). Tanks were normally stocked with 5 juvenile black tiger prawns and experiments were run over a period of 8-10 weeks. The prawns were weighed at the start of each experiment and at

the conclusion - in the first experiments prawns were approximately 0.5g each, while in the second set, the prawns were approximately 3g.

In the study of food sources, a total of five types of foods were supplemented to the normal diet of commercial pellet feed. These were chitin, cornflour, wheat germ, molasses, and mangrove leaves. Chitin, cornflour and wheat germ are complex carbohydrates which are metabolised slowly and provide surfaces for bacterial attachment. Molasses is a soluble type of carbohydrate and it should be broken down by bacteria at a faster rate than complex carbohydrates. Mangrove leaves were used as a naturally occurring reference material. It breaks down relatively slowly and provides good structural support for the attachment of bacteria.

The bacteriology of test tanks was determined by monitoring extracellular enzyme activity within the water and soil. *Vibrio* levels were also measured with agar plates. Tank conditions were routinely measured, including dissolved oxygen, pH, temperature, as well as dissolved and total nitrogen and phosphorus.

6.3.2.3 Results

The results for the first set of experiments are shown in Supplement #14. The second set of experiments produced similar results and together they will be presented to a scientific journal. Table 6.3.2.3.1 shows the average growth rates and survival rates for prawns in the first set of experiments. As a general comment, small prawns were used so that the conditions in the tanks were not stressful. All tanks had high survivals and reasonable growth except for the test in which prawns were fed a supplement of dead prawns. This had 0% survival.

Table 6.3.2.3.1 Results for survival and growth rates of prawns used in the first set of experiments.

Test	*Test conditions*	*Survival (%)*	*Average growth rate (%)*
Filtration system	Undergravel filter + pellet feed (control)	92	237
	Bioballs + pellet feed	88	250
	Airstone + pellet feed	90	255
Food	Wheat germ	80	243
	Corn flour	80	477
	Molasses	100	239
	Mangrove leaves	100	249
	Extra pellet feed	100	550
	Dead prawn	0	n.a.
Addition of Vibrio broth	Green Vibrio	100	319
	Black Vibrio	60	202
	Yellow Vibrio	80	110
	White Vibrio	100	281

a) Water quality parameters. Observations and measurements of tank conditions are partly summarised in the Table in Supplement #14 and Table 6.3.2.3.1. Measurements of the levels of presumptive *Vibrio* bacteria were generally low

(<500cfu/mL), except for consistently high levels in the tanks that were fed dead prawns (1,000-20,000 cfu/mL). Total nitrogen and dissolved ammonia levels were generally low throughout the experiment (<1000 μg/mL), except tanks in which only air stones were used had significantly higher levels. Dissolved phoshorus and total phosphorus increased steadily in all tanks from approximately 300 to 3500 μg/mL.

b) **Filtration**. In Supplement #14 a Table summarises the observations and pond conditions during the experiment and graphs of results for various representative tanks are given. Firstly, the extracellular enzyme profiles for three types of filtration systems are presented. Tank 12 used no filtration - only an airstone to aerate the tank. This was a control because it was analogous to farm situations. Bioballs were used in tank 20 and an undergravel filter was used in tank 14. The extracellular enzyme profiles for most substrates were significantly higher for the control tank than for the tests with bioballs and the undergravel filter. Leucine, the substrate for protease activity, is the key bioindicator for water quality in prawn farming. In the control tank protease activity was regularly around 1500 nmol/L/hr, while for bioballs it was 200-500 nmol/L/hr and for the undergravel filter it was 100-200 nmol/L/hr. Similar findings were obtained for the duplicate tank and the replicate experiment. Consequently, all of the food tests and other experiments were carried out with tanks equipped with an undergravel filtration system.

c) **Extreme conditions**. Two types of tests were carried out in order to measure the extracellular enzymes of tanks under extreme conditions. Firstly prawns received a supplement of dead prawns (eg tank 10) and it is clear that in those tanks most extracellular enzyme profiles were very high. Esterase activities (ie acetate, propionate, butyrate) were 1000-5000 nmol/L.hr, while protease activities (ie leucine, guanidinaobenzoate) were 250-1000 nmol/L/hr. There was 100% mortality in this test. In the second type of test, extra commercial pellet was given (tank 9). The enzyme profiles were elevated, but not as high as with added dead prawns.

d) **Food supplements**. The comparative effects on the extracellular enzyme profile are shown for a range of food supplements, including mangrove leaves (tank 4), wheat germ (tank 5), corn flour (tank 6), and molasses (tank 7). In all cases, the enzyme activities were relatively low for most substrates. Towards the second half of the time course, acetatase activity climbed to levels of approximately 600-800 nmol/L/hr for all food supplements. Protease activity (ie leucine, guanidine benzoate) remained low (50-200 nmol/L/hr) throughout for each test.

e) **Protease activity**. Factors which affected protease activity (ie leucine breakdown) were considered to be the most important outcome of these experiments. Results for the duplicates for each type of test are presented graphically as follows. Firstly, the effect of water filtration on protease activity is shown. The lowest activity was obtained for undergravel filtration, followed by bioball and then airstone. Secondly, the effect of feeding dead prawns is shown to cause significantly higher levels of protease activity than the control. Thirdly, various types of food supplements are shown and protease activity was consistently low, except for one of the tanks that received extra pellet feed. Finally, the effect of regularly adding *Vibrio* cultures, in the form of 2mL of broth, to each tank is shown. Measurements of protease activity were taken 3 to 4 days after the *Vibrio* broth was added. On occasions protease activity rose

to high levels (200-1200 nmol/L/hr) while for the controls (ie undergravel filter) the levels were <200 nmol/L/hr.

f) **Probiotics**. One commercial brand of probiotics was tested in the second set of tank experiments in the manner recommended by the supplier. It had an extracellular enzyme profile that was not greatly different to the *Vibrio* sp. that were isolated by ourselves. Included in Supplement #14 is one typical example of the effect of the probiotic on the extracellular enzyme profile of a tank. It shows the time course from t=0 to 24 hrs. Protease activity (ie leucine substrate) was the dominant enzyme, increasing from 500 to 2,800 nmol/L/hr following administering of the probiotic. Chitinase activity (ie glucopyranoside substrate) and esterase activity (ie acetate substrate) were also elevated by the addition of probiotic. Within 24 hrs the levels of enzyme activity had returned to pre-test levels for all enzymes. This type of result was consistent with those obtained for 15 such tests with the probiotics as well as supplements of *Vibrio* broths.

6.3.2.4 Conclusions from tank tests

1) The tanks tests demonstrated that these types of mesocosms were very useful tools for investigating prawn bacteriology. The technique of measuring extracellular enzyme activity provided significantly better information on the bacterial status of the mesocosm than traditionally measured parameters, such as TCBS agar counts. Extracellular protease activity (as measured with leucine substrate) was a clear bioindicator of the bacteriology of a tank.

2) The effect on tank bacteriology of adding food supplements was accurately monitored. Low value foods, with a high carbon to nitrogen ratio, had no deleterious effects and did not increase protease activity in tanks. In fact protease activity was at a very low level for those treatments. On the other hand, adding dead prawns as a supplement resulted in high mortality and very high levels of protease activity, as well as high levels for other enzymes. Overfeeding with commercial pellet resulted in the highest growth rate, but by the end of the experiment the protease activity had usually risen to high levels.

3) Adding a commercial brand of probiotic and various *Vibrio* broths caused temporary increases, lasting approximately 24 hrs, in extracellular enzyme activity, including protease activity. Hence, the probiotic did not appear to improve the extracellular enzyme profiles, though other parameters of water quality were relatively good and no mortalities occurred. Similar results for water quality were obtained for the addition of *Vibrio* broths.

4) The type of filtration system that was used had a significant effect on tank bacteriology. A treatment system based on conditioned marine sediment and use of an aerator (ie airstone), gave the worst results. That system is comparable to earthen pond aquaculture. In comparison, undergravel filtration gave the best result, followed by bioball filtration. This finding has significant implications for prawn farming and further research work should be carried out so that the concepts can be extended to pond aquaculture.

6.3.3 The effect of supplements of rice pollard on bacteriology of prawn ponds

6.3.3.1 Introduction

The findings from the tank tests (mesocosms) were highly significant and indicated that trials should be carried out in prawn ponds under farming conditions. Tru Blu Prawn Farm on the Clarence River was interested in collaborating with trials on food supplements, so for the 2001-2002 crop a series of experiments were carried out using the cheapest available low-value food – rice pollard.

6.3.3.2 Materials and methods

In the first trial pollard was distributed on the pond surface, but because of the high oxygen demand of raw organic matter, fermentation of the pollard occurred in the ponds. This problem had been encountered earlier in mesocosm tests, but because of the greater rate of aeration and stirring that occurred in our tank tests, it was overcome. In the pond situation, the best solution was for the pollard to be fermented in an aerated vat for approximately 7 days prior to distribution. Fig 6.3.3.2.1 is a photograph of an aerated vat of rice pollard.

From March to May 2002, testing of rice pollard was organized by the author with the assistance of Richard von Fister of Tru Blu. Some 5 ponds received fermented pollard and 5 other ponds were used as controls. All ponds received normal daily rations of commercial pellet feed. Four water samples were collected from each pond at various times after the addition of pollard and analysed for extracellular enzyme activity.

Fig 6.3.3.2.1 Rice Pollard was tested by Tru Blu Prawn Farm as a food supplement in 2001-2002. It was necessary to brew rice pollard in a vat prior to adding to ponds in order to prevent fermentation processes occurring in ponds.

6.3.3.3 Results and discussion of the effects of rice pollard

Partially fermented rice pollard was used by Tru Blu prawn farm from November to January of the 2001-2002 crop. According to the farmer and workers, pollard was usually applied on a weekly basis to all ponds during the first few months of the crop. The use of rice pollard reduced the level of cyanobacterial blooms. Also, water quality appeared to be very good and blooms were cleaner and fresher. The farmer did not set up any control ponds so statistical analysis was not possible. Addition of rice pollard to ponds was stopped once it was judged by the farmer that the prawns were large enough to feed on commercial pellet feed.

Tests carried out from March to May 2002, provided very interesting results. Firstly, the enzyme profiles of brewed pollard was compared to pond water in Figs 6.3.3.2.2 to 6.3.3.2.5. For sugars, pollard had significantly stronger levels of enzyme activity for most sugars (ie a-glucoside, b-glucoside, xyloside, galactoside, glucuronic acid, cellobioside), particularly b-glucoside. Pollard had a higher profile for only one chitinase (ie n-acetyl glucosaminide) but not for the others (diacetyl chitobioside and sulfoglucopyranoside). The findings for protease activity were also mixed, with leucine breakdown higher for pollard than for pond water, but not for guanidinobenzoate. There were no major differences between pollard and pond water for esterases (ie acetate, propionate, caprylate, butyrate). These findings were repeated in three tests and the findings were reproducible. It was concluded that brewing and fermenting resulted in the growth of bacteria that produced carbohydrases, some chitinases and proteases, but low levels of esterases. It is reasonable to suggest that during the brewing process, then main type of enzyme activity is the breakdown of complex carbohydrates into polymers and small chains of sugars. Hence, it is hypothesized that the outcome of pollard fermentation is the growth of bacterial colonies that have an enzyme profile that is relatively high in sugar and carbohydrate activity but lower in chitinase, protease and esterase activity. Since protease and chitinase activity is one of the key bioindicators for prawn culture, this result was very encouraging. It was hypothesized that the addition of brewed rice pollard would encourage the development of a beneficial bacterial community in prawn ponds.

In the experiment that followed, brewed pollard was added to 5 ponds at the rate of 50 to 100 L per pond. The extracellular enzyme profiles for the test ponds were measured prior to addition then 2, 24 and 72 hours after addition. The results for one test are shown in Fig 6.3.3.2.6 for sugars and chitin substrates and Fig 6.3.3.2.7 for proteins, esters, phosphates and sulfates (note that in those Figs the legend of Series 1, 2, 3, 4 refer to time =0, 2, 24, 72 hours). The results in Figs 6.3.3.2.6 & 6.3.3.2.7 are inconclusive - the changes are not significant. In repeats of the test, the changes in enzyme profiles were also inconclusive. It is possible that greater volumes of brewed rice pollard are required before significant changes in extracellular enzyme profiles in pond water can be measured.

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Fig 6.3.3.2.2 Extracellular enzyme activity for sugar substrates

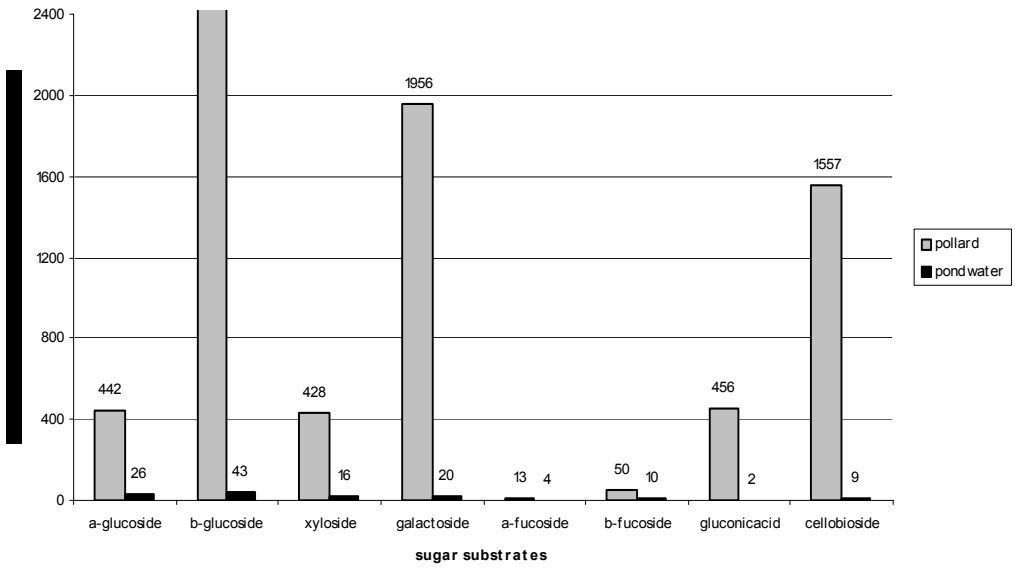


Fig 6.3.3.2.3 Extracellular enzyme activity for chitinases and proteases

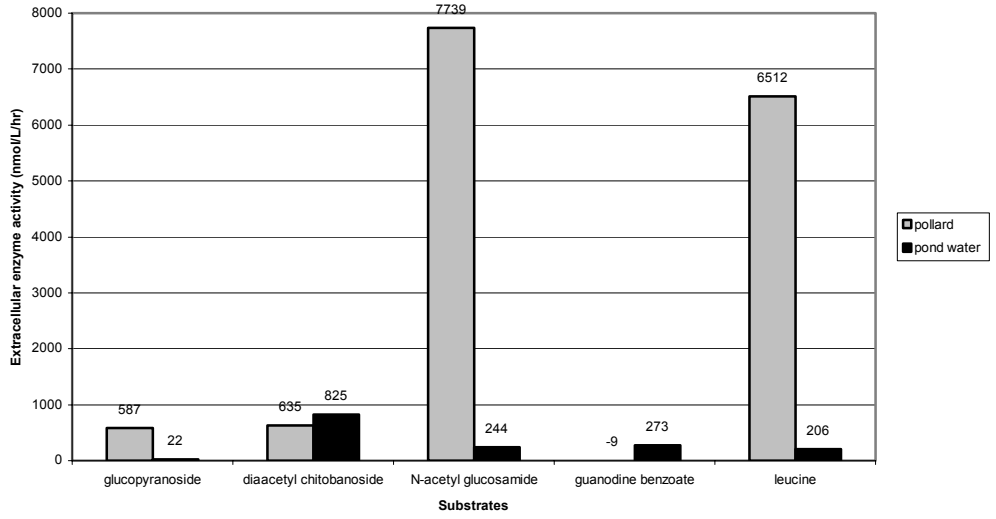


Fig 6.3.3.2.4 Enzyme activity for esterases

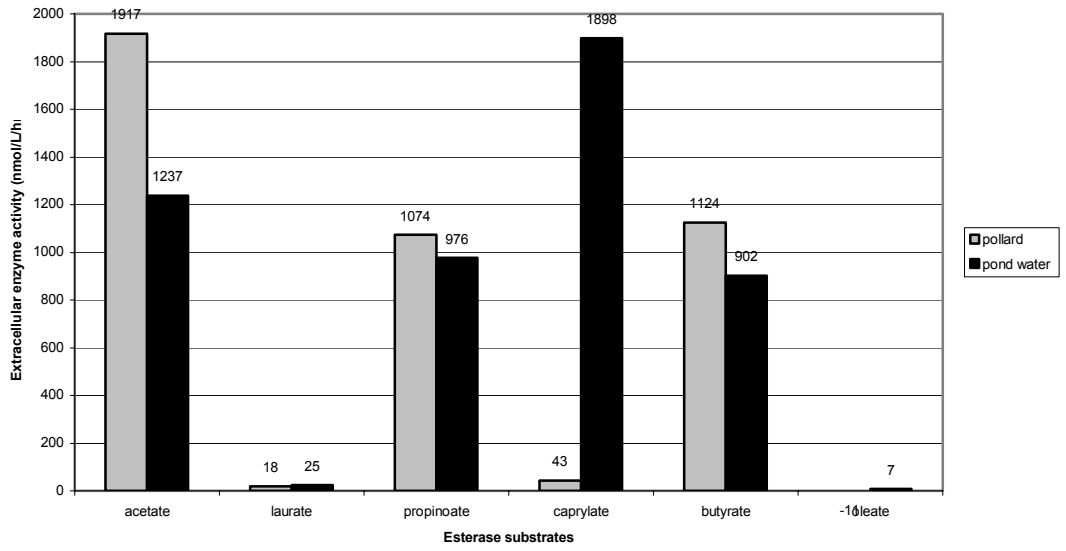


Fig 6.3.3.2.5 Extracellular enzyme activity of P and S

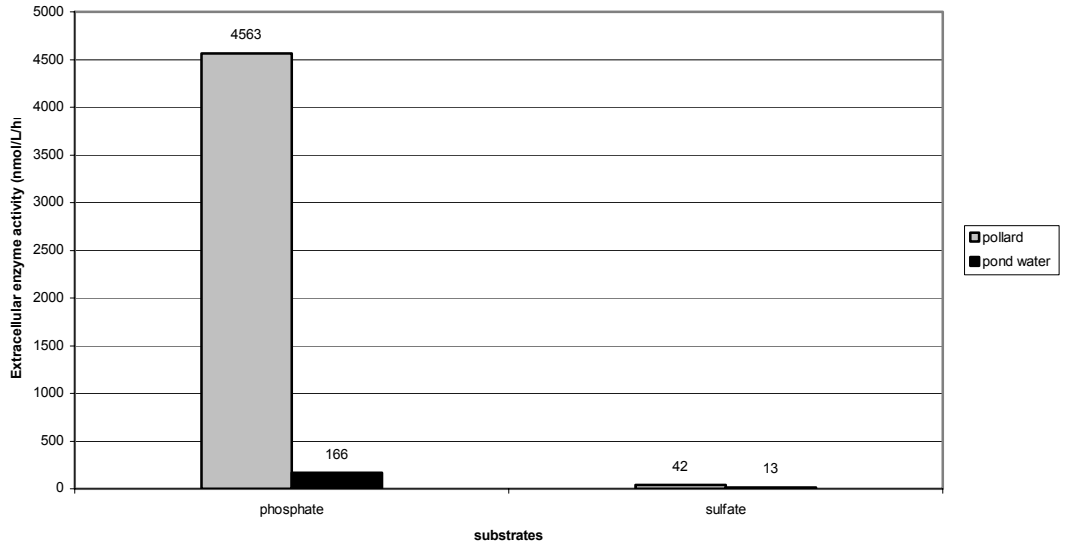


Fig 6.3.3.2.6 Effect of pollard on enzyme activities for sugars and chitin

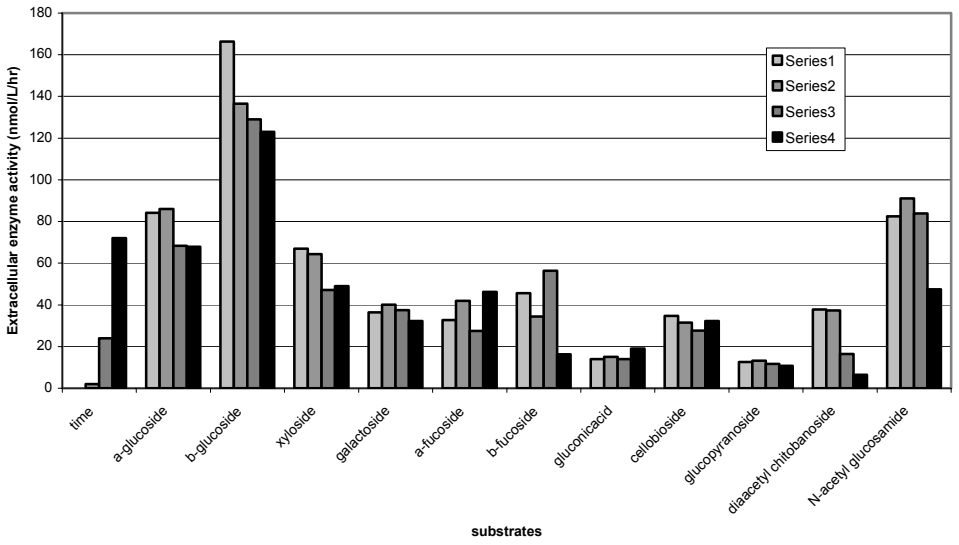
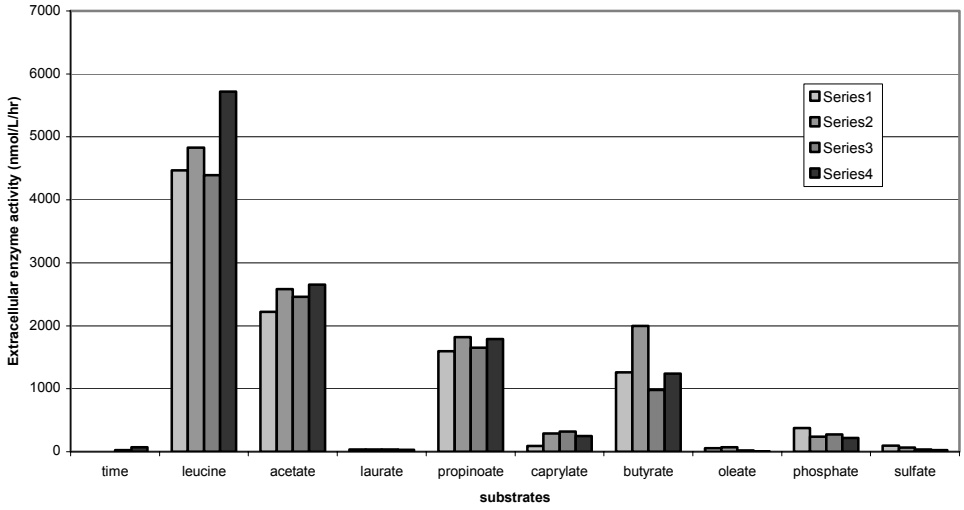


Fig 6.3.3.2.7 Effect of pollard on enzyme activities for proteases, esterases and organophosphates and sulfatases



Note: In Fig 6.3.3.2.6 and Fig 6.3.3.2.7 (above) the legends should be interpreted as: Series 1 is t=0; Series 2 is t=2hr; Series 3 is t=24hr, Series 4 is t=72hr.

6.3.4 Conclusions

The use of extracellular enzyme techniques with isolated cultures of bacteria, tank mesocosms and pond trials provided a new way of investigating the bacteriology of aquaculture systems. The technique enabled the bacteriology to be examined with higher resolution and at a more complex level than traditional methods. The key bioindicator for studying bacteriology in these systems was protease activity as determined by the substrate leucine, and to a lesser extent guanidinobenzoate.

The work shows that food supplements, with low food value and high carbon to nitrogen ratios, may be a very useful complement to commercial pellet feed. These foods reduced the level of protease activity in tank tests. It was hypothesised that prawns benefit from ingesting partially fermented, low-value food supplements (such as rice pollard), because in the fermentation process the raw organic material is digested by bacterial extracellular enzymes, absorbed and converted to higher value food. Preliminary results from a farm trial suggests that rice pollard minimises cyanobacterial blooms, improves water quality and may be a benefit to prawn growth. However, changes in the extracellular enzyme profiles when brewed pollard was added to ponds were inconclusive. Further trials with appropriate controls, larger quantities of supplement and a range of supplements are needed to rigorously test the benefits to pond productivity, FCR, survival and extracellular enzyme activity. (A similar situation exists for the use of probiotics.).

6.4 Investigations of prawn pathogens and disease

6.4.1 Cyanobacteria

During the course of the project, investigations into cyanobacteria were carried out at farms in FNQ and NSW in order to determine the frequency of blooms, the types of cyanobacteria, their extracellular enzyme profiles, links with prawn mortalities and test methods for reducing their occurrence.

6.4.1.1 Background

Until the 1960s cyanobacteria were called blue-green algae and generally classified as a primitive group of algae. Since then, the classification and taxonomy of cyanobacteria has been completely revised and every day the use of PCR and molecular genetic techniques is shedding new light on these primitive organisms. Nevertheless, it is accepted that cyanobacteria are actually photosynthetic bacteria rather than algae and they play important roles in many ecosystems – producing oxygen, fixing nitrogen and producing organic matter. Cyanobacteria can also cause problems by forming dense scums and blooms that produce noxious odours and lower the dissolved oxygen concentration. Also, some species produce poisonous (toxic) chemicals, mainly liver toxins (hepatotoxins) and nerve toxins (neurotoxins) that can kill fish, birds and mammals.

There are fundamentally three ways in which cyanobacteria occur in prawn ponds: as benthic blooms which cover the sediment, as planktonic forms that freely circulate in the water column, and as surface scums. The most commonly detected form of cyanobacteria in prawn farms in all of these appearances is *Oscillatoria*, however numerous other types have also been found.

a) **Benthic blooms of cyanobacteria** usually develop relatively slowly, in comparison to microalgal blooms, however once they occur they can be more persistent and worrisome. In the early stages, their growth may go unnoticed because the water colour appears to be quite strong, though appearing to darken each day. Secchi visibility gradually lengthens to 70cm or more, though the water colour mistakenly may suggest that there is still a vigorous microalgal bloom. In fact, the microalgal bloom is dieing off and the water colour is really due to the reflection of colour from the benthic cyanobacterial mat on the bottom of the pond. The next stage occurs several weeks after the benthic bloom started to develop. It usually occurs on a sunny day when oxygen bubbles become trapped in the thick benthic mat and they lift patches of benthos from the bottom.

b) **Planktonic blooms of cyanobacteria** are dominated by, in descending order of frequency, *Oscillatoria*, *Nodularia*, *Anabaenopsis* and *Microcystis*. *Oscillatoria* can be distinguished from the other species by collecting a sample of pond water in a china cup and letting is stand for a few minute or so. Filaments of *Oscillatoria* will collect as a mass of fine hairs (trichomes) at the bottom of the cup. The other species tend to settle as a surface scum – often leaving a green paint-like ring inside the cup. Microscopic examination will reveal the type of cyanobacteria.

c) **Surface scums of cyanobacteria** occur when either benthic mats or planktonic species collect on the surface and are blown to the windward areas of the ponds. The scums usually rot and cause deterioration of the sediment – the blackness of the sediment is caused by the monosulfide of the reduced sediment. Frequently the rotting scum produces noxious odours, and a pigmented ooze with colours ranging from reddish-purple (*Oscillatoria*) to blue (*Microcystis*).

Information on: Microcystins and Nodularin.

There are approximately 65 different types of Microcystins. All Microcystins are small proteins (polypeptides) having 7 amino acids and their structure is either cyclic or ringed. Nodularin has a similar structure to Microcystin but has only 5 amino acids. The names reflect the fact that the toxins were first discovered in members of the genera *Microcystis* and *Nodularia*, but these toxins have since been found in many genera of cyanobacteria.

Microcystins and Nodularin are hepatotoxins which inhibit protein phosphatases (resulting in an excess of phosphates on cellular proteins). This has a major impact on liver cells because the toxins accumulate in the hepatocytes (ie main type of liver cell). The microfilaments (ie proteins that normally form the cytoskeleton or internal skeleton of cells), collapse and the hepatocytes can no longer maintain their shape. As a consequence, the hepatocytes shrink, leaving spaces between cells (ie sinusoids). Blood seeps from capillaries into these spaces and blood accumulates there. This leads to local tissue damage in the liver and shock as well as death if the toxin concentration is high enough. When an animal has died from ingesting these hepatotoxins, the liver and kidney are enlarged because of the internal bleeding (ie haemorrhage) and they are heavily mottled (ie surface patches of blood).

6.4.1.2 Types of cyanobacteria in prawn farms

A weekly survey of the planktonic types (ie in water column) of algae in Far North Queensland farms was carried out by Daniel Ivanoff on 214 ponds in year 2000 and the results are summarised in Table 6.4.1. It shows that cyanobacteria were the second most abundant form of algae; the types of algae were: (in descending order) non-motile green algae (eg *Pavlova* sp.), cyanobacteria, flagellates, diatoms and dinoflagellates. There were very few correlations between cell counts for the 6 types of plankton, except for a significant correlation between non-motile green algae and total protozoans (at 0.01 level), and between cyanobacteria and flagellates (0.05 level).

Table 6.4.1 Statistics for survey of abundance of planktonic algae in ponds

Descriptive Statistics

	Mean	Std. Deviation	N
Total Flagellates (c/mL)	56048.54	73987.627	214
Total non-motile green algae (c/mL)	278085.85	957840.909	212
Total Diatoms (c/mL)	35597.77	103969.873	213
Total Dinoflagellates (c/mL)	6055.03	20849.549	214
Total Cyanobacteria (cells/mL)	131363.8498	428671.59359	213
Total Protozoans	828.24	3515.694	213

Correlations

		Total Flagellates (c/mL)	Total non-motile green algae (c/mL)	Total Diatoms (c/mL)	Total Dinoflagellates (c/mL)	Total Cyanobacteria (cells/mL)	Total Protozoans
Total Flagellates (c/mL)	Pearson Correlation	1	.090	.045	-.055	.144(*)	-.037
	Sig. (2-tailed)	.	.192	.510	.426	.036	.593
	N	214	212	213	214	213	213
Total non-motile green algae (c/mL)	Pearson Correlation	.090	1	.059	-.058	-.027	.195(**)
	Sig. (2-tailed)	.192	.	.393	.397	.692	.004
	N	212	212	211	212	211	211
Total Diatoms (c/mL)	Pearson Correlation	.045	.059	1	-.077	.018	-.040
	Sig. (2-tailed)	.510	.393	.	.265	.791	.560
	N	213	211	213	213	212	212
Total Dinoflagellates (c/mL)	Pearson Correlation	-.055	-.058	-.077	1	-.039	.042
	Sig. (2-tailed)	.426	.397	.265	.	.569	.545
	N	214	212	213	214	213	213
Total Cyanobacteria (cells/mL)	Pearson Correlation	.144(*)	-.027	.018	-.039	1	.028
	Sig. (2-tailed)	.036	.692	.791	.569	.	.690
	N	213	211	212	213	213	212
Total Protozoans	Pearson Correlation	-.037	.195(**)	-.040	.042	.028	1
	Sig. (2-tailed)	.593	.004	.560	.545	.690	.
	N	213	211	212	213	212	213

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Table 6.4.2 Non Parametric stats on dye vs cyanobaceria

Kruskal-Wallis Test results:
use of dye significantly reduces both surface mats and benthic cyanobacteria.

Ranks

	use of dye	N	Mean Rank
B-G filaments (0=nil, 5=>75% pond)	dye	98	2096.60
	no dye	4183	2142.04
	Total	4281	
B-G surface mats (0=nil,5=mats 50%)	dye	98	2305.54
	no dye	4177	2134.07
	Total	4275	
B-G benthic (0=nil, 5 all pond bottom)	dye	94	1715.13
	no dye	4109	2110.85
	Total	4203	
B-G plankton (0=nil, 5=blackwater)	dye	94	2093.90
	no dye	4089	2091.96
	Total	4183	

Test Statistics(a,b)

	B-G filaments (0=nil, 5=>75% pond)	B-G surface mats (0=nil,5=mats 50%)	B-G benthic (0=nil, 5 all pond bottom)	B-G plankton (0=nil, 5=blackwater)
Chi-Square	.288	6.582	13.874	.001
df	1	1	1	1
Asymp. Sig.	.592	.010	.000	.977

a Kruskal Wallis Test
b Grouping Variable: use of dye

Figures 6.4.1 to 6.4.5 illustrate the cell counts for the various types of algae with respect to age of crop. Fig 6.4.1 shows that planktonic forms of cyanobacteria started to occur in some ponds approximately 60 days after stocking and from then onwards, ponds either had high numbers (>400,000 cells/mL) or were relatively free of planktonic cyanobacteria. In general, the other species of algae tended to be present in varying densities in most ponds throughout the growout. The main types of cyanobacteria were filamentous, including *Oscillatoria* sp. (found in 14% of samples) followed by *Pseudoanabaena* sp. and *Anabaena* sp. (in 7% of samples). On rare occasions other forms were observed, and these included *Microcystis* sp and *Nodularia* sp (in NSW farms). Figure 6.4.6 illustrates the various forms of cyanobacteria that were observed in prawn ponds during the project.

Fig 6.4.1 Total cyanobacteria in ponds

Ponds were surveyed weekly

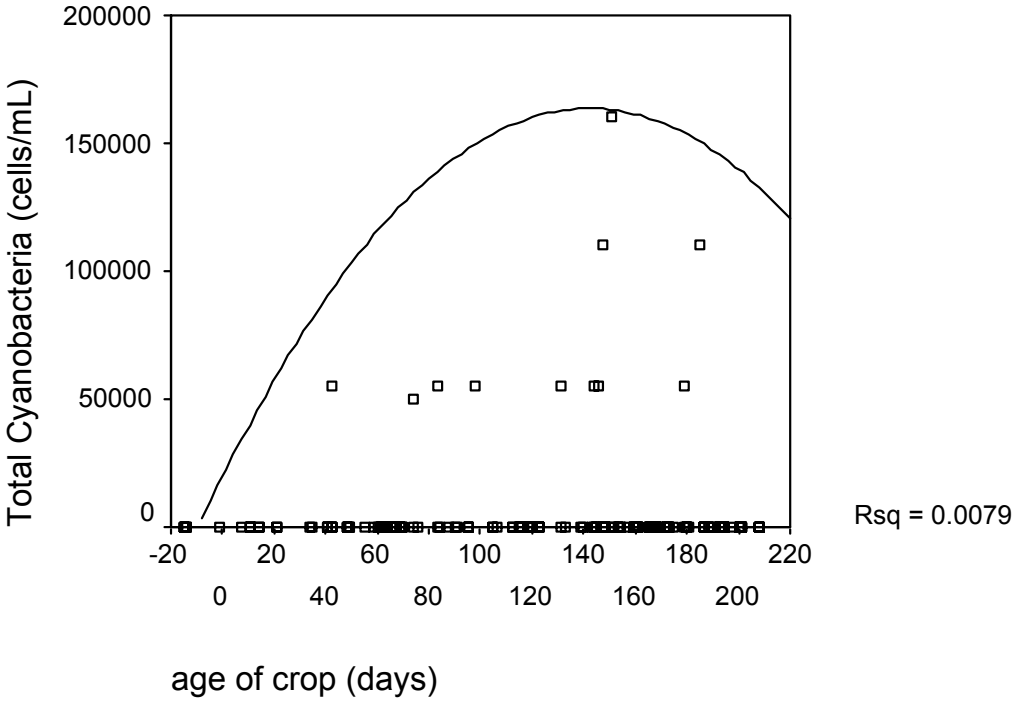


Fig 6.4.2 Total flagellates in ponds

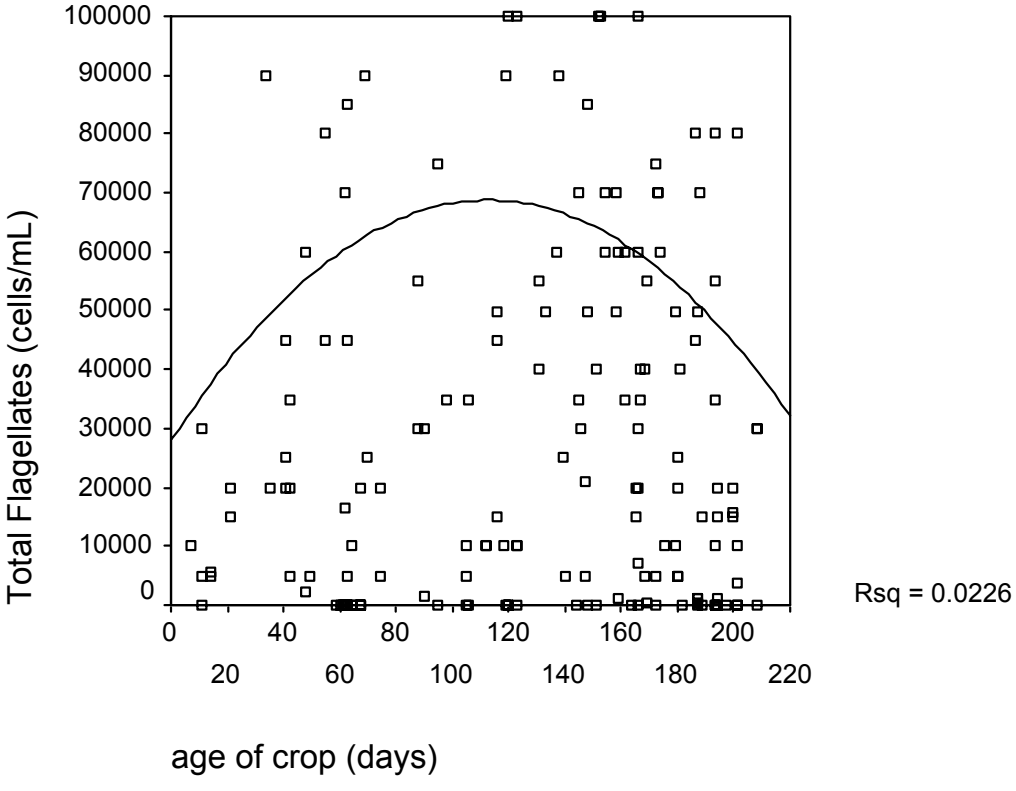


Fig 6.4.3 Total non motile green alage

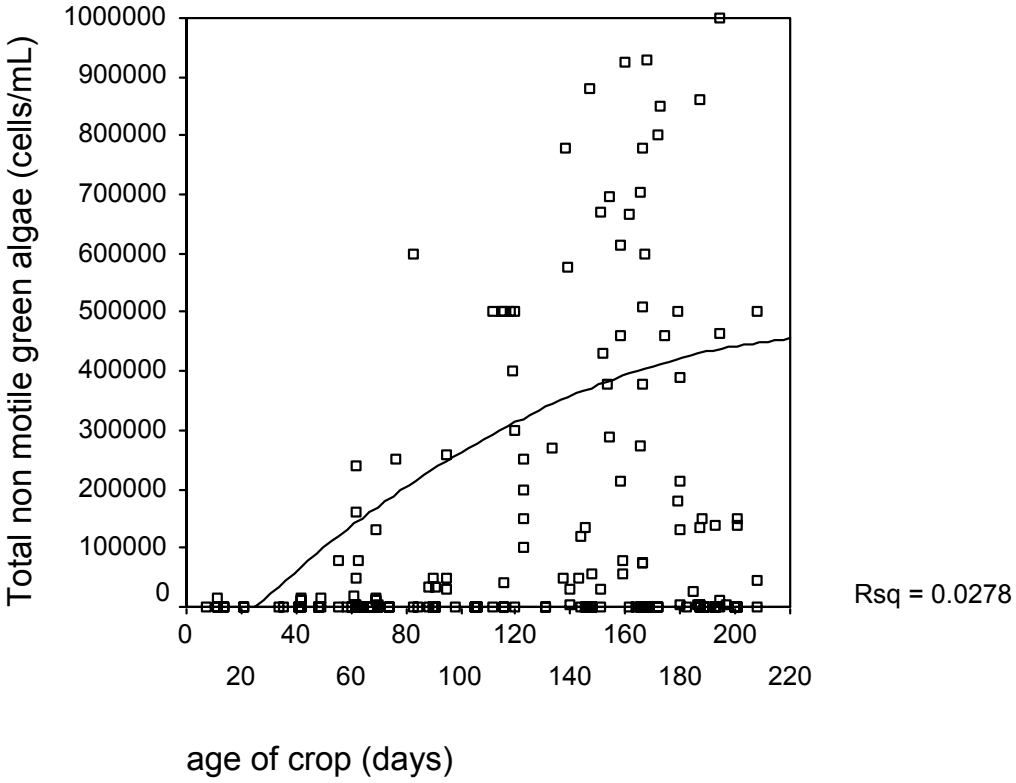
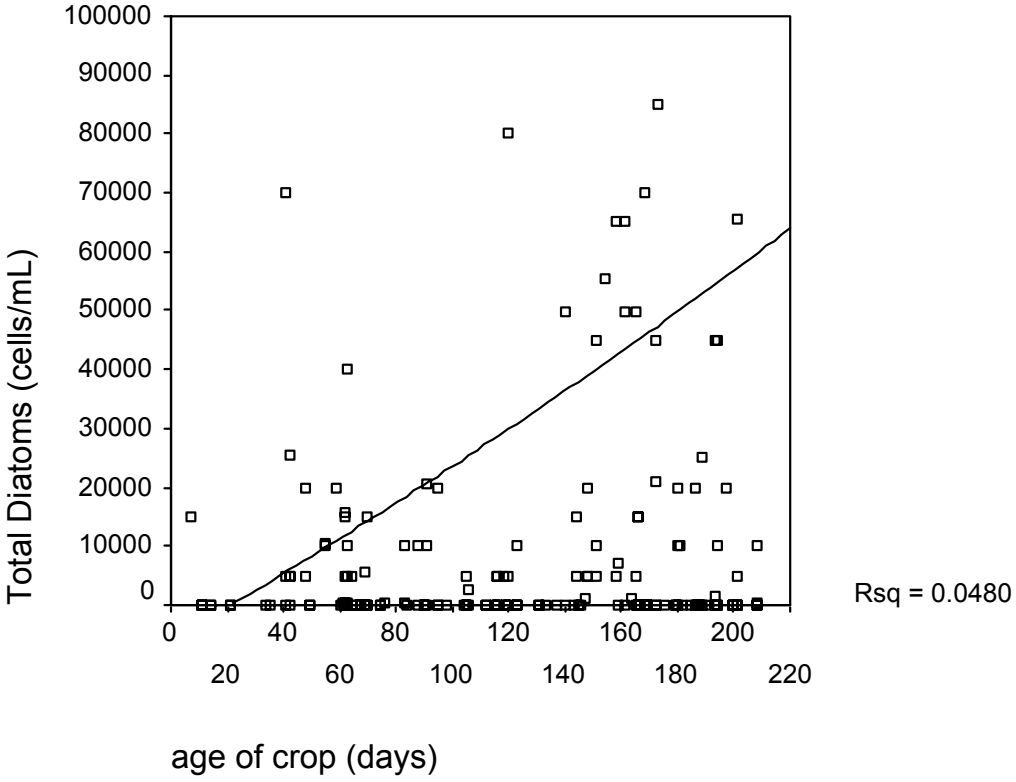


Fig 6.4.4 Total diatoms in ponds



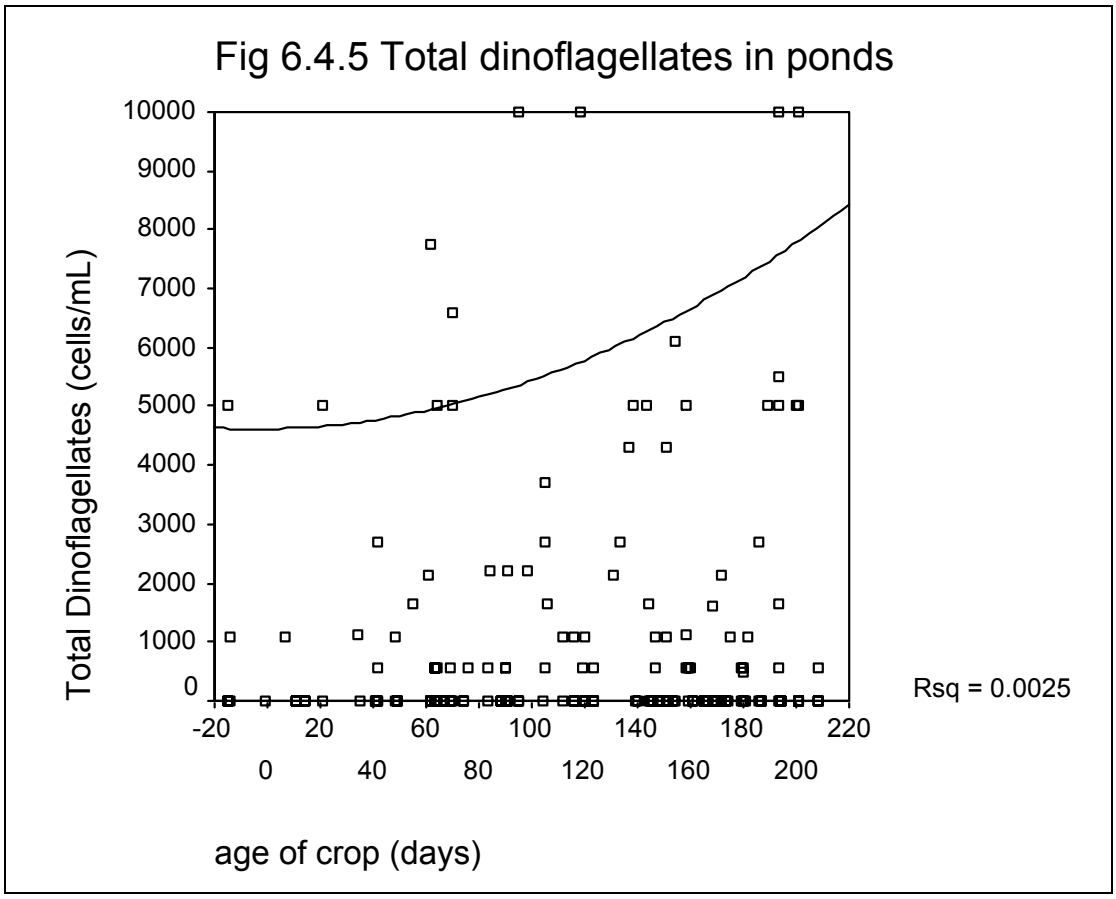
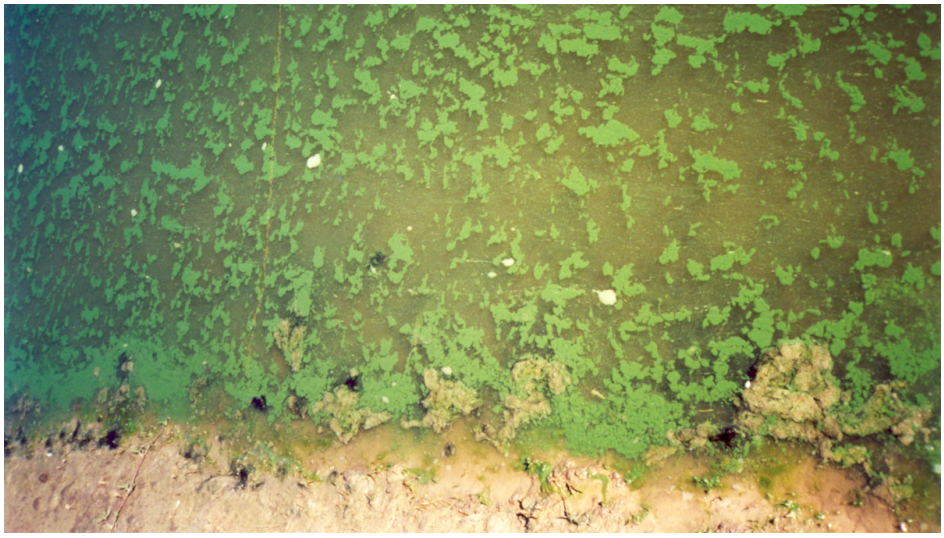


Fig 6.4.5 Total dinoflagellates in ponds
Total Dinoflagellates (cells/mL)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-20
0
20
40
60
80
100
120
140
160
180
200
220
Rsq = 0.0025
age of crop (days)

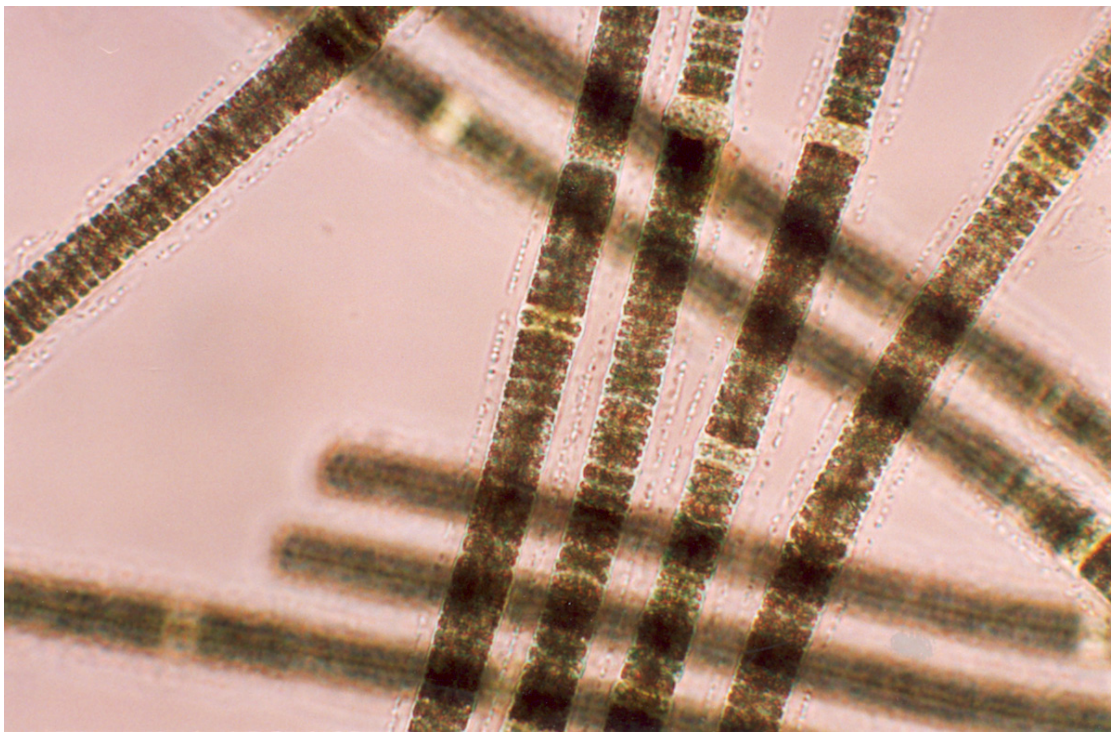
Figure 6.4.6 Field observations and microscopic observations of cyanobacteria in prawn farms.

a) Field observations: (top) green scum of toxic planktonic *Nodularia spumigena* at the edge of a pond (Northern NSW April 2001); (bottom) surface mats of Oscillatoria in an effluent drain.

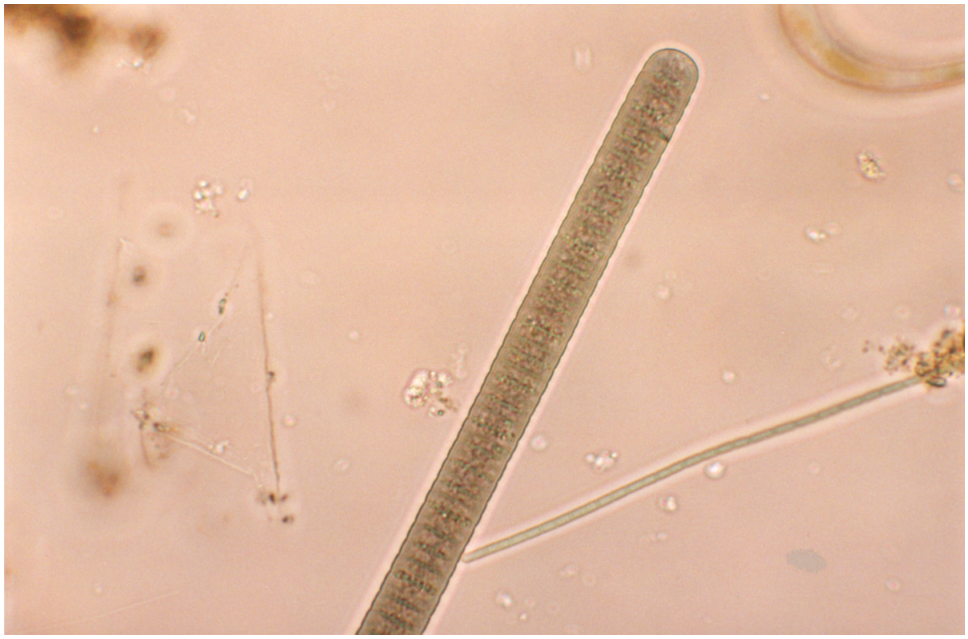


b) Filaments (trichomes) of *Nodularia spumigena* in the scum shown in a) above (100x magnification).

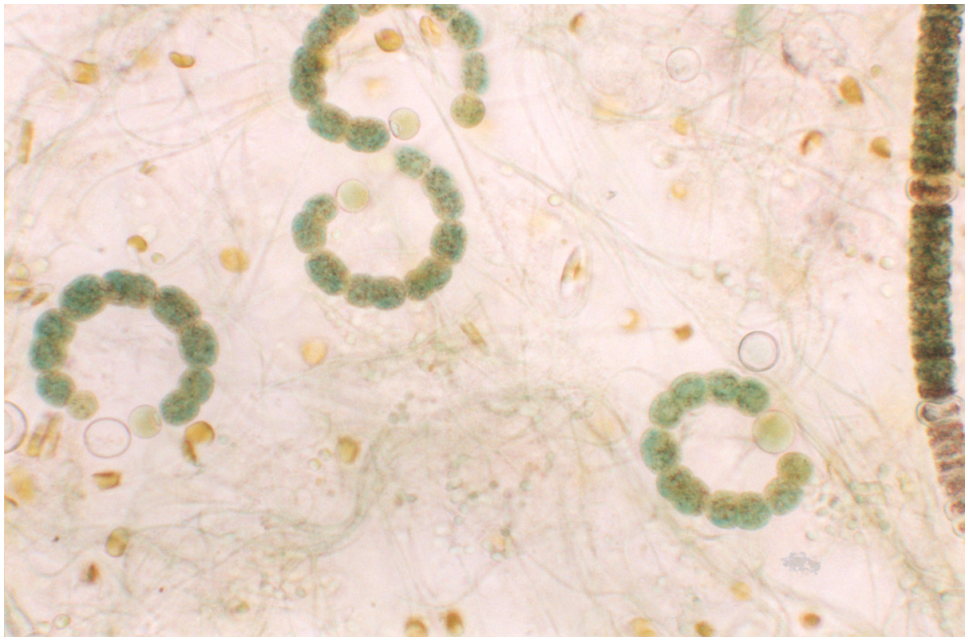
c) Light photomicrograph of *Nodularia spumigena.*
Trichomes from a) and b) above, showing grey-green vegetative cells, clear heterocysts (where nitrogen is fixed) and a gelatinous coat (400x).



d) Examples of two types of *Oscillatoria*.
The species in the centre has a trichome 10µm wide and has vegetative cells 2µm long, while the thinner species is 1µm wide and its cells are 2.5µm long (400x). *Oscillatoria* are the most common types of cyanobacteria at prawn farms. All *Oscillatoria* lack heterocysts.

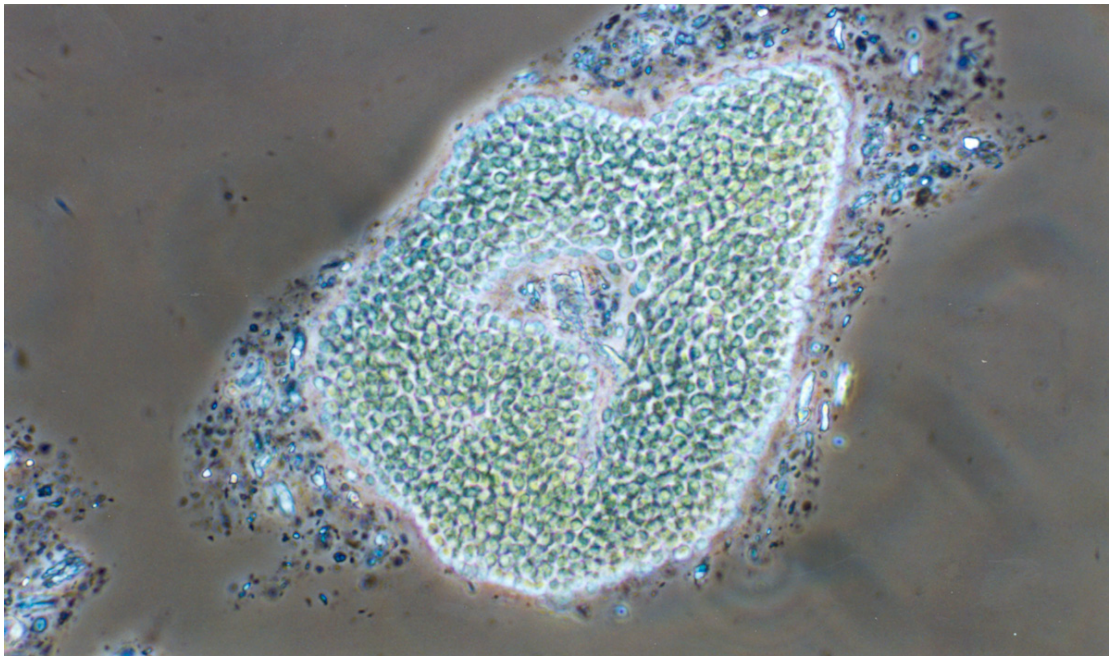


e) Examples of *Anabaeopsis* sp.
Each of the four coils has a alkinete (reproductive cell) at one end and a heterocyst (nitrogen fixing cell) at the other. A trichome of *Nodularia spumigena* can also be seen as well as a very thin filamentous species and brown unicellular algae in this mixed culture (400x).

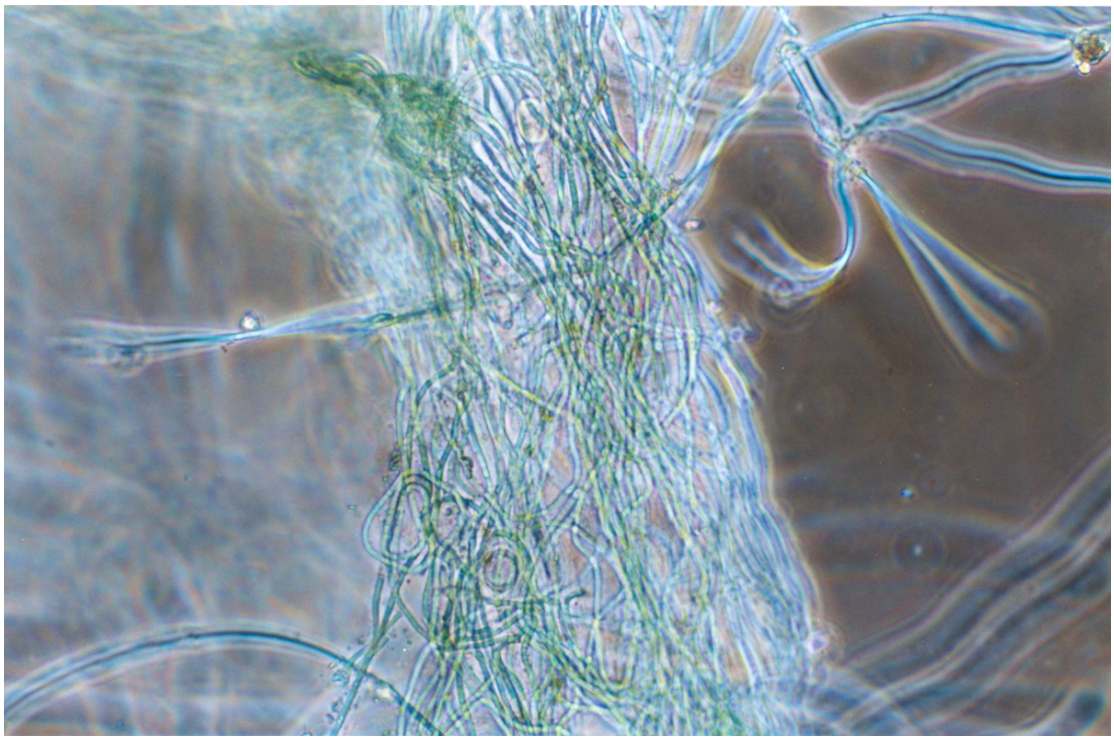


f) Example of Microcystis sp.

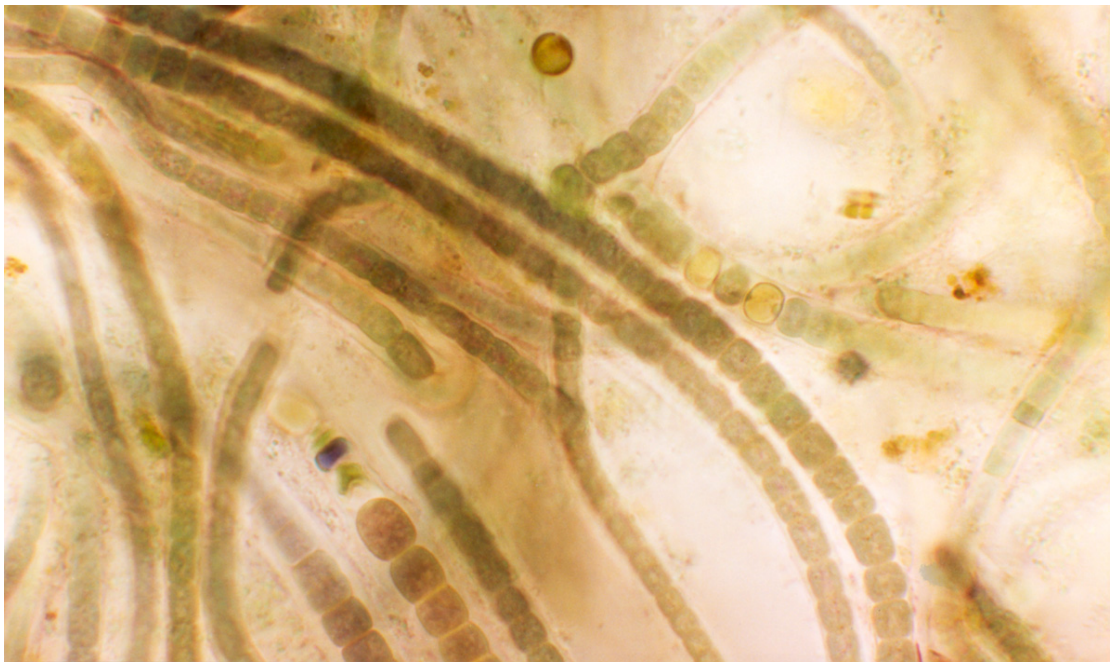
Microcystis was observed on only rare occasions – the colonies were usually irregular in shape and contain numerous small spherical cells. In this example there is a collection of debris attached to the colony's gelatinous matrix (400x).



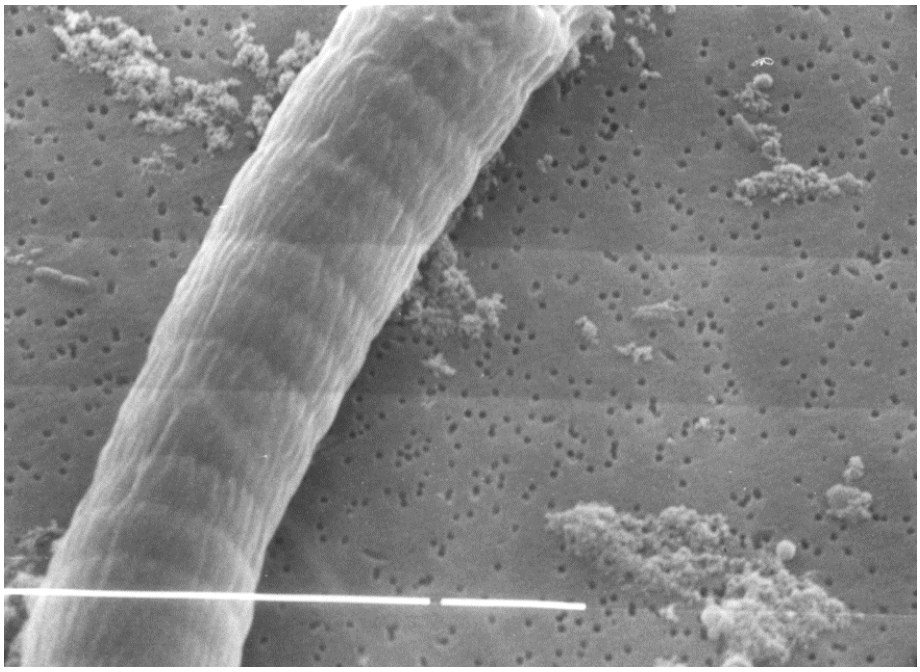
g) Example of *Pseudoanabaena* sp in subculture (400x).



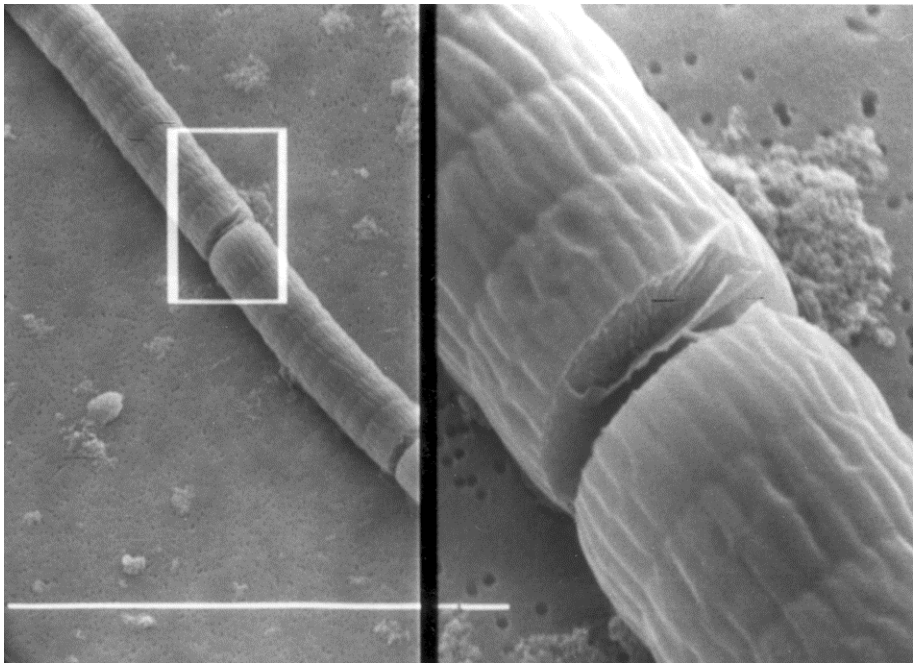
h) Example of Oscillatoriales in subculture (x400).
After inoculation, the growth of cells was slow, usually short-lived and their morphology (appearance) varied greatly from the original inoculum. Although numerous variations in growth conditions (ie media composition & preparation, light intensity, photoperiod, technique, etc) were used, no suitable method for long-term growth and storage of most cyanobacterial isolates was found.



i) Electron micrograph of *Oscillatoria* sp.
The trichome is 6.5µm wide and each cell is 2µm long, as indicated by the banded surface. The specimen was mounted on support, critically point dried and gold coated. The small scale bar is 5µm long (Scanning electron microscopy x4,000).



j) Electron micrograph of a specimen of *Oscillatoria* in the process of division.
The trichome is 7.5µm wide and cells are 2µm long. (Scanning electron microscopy: left hand side x1000, right hand side x5,000).



6.4.1.3 Prawn mortalities and morbidity associated with cyanobacterial blooms

Studies by other researchers on freshwater water supplies and water bodies, have shown that many of the types of cyanobacteria that were detected in our project are toxic and can cause mortalities in some fish, mammals and birds. Also, prior to this project, the author reported that some planktonic and benthic cyanobacterial blooms of Oscillatoriales had toxic effects on *P. monodon*, *P japonicus* and *Artemia salina* (Smith 1996). In that report, it was noted that because species of Oscillatoriales, including *Oscillatoria*, *Lyngbya*, *Nodularia* and *Spirulina* were ubiquitous in prawn farms, the findings have significant implications for the aetiology of disease in the prawn farming industry. Unfortunately the toxins that caused mortalities in those incidents were not identified. A similar problem was previously encountered by Lightner (1975) in his observation of haemocytic enteritis of *P. stylirostris* associated with a bloom of *Spirulina* sp. The identity of the toxin/s was not determined in that incident.

In this project there were incidences in which prawn mortalities and slow growth of prawns were associated with blooms of cyanobacteria and in these cases hepatotoxins were identified. Those incidents are summarised here.

A) Nodularin found in blooms of *Nodularia spumigena*.

In April 2000, a bloom of *Nodularia spumigena* (dominant species) and *Microcystis* sp. (minor species) occurred in two ponds at a farm on the Clarence River. Water quality parameters were not exceptional – Secchi disk visibility was 20-40cm, salinity 21-22ppt, pH 7.3-8.4, temperature 19-20^{o}C, dissolved oxygen 5.7-9.0mg/L, and level of presumptive *Vibrioanacae* bacteria was 3,800±2,800cfu/mL (n=5). Affected ponds had green planktonic filaments as well as bright green paint-like scum. In the pond with the densest bloom, the farmer applied an algicide, simerzine, which caused the bloom to disappear and the prawns to die. Cyanobacteria were collected from the second pond and used for subculture, photography, toxicity tests, and toxin analysis. Samples were centrifuged so that the pellet and supernatant could be separated and freeze dried. Mouse bioassay tests were performed by intra-peritoneal injection (1mL) of filtered freeze-dried material re-suspended in saline. The pellet fraction was sub-lethal and hepatotoxic, while the supernatant fraction was hepatotoxic and lethal in 54 minutes. Necropsy (dissection) of mice tested with the supernatant fraction revealed that livers were heavily mottled, patchy, dark and enlarged. Kidneys were also heavily mottled. HPLC analysis of the supernatant fraction revealed the hepatotoxin, Nodularin was present at 30µg/L pond water.

In 2001 and 2002, collection of water samples was carried out on a weekly basis and microscopic analysis showed that 5% of ponds had *Nodularia spumigena* present in the final 3 months of the crop (March to May). *Anabaenopsis* sp and *Microcyistis* sp. occurred at lower frequencies during these times. This period also corresponded to increases in rainfall and decreases in river salinity.

These findings have important implications for understanding the aetiology of environmental diseases in *P. monodon*, as well as the health of workers at prawn farms where toxic blooms of cyanobacteria occur. The results and findings were

presented to Fifth International Conference on Toxic Cyanobacteria, Noosa QLD 2001 (see Appendix 5).

Table 6.4.2 Detection of Microcystins: summary of findings for mouse toxicity and toxin detection.

Three blooms were separated into pellet and supernatant, freeze dried then tested. A) For mouse biotoxicity tests, 0.2g was reconstituted in 5 mL Millipore water, sonicated, filter sterilized and 1mL was injected Intra Peritoneal into each mouse. Mice were observed for 24 hrs or until death, then livers and kidneys from each mouse were examined. B) For toxin detection, the freeze-dried pellets and supernatants were reconstituted in Millipore water and analysed for microcystin, by protein phosphatase, HPLC-UV diode array and LC-MS.

Sample Number	Type of cyanobacterial bloom	Fraction of freeze-dried extract	Result of mouse toxicity test	Result of toxin tests for microcystin
1	Mixed Spirullina & Oscillatoria	Pellet (1)	Livers & kidneys were patchy – possible toxic	No microcystin detected
		Supernatant (2)	Livers & kidneys were patchy – possible toxic	Detected one type of MicrocystinRR
2	Microcystis	Pellet (3)	Livers & kidneys were mottled – possible toxic	Detected two types of microcystins: MicrocystinLR, MicrocystinLA
		Supernatant (4)	Mice appeared ill and livers slightly enlarged, heavily mottled and kidneys mottled – sub-lethal hepatotoxin	Detected three types of microcystins: MicrocystinYR, MicrocystinLR, MicrocystinLA
3	Oscillatoria	Pellet (5)	Mice appeared very ill and livers were heavily mottled and kidneys were mottled – possible toxic.	Detected four types of microcystins: MicrocystinRR, MicrocystinYR and 2 other unknown microcystins.
		Supernatant (6)	Liver patchy but close to normal – possible toxic.	Detected four types of microcystins: MicrocystinYR, MicrocystinLR Microcystin LA and 1 other unknown microcystin.

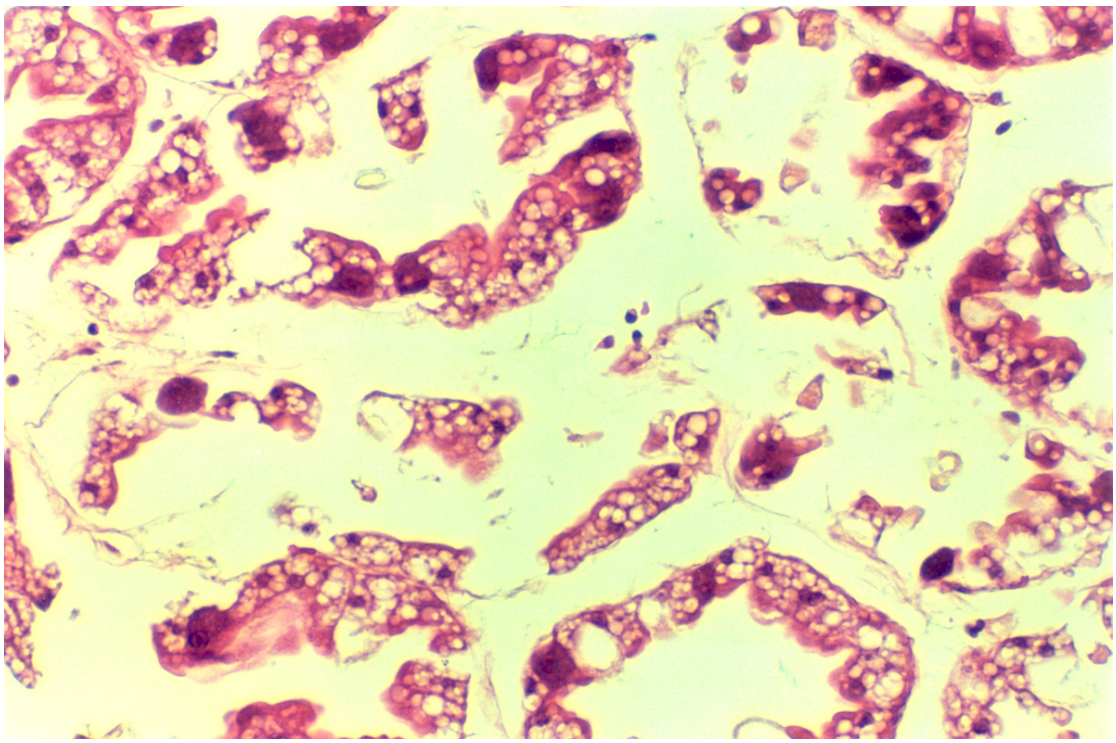
B) Microcystins identified in other cyanobacterial blooms

Samples were collected from numerous cyanobacterial blooms throughout the project and they were frozen upon collection then processed as follows. Samples (approximately 1litre) were thawed, homogenized and centrifuged at 4^{o}C. The supernatant and pellet were freeze-dried separately and stored at –80^{o}C. Challenge tests with *Artemia* were as outlined in (Smith 1996). Freeze-dried material was resuspended in sterile 50% seawater (500mg/L) and challenge tests with freshly hatched Artemia were performed. Three cyanobacterial blooms that gave >90%

mortalities of Artemia within 24hrs (compared to <10% in controls) were tested for the presence of microcystins. Freeze-dried samples were sent to a collarator, Dr Ambrose Furey (Research Manager, Ecotoxicology Research Unit, Cork Institute of Technology, Cork, Ireland) and in October 2002 a report from Dr Furey stated that 5 of the samples contained microcystins (see graphs in Supplement #17). The results for toxin detection as well as mouse toxicity tests (carried out 7/4/2000) are summarized in Table 6.4.2. It shows that a range of hepatotxins were present in the samples.

Some 6 separate challenge tests were carried out in which healthy prawns were injected with filtered extracts from toxic cyanobacterial blooms. Prawns were sacrificed within 24hrs following intramuscular injection. Histopathology (Fig 6.4.6.2) revealed tissue damage to the hepatopancreas was characterized by tubules having relatively thin walls and cells appeared to have sloughed off into the lumen (ie broken away from the basement membrane). The parenchyma tissue between the tubules is often lost. In extreme cases the hepatopancreatic cells of the tubules "rounded up" and the structure of the tubules was severely damaged. These findings have not been previously reported for prawns, though they are consistent with the action of Microcystin on mammalian liver tissue (see Information Box on Microcystin & Nodularin – section 6.4.1).

Fig 6.4.6.2 Examples of damage to hepatopancreatic tubules in prawns injected with cyanobacterial extracts (x400).
In this case, the prawn was injected (intra muscular) with filtered supernatant from an *Oscillatoria* bloom which contained microcystins (see Table 6.4.2 – sample #3 contained MicrocystinYR, MicrocystinLR Microcystin LA and 1 other unknown microcystin).



6.4.1.4 Extracellular enzymes in cyanobacterial blooms

Fig 6.4.7 (a to s) shows the extracellular enzyme profiles for a range of bloom samples of approximately equal cell densities, from ponds, hatchery cultures and laboratory cultures. The values are the means for different sources (n=5 to 16), except for the *Nodularia* pond sample which was measured in duplicate on samples taken on each consecutive days (ie n=4). This *Nodularia* bloom occurred in a prawn pond on the Clarence River and its toxicity has been reported (Appendix 5). Extracellular enzyme activities are included for reference materials (ie hatchery samples of suspensions of freshly hatching *Artemia* eggs and new filtered seawater). Fig 6.4.7 (a to e) shows that the *Nodularia* bloom had significantly higher enzyme activities for six types of sugars than the 8 other types of bloom samples. As for esterase and lipase activity, there is no bloom that had significantly higher enzyme values although hatchery cultures of *Pavlova* generally had the highest mean values (Fig 6.4.7 g, h, i, m, n, s). The two blooms with highest protease activity were the *Nodularia* pond culture (Fig 6.4.7 j) and *Pavlova* hatchery culture (Fig 6.4.7l). The *Nodularia* pond culture had chitinase enzyme activities that were more than 100 times higher than the other blooms (Fig 6.4.7 k, o, p). The *Nodularia* bloom also had significantly higher phosphatase and sulfatase activities than the other blooms.

The results in Fig 6.4.7 indicate that, in comparison with the other algal blooms, the toxic *Nodularia* bloom had the most striking extracellular enzyme profile. However, the *Nodularia* laboratory culture, which was grown from the same pond bloom had a much lower enzyme profile. Examination of Fig 6.4.6 c shows that there is gelatinous coat with motile microbes (ie bacteria) attached. Whereas *Nodularia* in laboratory culture did not appear as healthy as freshly collected pond samples (trichomes were generally shorter with occasional damaged cells, the gelatinous coat was not distinct, and vegetative cells were pale and off-coloured). The findings with respect to the differences in extracellular enzyme profiles, suggest that in laboratory culture either *Nodularia* substantially reduced its enzyme profile and/or the change was due to the loss of the motile microbes that were attached to the gelatinous coat of the pond bloom. Further work on investigating the extraceullar enzyme profiles of algal blooms is needed to understand this issue.

Importantly for the farming industry, the extracellular enzyme profiles for filamentous green algae and seagrass were substantially lower than that of the toxic *Nodularia* bloom. This supports long held personal observations that filamentous green algae is a beneficial type of organism and much superior to cyanobacteria for culturing prawns. With regards the results for algal cultures in prawn hatcheries, *Pavlova* appeared to have the highest extracellular enzyme profiles and this aspect may need to be considered when delicate stages of prawn larvae are cultured.

6.4.1.5 Investigation of methods for reducing occurrence of cyanobacteria.

Some cyanobacterial blooms at prawn farms are toxic to prawns (Smith, 1996) as well as mammals (by mouse bioassay). The work in this project has now shown that some strains of cyanobacteria contain known hepatotoxins (6.4.1.4). Hence, it is important to develop strategies for minimizing their occurrence. Trial-and-error over the previous 10 years has shown that the occurrence of cyanobacterial blooms is encouraged by addition of soluble fertilizers and decreases in salinity (ie during

periods of rainfall). Drying out of ponds between each crop is an important method for slowing the onset of cyanobacterial blooms. Chemical inhibitors, such as simerzine have been used by farmers on occasions to destroy cyanobacterial blooms, but there are problems with residual chemicals in prawn tissue. Simerzine is not recommended.

During the project two experiments were carried out to test the effectiveness of management practices on cyanobacterial blooms. In one approach, three farms in sub-tropical FNQ tested green/blue dyes at various times, but mainly in early stages of the growout. Non-paramentric statistical tests are shown in Table 6.4.2 and the results show that the addition of green/blue dyes significantly reduced the occurrence of cyanobacterial surface mats (<0.01 level) and cyanobacterial benthic blooms (<0.001 level). It had no significant effect on planktonic forms and filamentous forms of cyanobacteria.

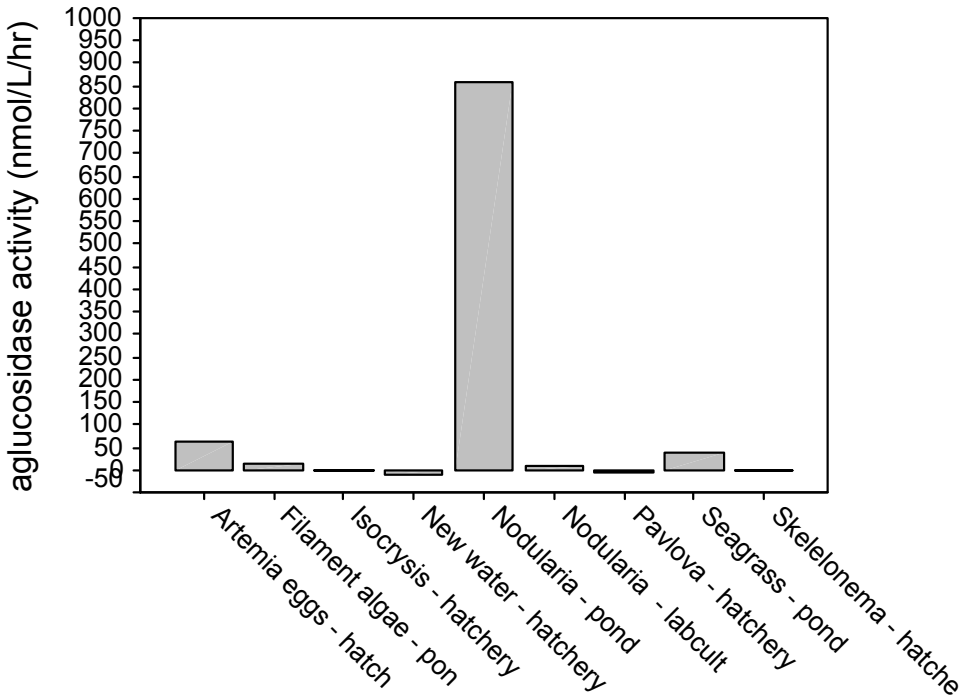
In the second approach, rice pollard was used as a bacterial supplement for the 2001-2 crop at one farm on the Clarence River (see Section 6.3). The supplement was added to most ponds during the first 60 days of the crop and, in selected ponds, towards the end of the growout. Rice pollard was chosen because of its cheapness, availability and high carbon to nitrogen ratio. Observations suggest pollard significantly lowered the incidence of cyanobacterial blooms. In Section 6.3 the effects of rice pollard on extracellular enzyme activity are described and discussed. It is an area that needs follow up experimentation.

6.4.1.6 Conclusions about cyanobacteria in prawn farms

The findings of the project have significantly improved our understanding of cyanobacteria in prawn farms. Experiments have shown for the first time that some blooms of various genera in Australian prawn farms contain known hepatotoxins (Nodularin and Microcystins). Also, in support of earlier work by the author, some blooms were toxic to prawns and mice. The histology of prawns used in challenge tests revealed that these toxins caused damage to the hepatopancreas consistent with its effects on mammalian liver. Further analysis of the histology may provide more information on the effects of these hepatotoxins on prawn tissue.

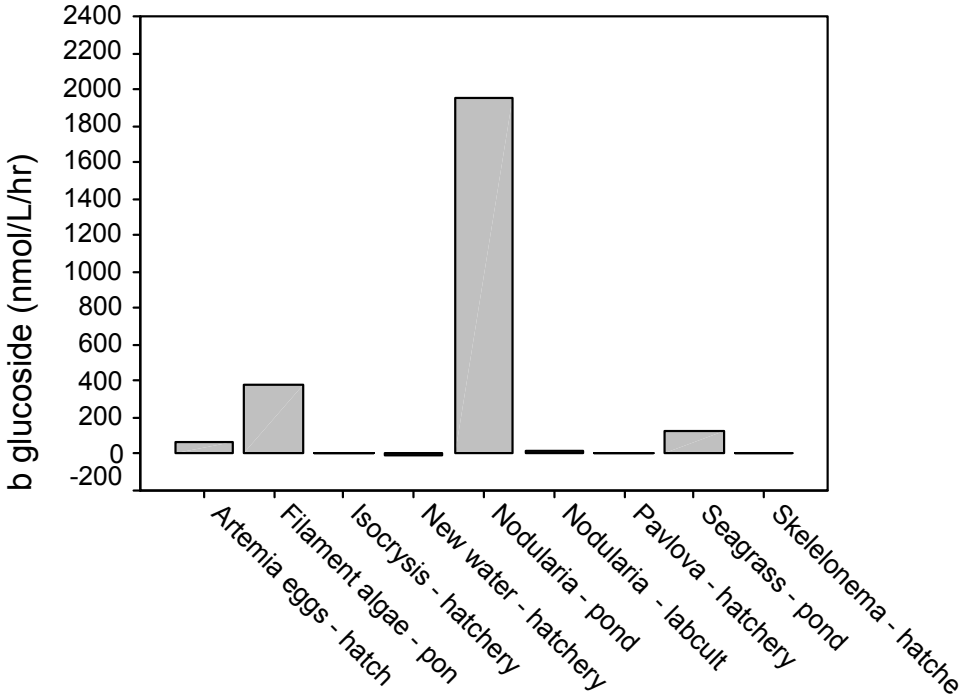
The study has shown that two management practices are capable of reducing the incidence of cyanobacterial blooms: a) the use of green/blue water-soluble dyes and b) addition of pollard as a bacterial supplement. In view of issues relating to the long term health of farm workers, as well as that of consumers of prawns, it is vital that further work be carried out to improve on the methods that were developed in this study to minimize the occurrence of cyanobacterial blooms.

Fig 6.4.7a



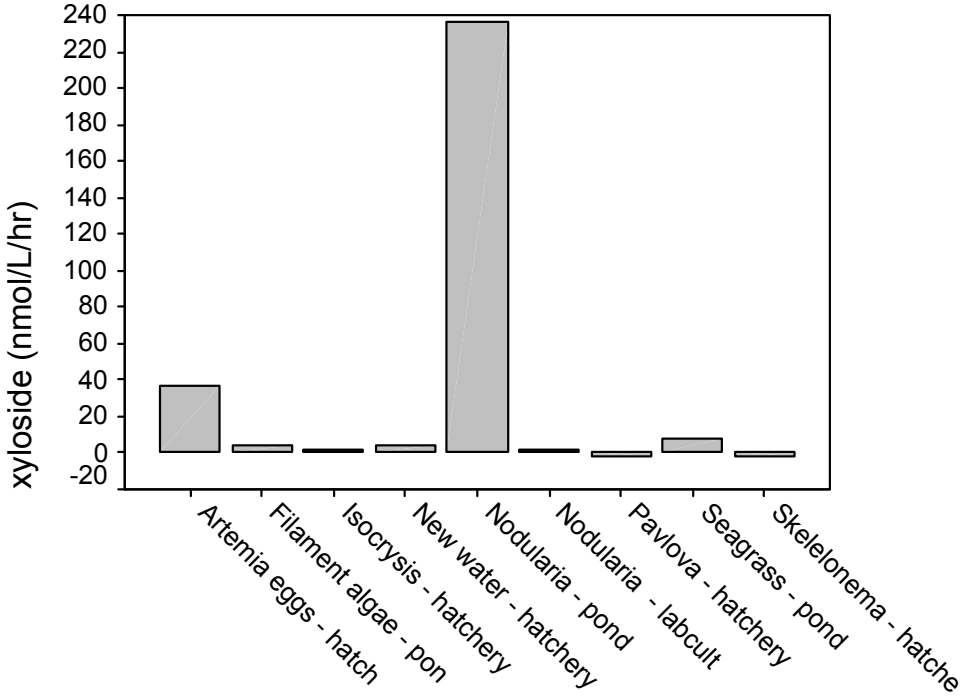
Bloom samples (from pond, hatchery or lab culture)

Fig 6.4.7b



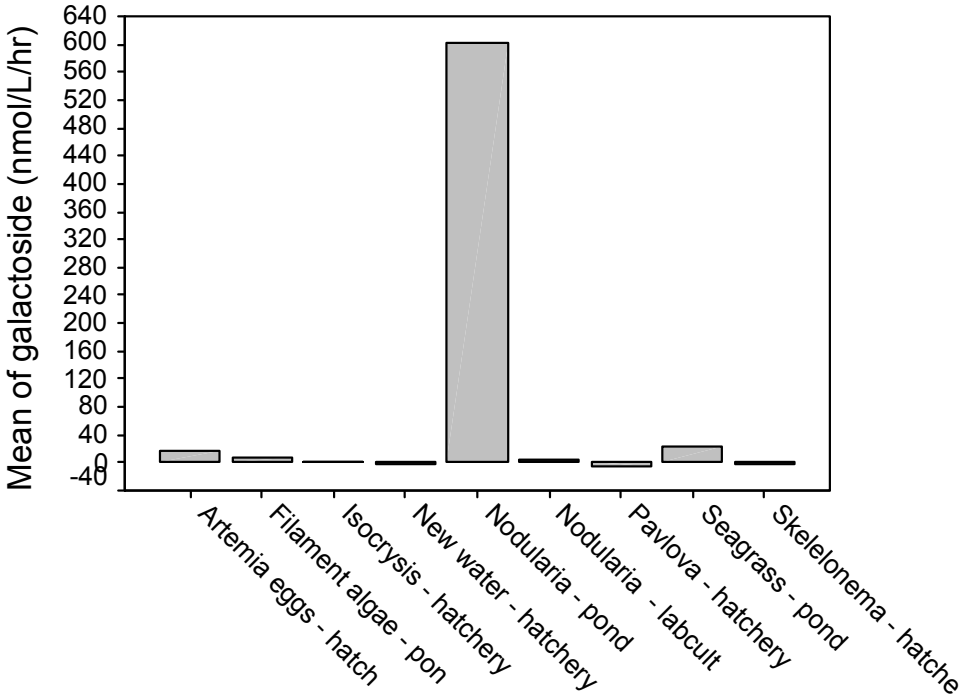
Bloom samples (from pond, hatchery or lab culture)

Fig 6.4.7c



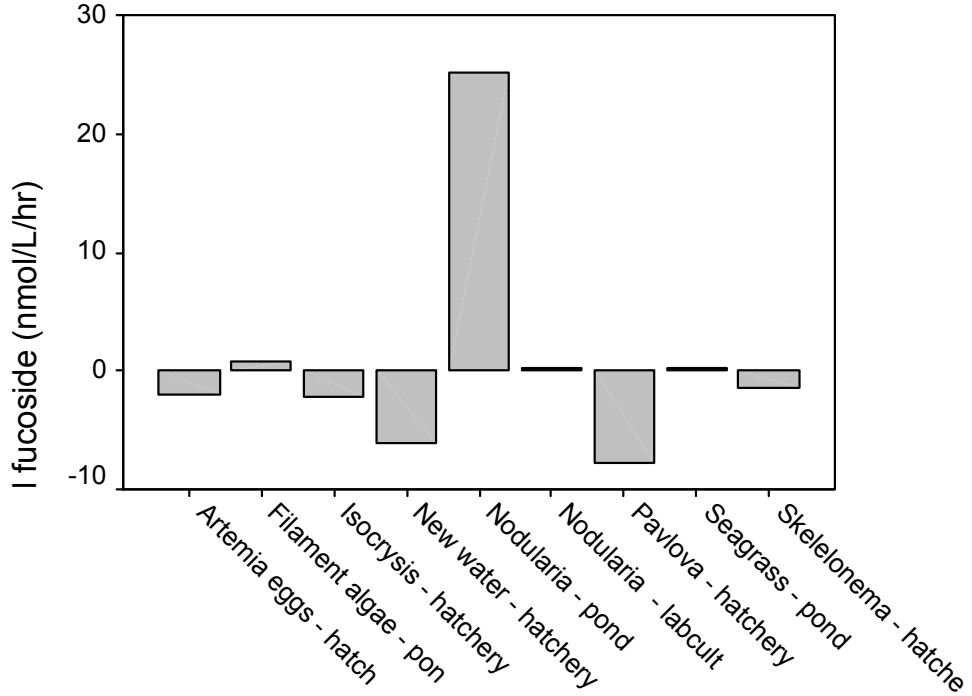
Bloom samples (from pond, hatchery or lab culture)

Fig 6.4.7d



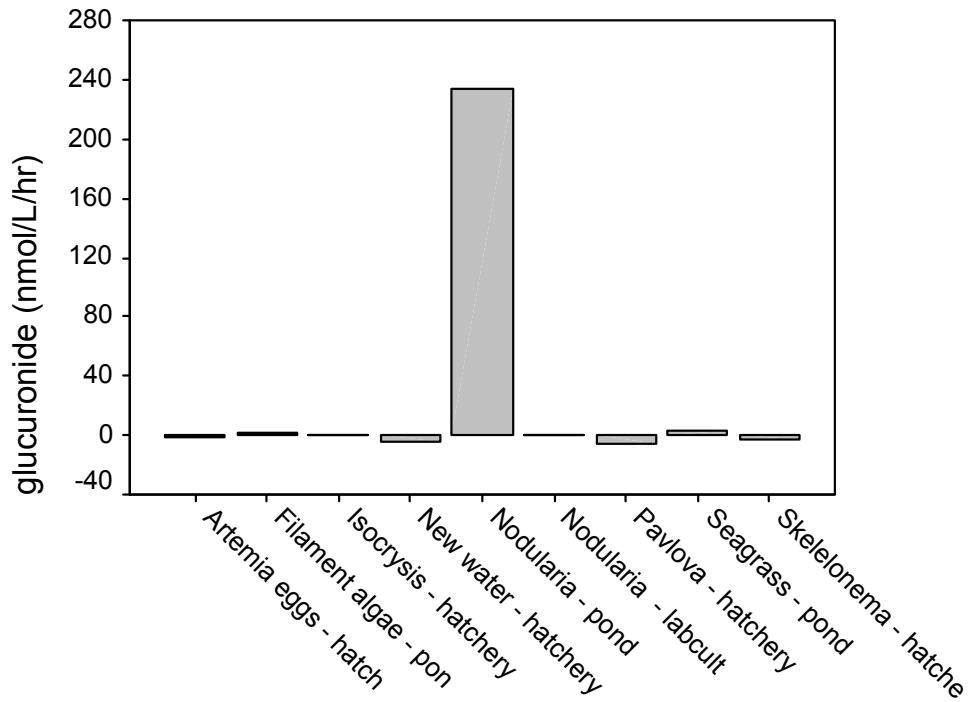
Bloom samples (from pond, hatchery or lab culture)

Fig 6.4.7e



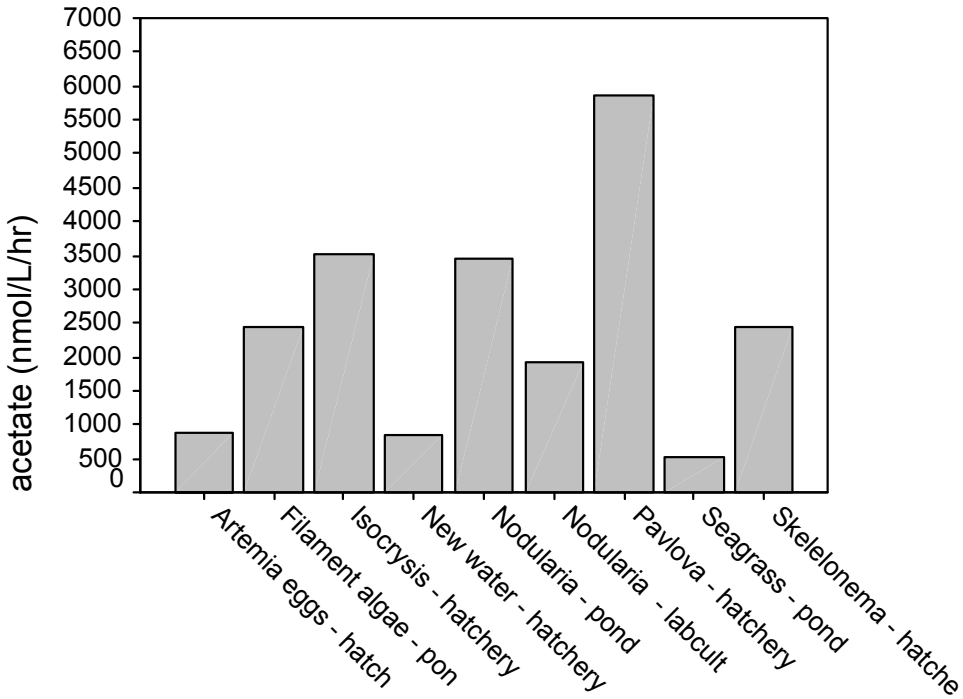
Bloom samples (from pond, hatchery or lab culture)

Fig 6.4.7f



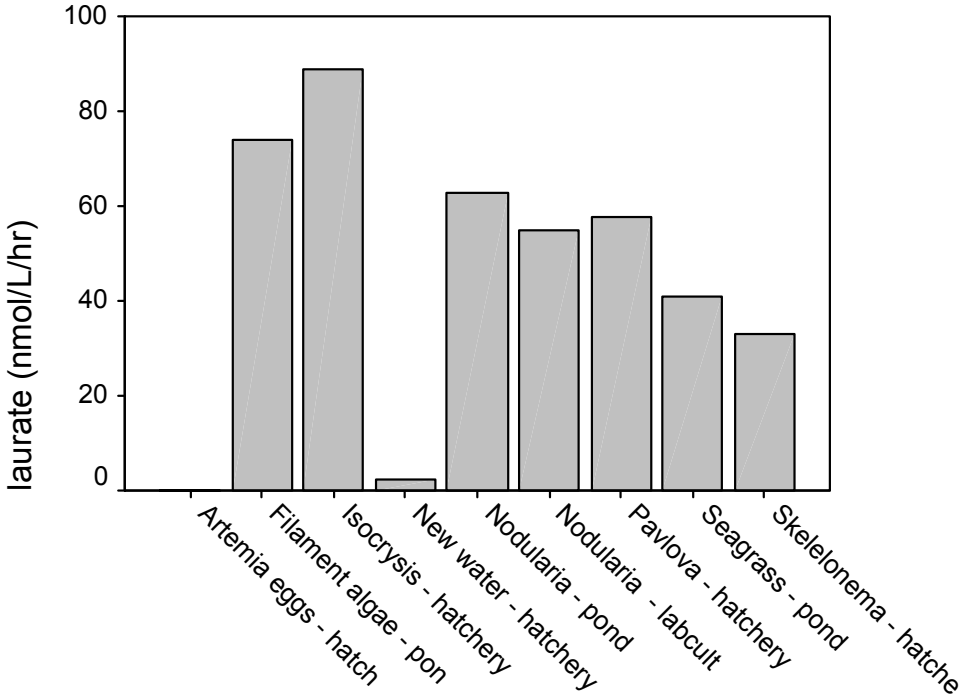
Bloom samples (from pond, hatchery or lab culture)

Fig 6.4.7g



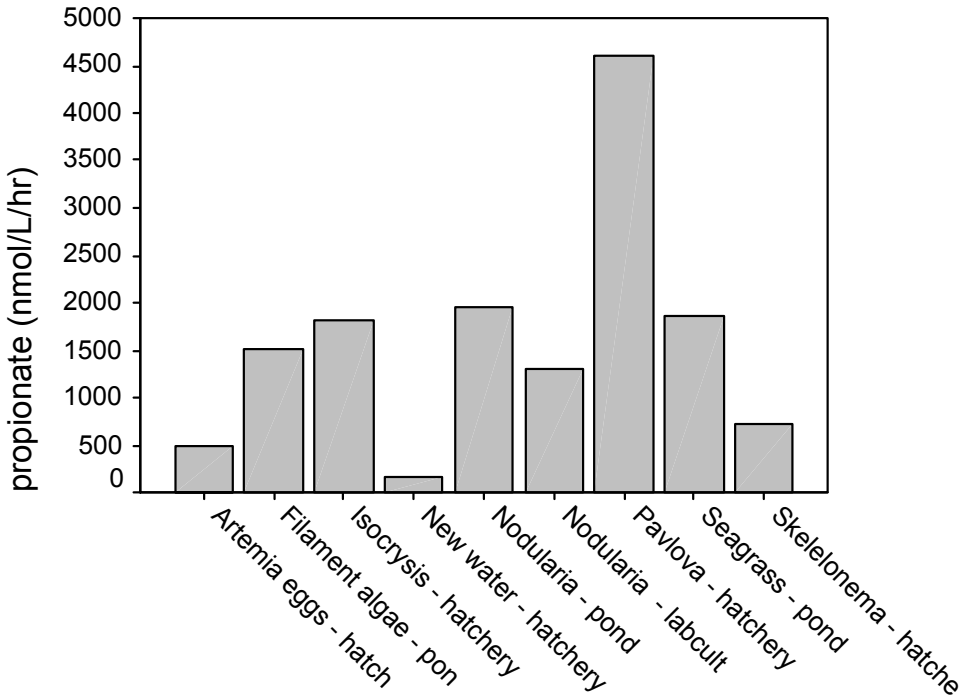
Bloom samples (from pond, hatchery or lab culture)

Fig 6.4.7h



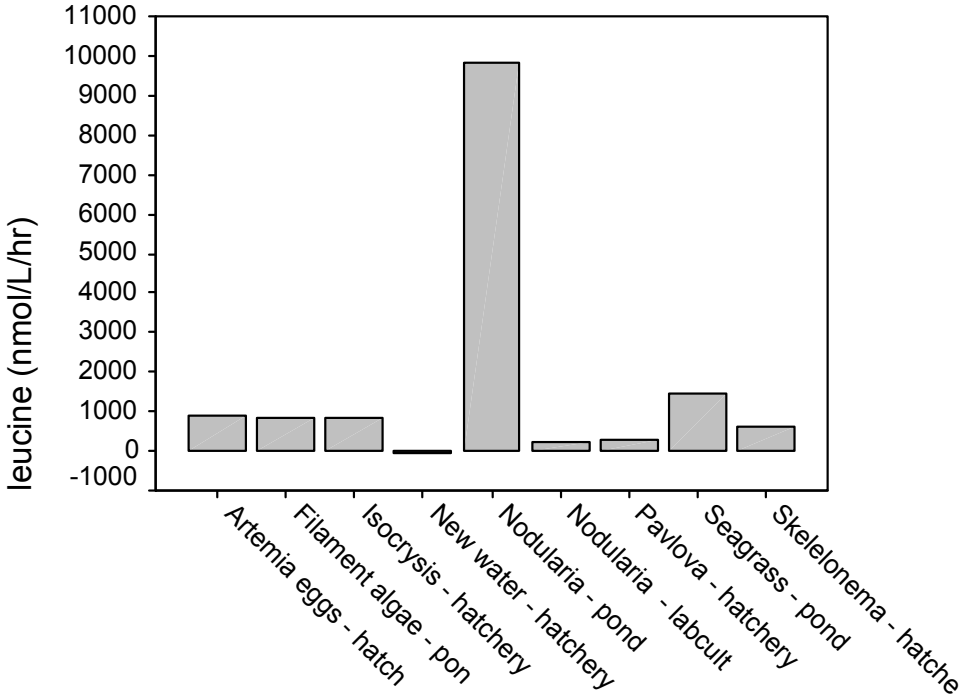
Bloom samples (from pond, hatchery or lab culture)

Fig 6.4.7i



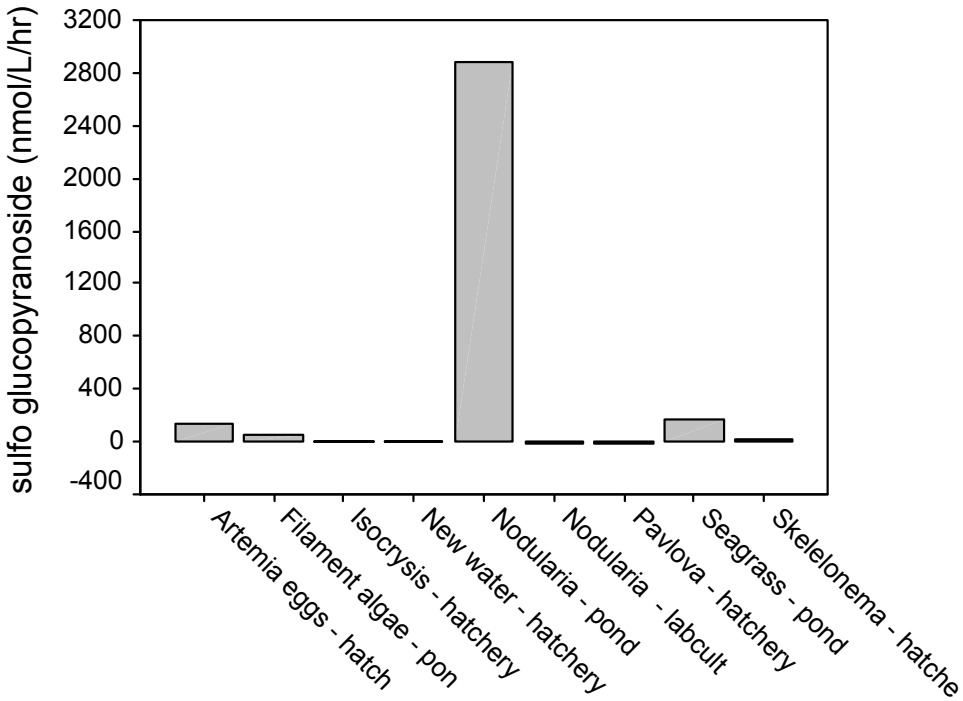
Bloom samples (from pond, hatchery or lab culture)

Fig 6.4.7j



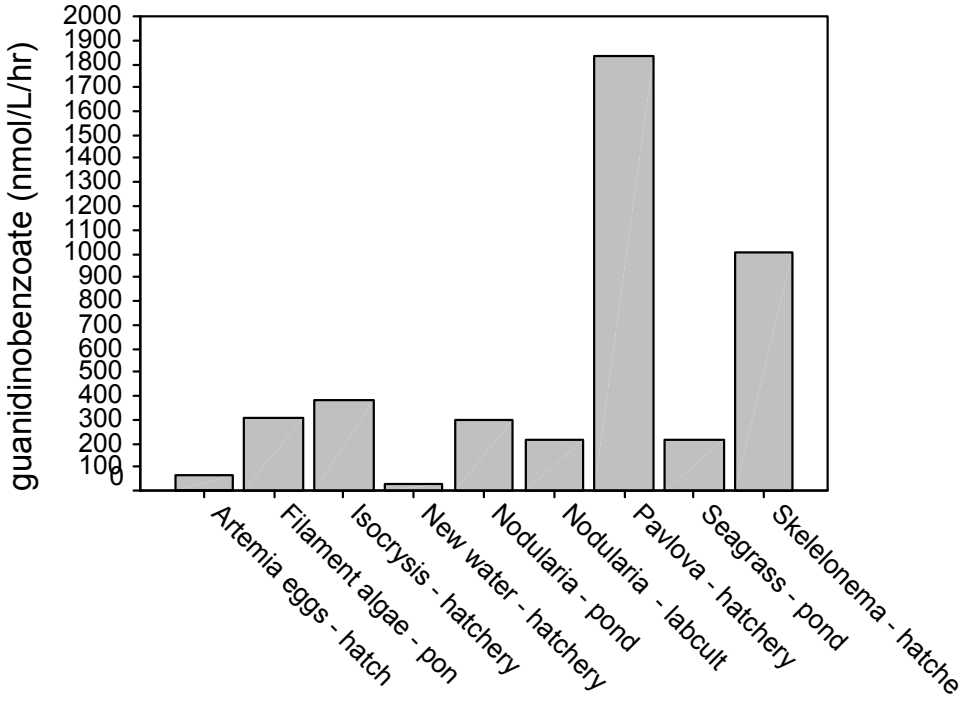
Bloom samples (from pond, hatchery or lab culture)

Fig 6.4.7k



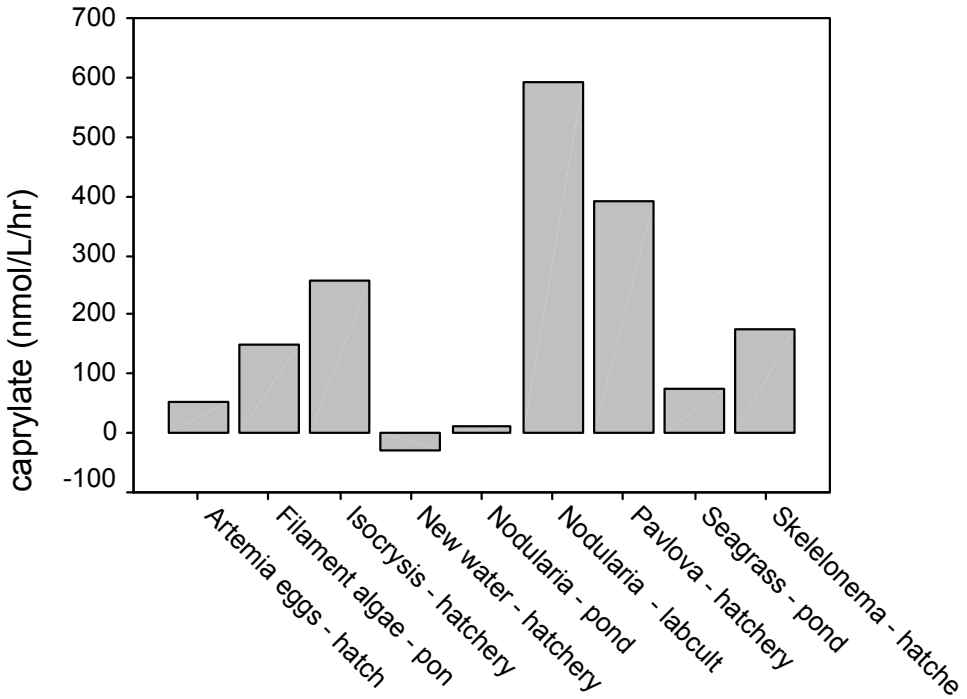
Bloom samples (from pond, hatchery or lab culture)

Fig 6.4.7l



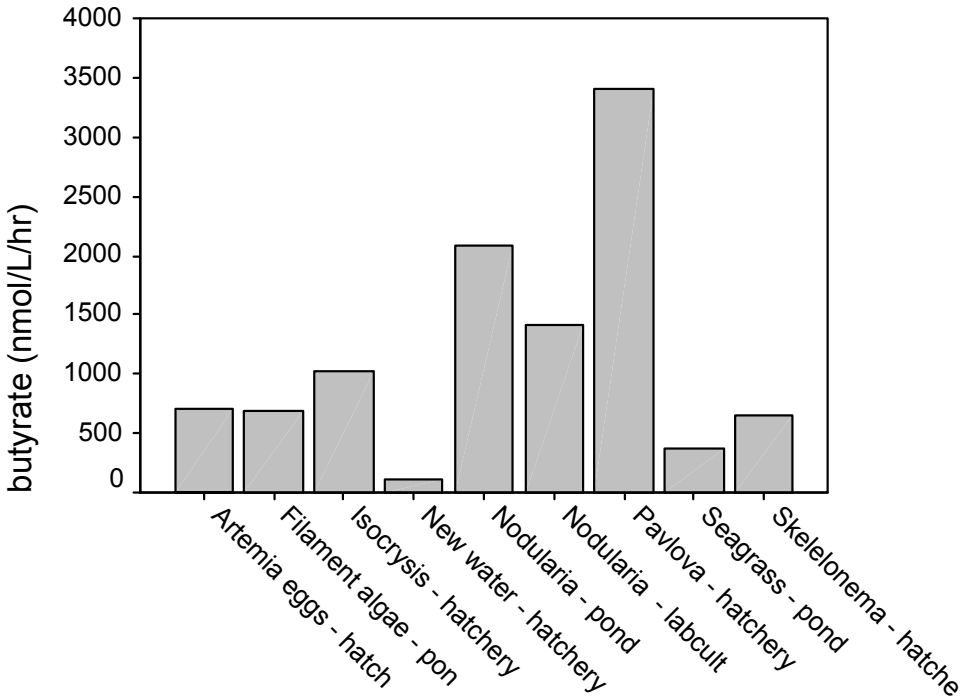
Bloom samples (from pond, hatchery or lab culture)

Fig 6.4.7m



Bloom samples (from pond, hatchery or lab culture)

Fig 6.4.7n



Bloom samples (from pond, hatchery or lab culture)

Fig 6.4.7o

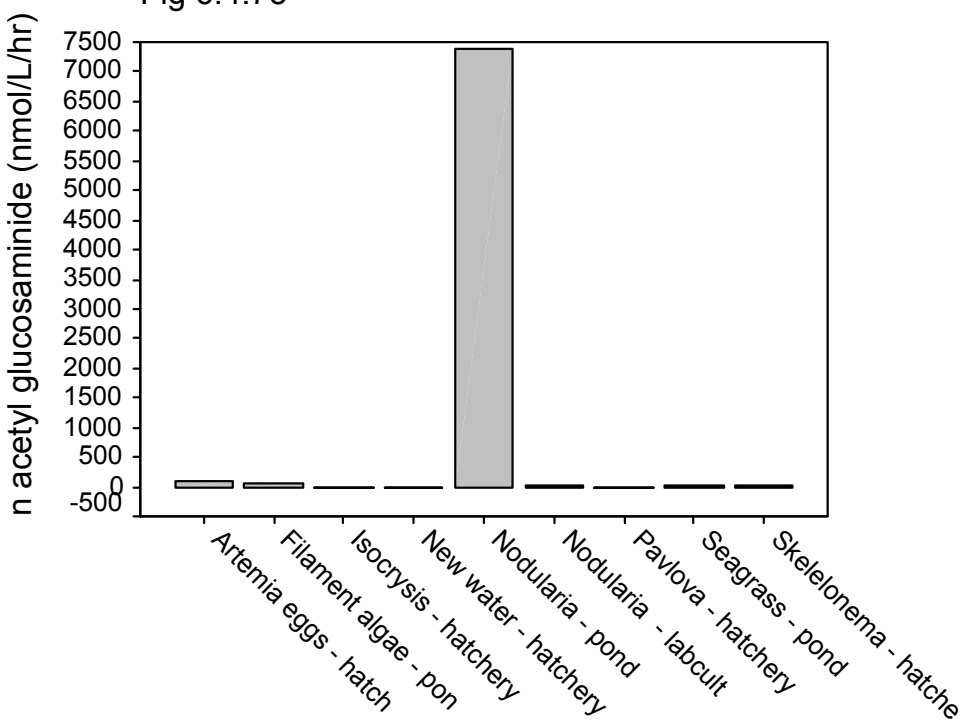


Fig 6.4.7p

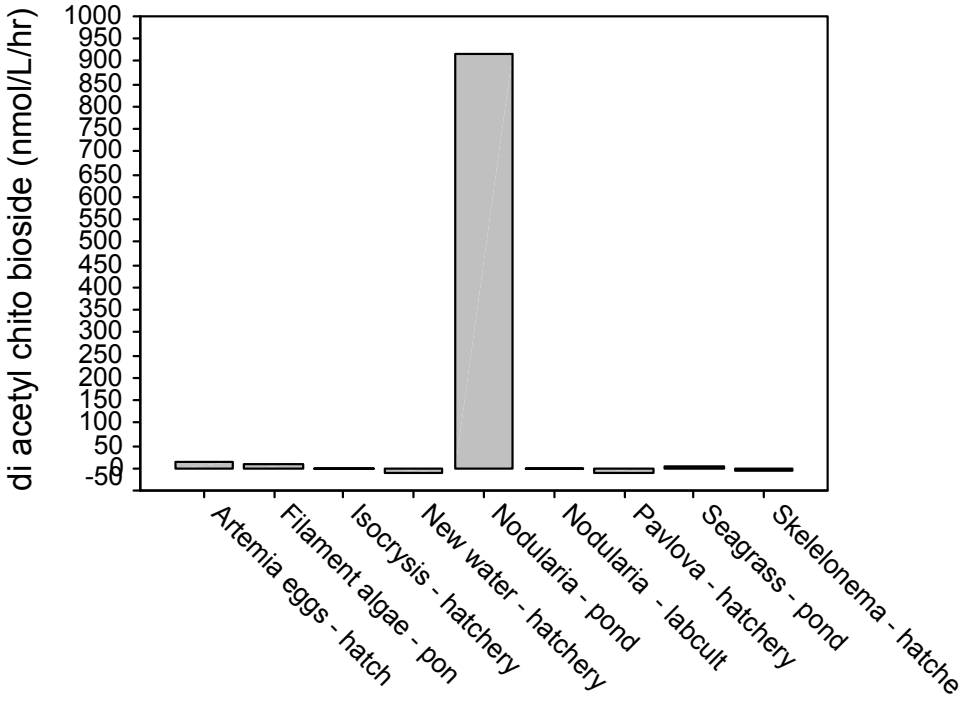
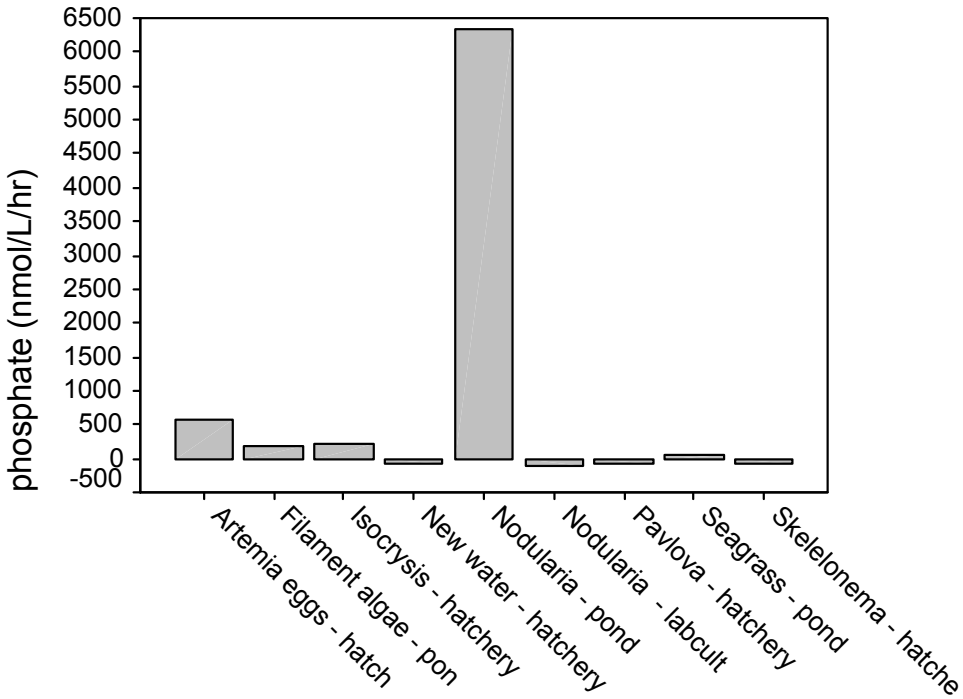
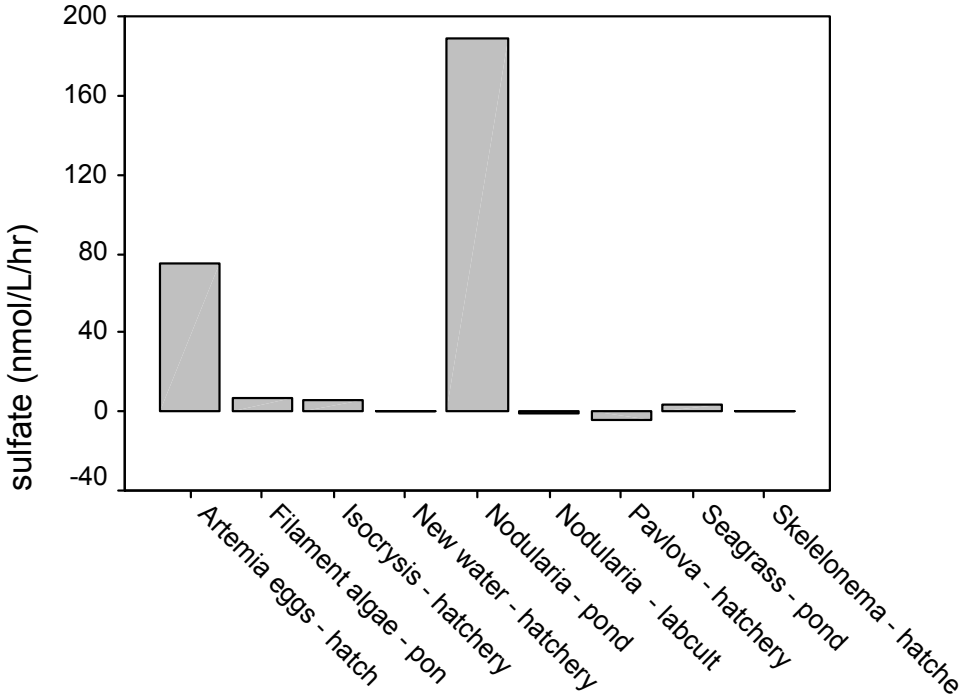


Fig 6.4.7q



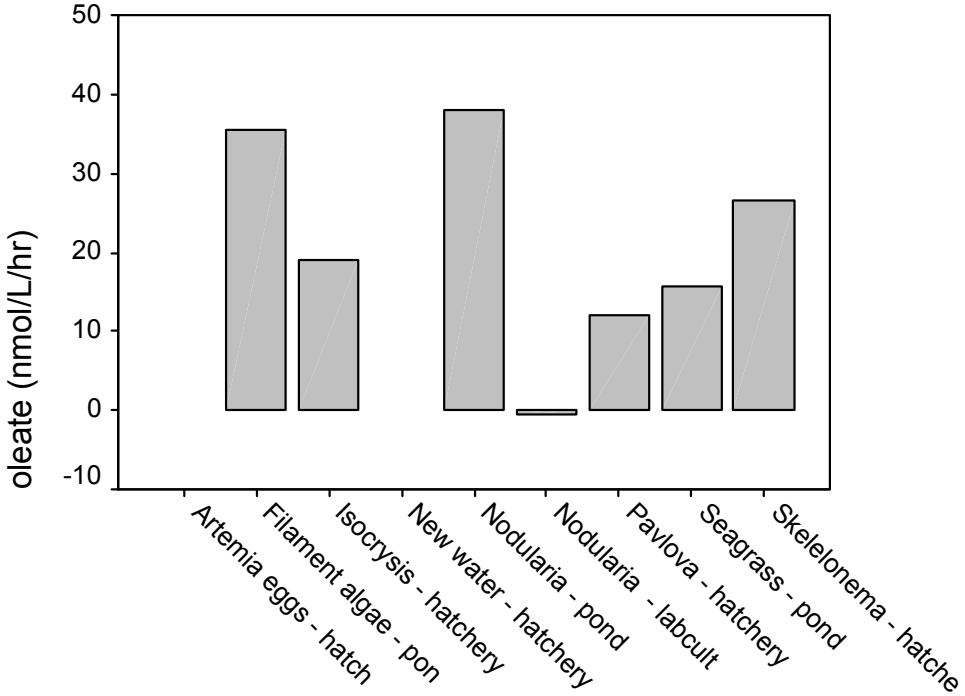
Bloom samples (from pond, hatchery or lab culture)

Fig 6.4.7r



Bloom samples (from pond, hatchery or lab culture)

Fig 6.4.7s



Bloom samples (from pond, hatchery or lab culture)

6.4.2 Vibriosis

6.4.2.1 Introduction

Vibriosis is a widespread disease of crustaceans that can affect prawns in hatcheries, nurseries and growout stages. It is caused by *Vibrio* bacteria such as *V. harveyi*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus* as well as a range of other species of vibrios. It is often called "Seagull disease" because moribund prawns usually come to the surface and edge of ponds where large numbers of seagulls and other birds take their fill. The clinical signs of vibriosis are: accumulation of Gram negative bacteria in tissues of prawns (as seen by histological examination), or measurement of high levels of *Vibrio* bacteria in haemolymph and tissue (as measured with TCBS agar). From the farmer's point of view, the signs of vibriosis are non-specific and include: a sudden loss of appetite, prawns resting at the edge of ponds, lethargy, reddening of appendages and body, necrotic cuticles (ie black marks on shell), fouling of the gills and body, and abnormal moulting.

In this study a number of cases of vibriosis were investigated by traditional methods of histology and plate counts. Also, extracellular enzyme techniques were used to examine the processes that pathogenic bacteria were carrying out. The findings provide a new way of understanding the aetiology and signs of the disease. Also, it provides a method for trying to minimize its occurrence.

6.4.2.2 Cases of vibriosis at prawn farms

During the project a number of types of vibriosis were observed and studied. Appendix 5 lists a summary report that was presented to the Australian Prawn Farmers Association in July 2000 and it contains descriptions of some of these forms of vibriosis. The following sections further describe the findings.

I) Septic Hepatopancreatic Necrosis (SHPN) was the first type of vibriosis that was investigated during the project and it occurred in a pond on a farm in Far North Queensland in November 1998. Prawns showed non-specific signs of disease, including a loss of appetite, so specimens were preserved and histological examination was carried out. Fig 6.4.2.1 is an example of sections through the hepatopancreas showing loss of its normal tubular structure and organization. Tubules were inflamed with masses of heamocytes that were often melanised and necrotic. Plaques (thick colonies) of Gram-negative bacteria were seen within the lumen (Fig 6.4.2.1). The histopathology was consistent with descriptions and photomicrographs by Lightner (1996).

II) Shell disease was the second, and more common, type of vibriosis that was investigated. This was characterized by black necrotic marks on the cuticle, appendages and tail fan (uropods) – see Fig 6.4.2.2a. Farmers usually referred to these forms of vibriosis as "winter marks, "lightning strikes" and "tail rot". This type of vibriosis acts slowly on prawns – causing a gradual loss of appetite, reduced growth and wasting. Also, it can significantly reduce the market price of affected prawns. Histological examination of diseased prawns revealed the presence of both Gram-negative and Gram-positive bacterial colonies on the cuticle (Fig 6.4.2.2 b,c). In fact

the bacterial colonies appeared to be digesting the cuticle and progressing into the underlying cellular layers. This observation suggests that the bacteria may be secreting extracellular enzymes – chitinase and protease – to breakdown the cuticle and underlying tissues. This aspect is examined further in the next section. Fig 6.4.2.2.d shows haemocytes infiltrating the tissue layers beneath the cuticle and Gram-negative bacteria lining the areas where breakdown of the tissue structure has occurred. Some of the nuclei of the haemocytes have undergone apoptosis (cell death).

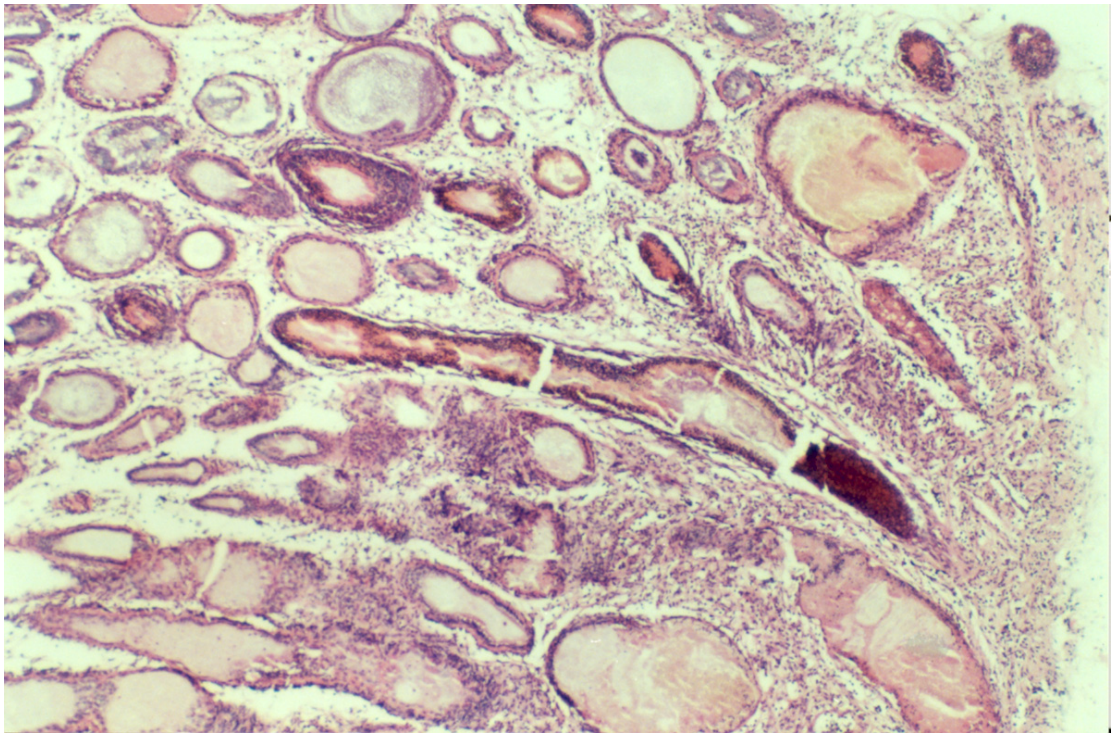
III) Gill disease such as "brown gill", "black gill" or "fouled gill", was a common problem and it can cause lethargy, loss of appetite, reduced growth and mortalities. Fig 6.4.2.3 shows comparisons between healthy and affected gills as seen with the Scanning Electron Microscope as well as views of biofilms and bacterial activity as seen with the Light Microscope. A biofilm of bacteria consisted of *Zoothamnium* spp., *Leucothrix*, mucous and accumulated debris. This fouling gives the appearance of brown or dirty gills. Staining with Alcian Blue confirmed that the detritus between the gills is embedded in a gel of mucous. The overall effect of these biofilms is to lower the dissolved oxygen level in the gills and, as a consequence, the dissolved oxygen in the haemolymph of the prawn. Also, examination of brown gills with the light microscope reveals that vibriosis occurs in the gills. Bacterial pathogens invade the surface of the gills and haemocytes infiltrate the gills in an immune response. As with shell disease, the bacteria are attached to the surface of the cuticle and it is most likely that they penetrate the chitin by secreting extracellular enzymes to breakdown the chitin.

IV) Eye disease was investigated and findings have already been published in "Diseases of Aquatic Organisms" (Smith 2000) (see Appendix 5). The results are further discussed in Section 6.4.4.

V) Broodstock and hatchery vibriosis was investigated at three hatcheries during the project. The results were presented in a summary report to the Australian Prawn Farmers Association in July 2001 (see Appendix 5). The most important findings were: a) the methods used to transport spawners were very stressful and conditions were conducive to development of vibriosis, b) spawner-holding tanks and artemia-culture tanks had unacceptably high levels of *Vibrio* spp. and high levels of chitinase enzymes, and c) the use of probiotics and UV pretreatment of intake water appeared to have no measurable positive benefits for larval tanks.

Fig 6.4.2.1 Septic Hepatopancreatic Necrosis (SHPN) of the hepatopancreas caused by *Vibrio* spp.

a) The hepatopancreas has lost its normal tubular structure (H&E stain 100x magnification).



b) The tubules of the hepatopancreas are surrounded by haemocytes and the lumen is filled with masses of Gram negative *Vibrio* bacteria (H&E x400).

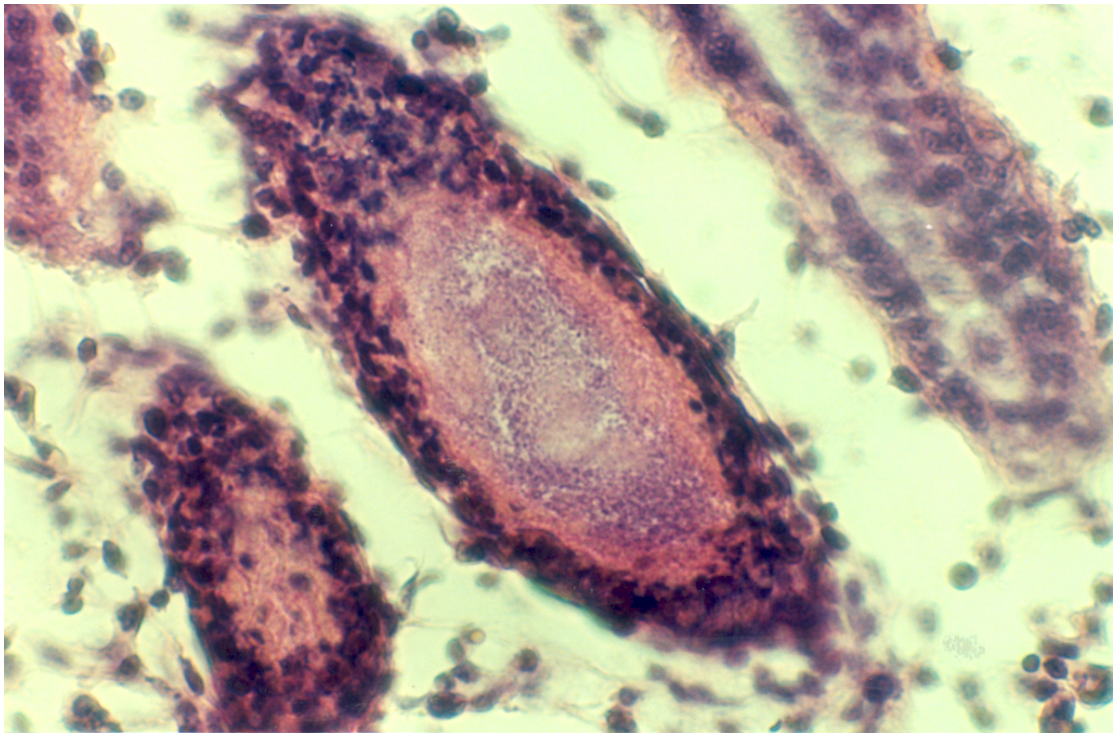
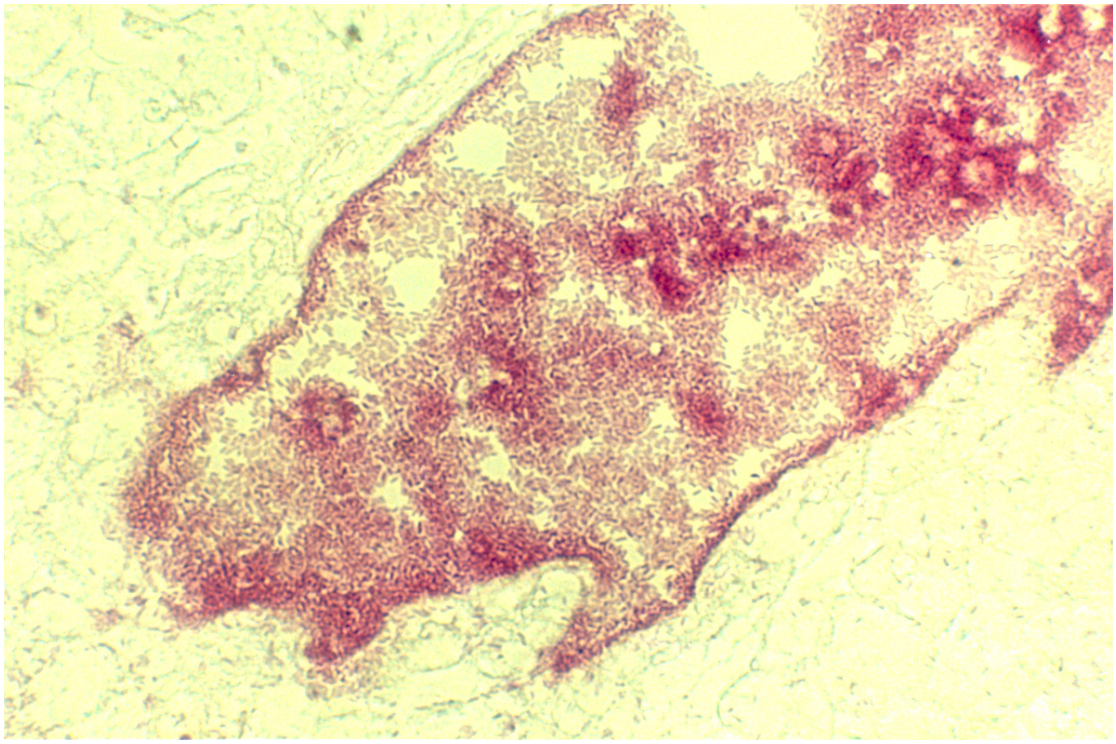


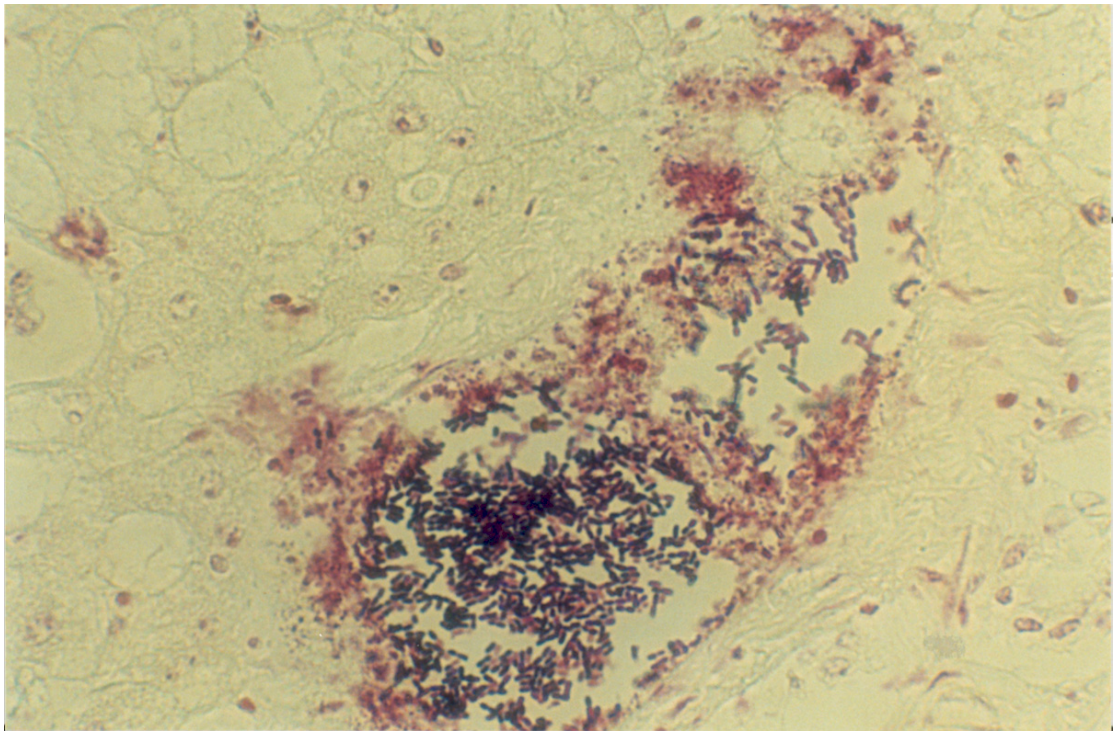
Fig 6.4.2.2 Examples of Vibriosis of the cuticle (shell-disease).

a) Three farmed prawns showing black necrotic marks on the shell (or cuticle). These marks are caused by *Vibrio* spp and other types of bacteria.

b) A colony of Gram-negative bacteria within the cuticular layer and tissue immediately beneath the cuticle (H&E stain 400x).



c) A mixed colony of Gram-negative and Gram-positive bacteria within the cuticular layer and underlying tissue (H&E stain 400x).



d) Haemocytes infiltrate the tissue that underlies the cuticle in an inflammatory response to the bacterial pathogens. Gram-negative bacteria line the large vacuoles of tissue damage (H&E 400x).

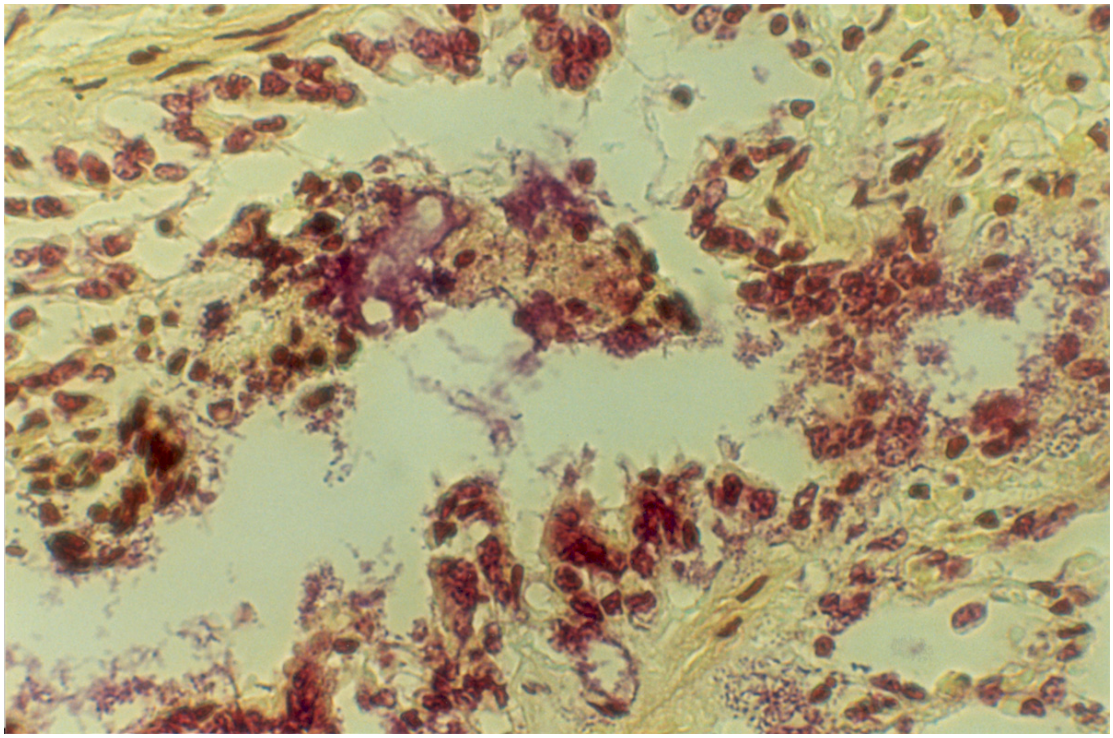
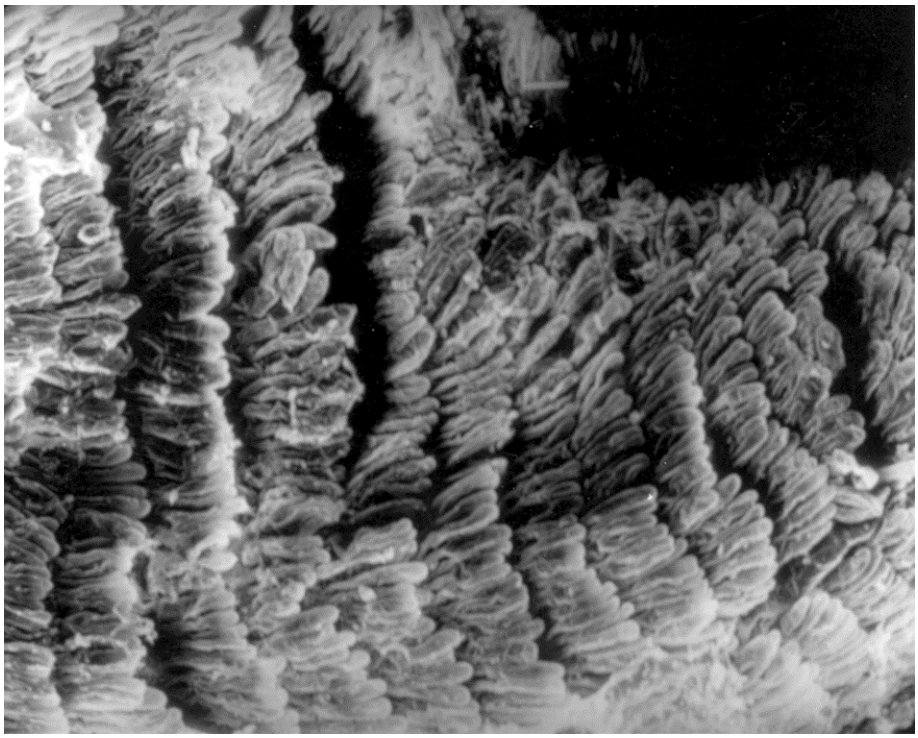
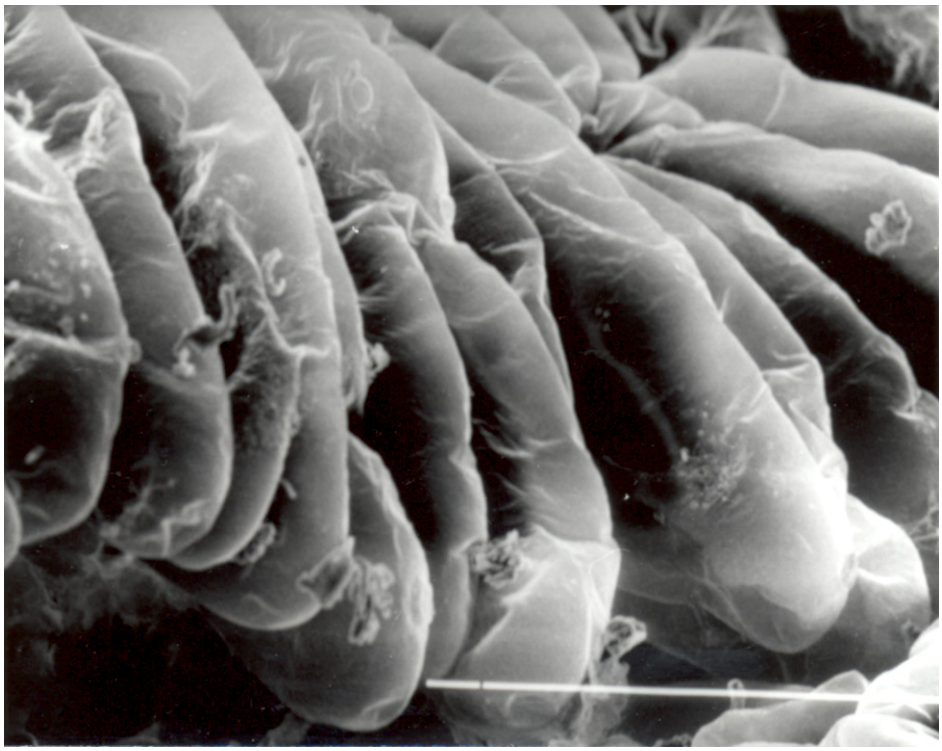


Fig 6.4.2.3 Appearance of gills under the Scanning Electron Microscope and Light Microscope

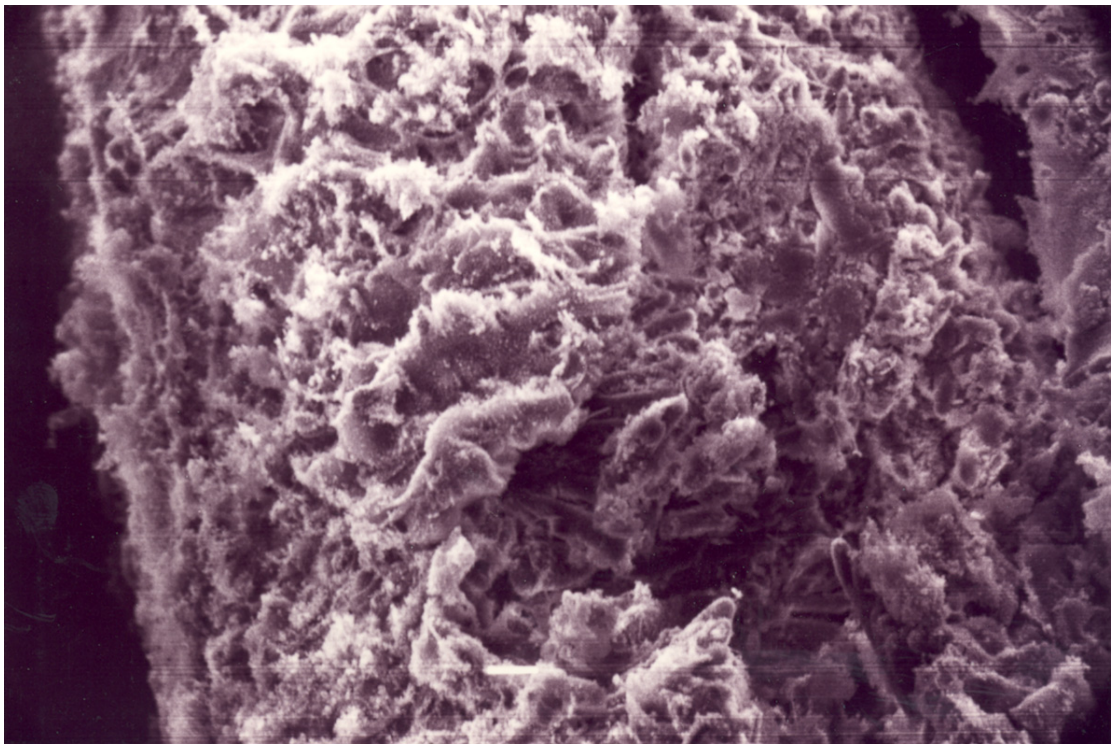
a) Healthy gills of *P. monodon* (Scanning Electron Microscope x100).



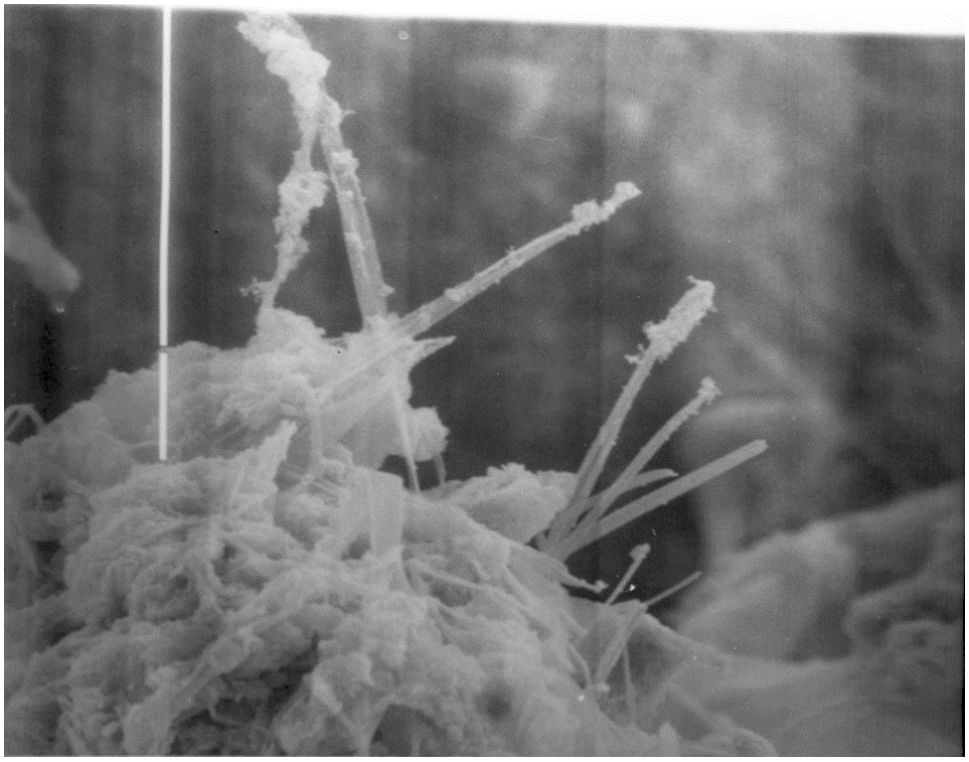
b) Healthy gills of *P. monodon* (Scanning Electron Microscope x2000).



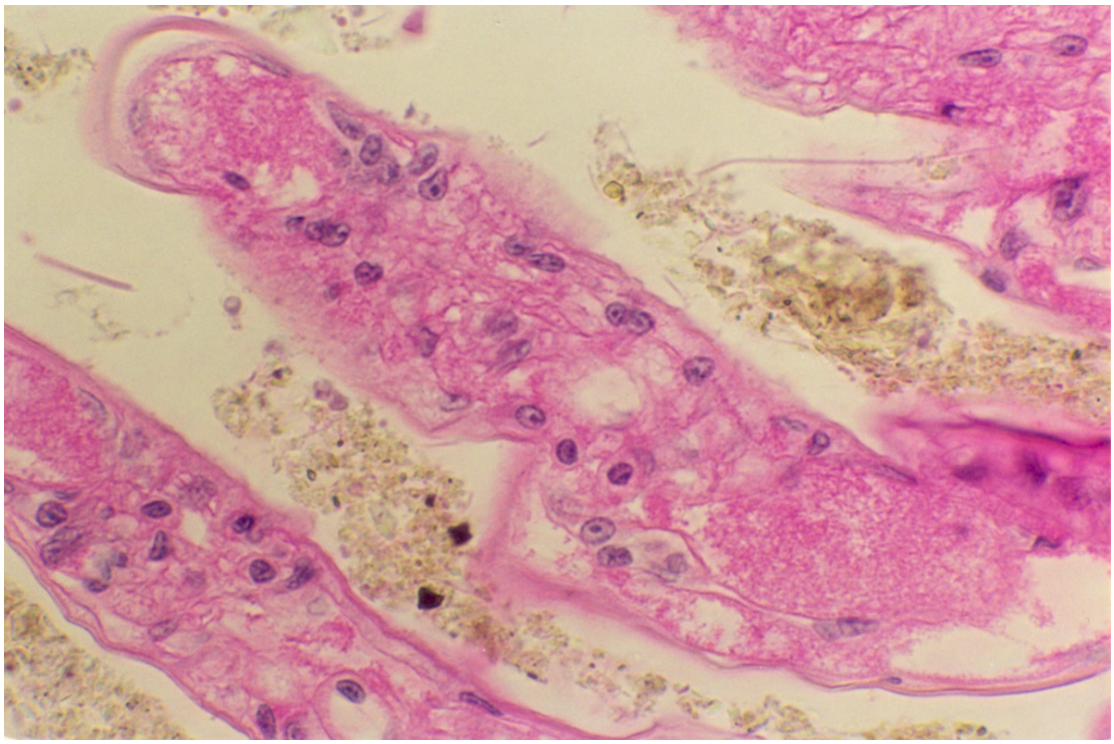
c) "Brown gills" of *P. monodon.*
Notice the biofilm almost completely covers the surface of the gills (Scanning Electron Microscope x100).



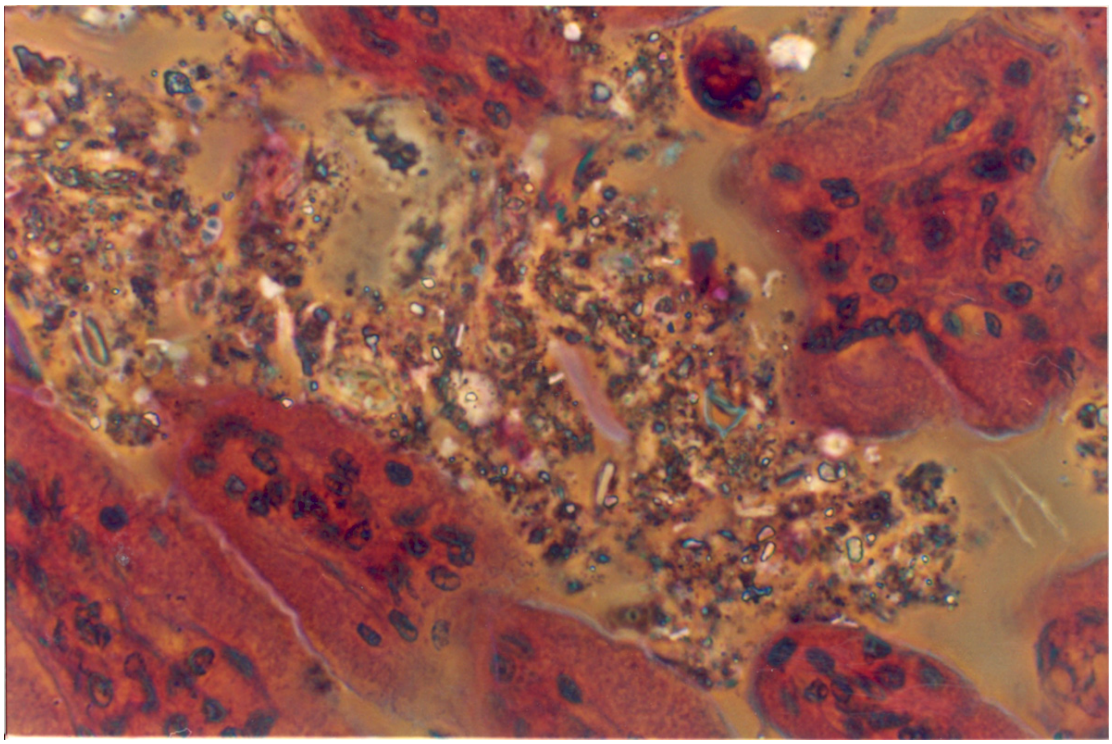
d) "Brown gills" of *P. monodon*.
A biofilm covers the gills making it impossible to see the surface of the gills clearly (Scanning Electron Microscope x2000).



e) A prawn with "brown gills".
Gill filaments are surrounded by a biofilm which contains bacteria, mucous and sediment particles (H&E stain 400x).

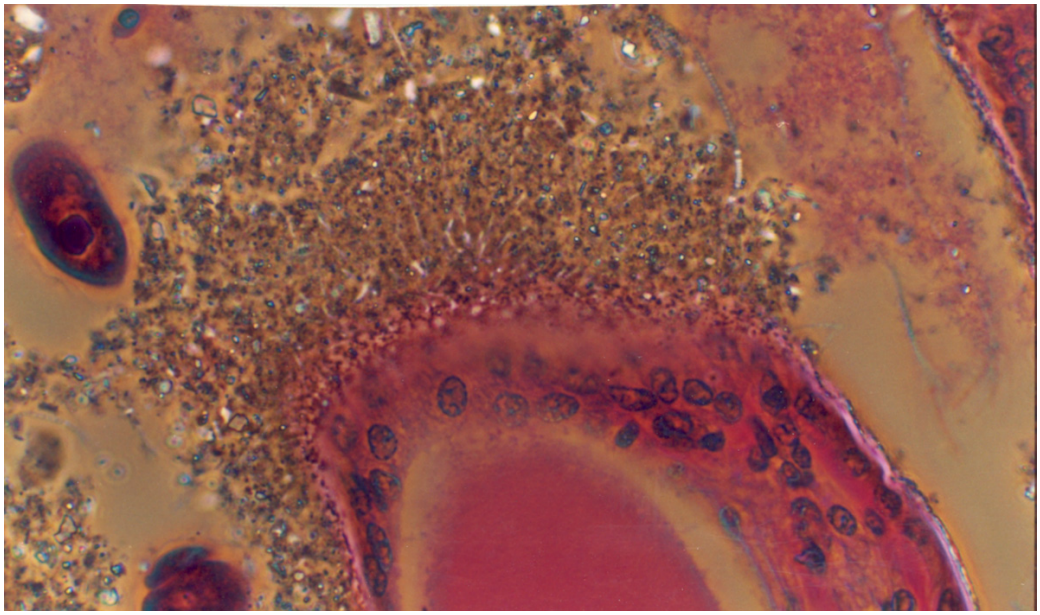


f) A prawn with "brown gills".
Gill filaments are surrounded by a biofilm which contains bacteria, mucous, *Zoothamnium* spp. (see dark red oval shaped object - top right), sediment particles and various types of microalgae (H&E stain 400x).



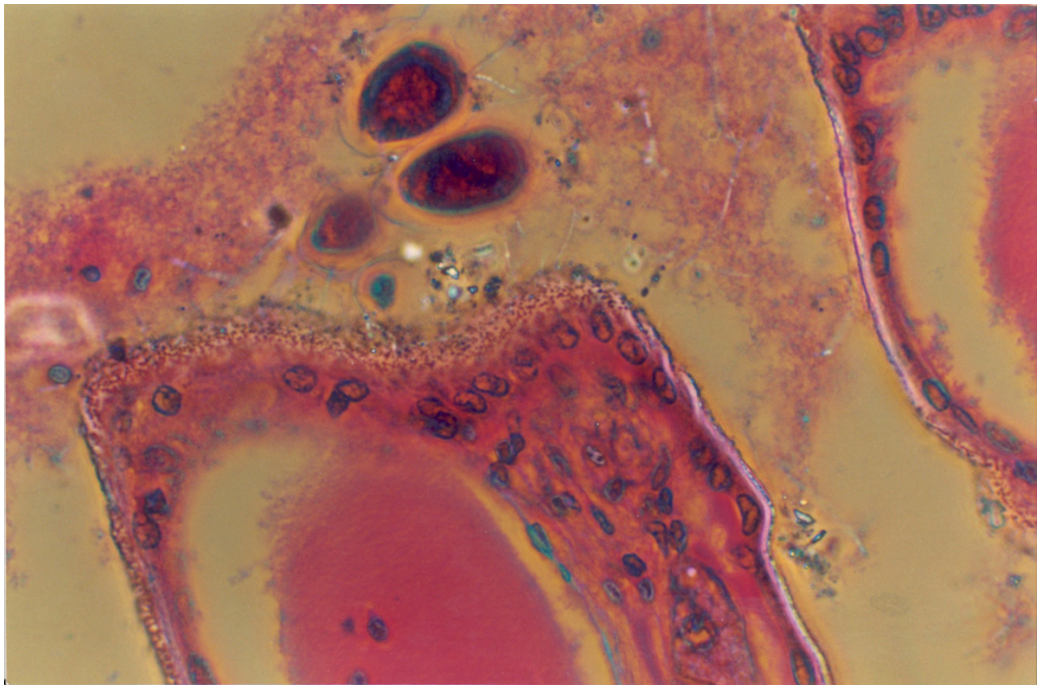
g) Gill filaments showing attachment of bacteria.

A biofilm of bacteria is attached to the surface of the gill filament. The immune response is characterized by haemocytes concentrating in the tissue beneath the site of attachment of the biofilm. *Zoothamnium* sp. can also be seen. (H&E 400x).



h) Gill filaments showing erosion of gill filaments by bacteria.

Erosion of the gill filaments is caused by bacteria, which are probably secreting extracellular enzymes to digest the chitin in the cuticle. Haemocytes concentrate in the underlying tissue and *Zoothamnium* sp. can be seen. (H&E 400x).



6.4.2.3 Extracellular enzyme profiles of vibrios and reference materials

Extracellular enzyme profiles were measured for prawn tissue, isolated cultures of bacteria and various reference materials. The results were very informative. Fig 6.4.2.4 provides a comparison of mean activities for 18 enzyme profiles for 10 different types of materials. The cyanobacterial bloom was included here because, as shown in Section 6.4.1, this type of bloom generally had the highest enzyme activities of all of the phytoplankton blooms that were investigated. Various types of prawn samples were tested throughout the project (n=8 to 12) and these included: prawn faeces, prawn moult, 'tail fan with tail rot' (ie vibriosis) and 'tail fan without tail rot' (no vibriosis). Tank detritus is composed of microalgae and bacteria – it accumulates overnight at the bottom of prawn tanks. Also, four types of presumptive *Vibrio* bacteria were selected as reference bacteria according to the colour of their colony on TCBS agar (ie black-green, green, pale green and yellow). These vibrios were cultured from water and sediment samples from ponds in Far North Queensland. The results are the mean activities for data for two colonies for each colour collected each week over a four-month period (n=16 to 18 for each type of *Vibrio*).

Fig 6.4.2.4 illlustrates some important new findings which are summarized as follows.

1) Enzyme activity for sugars (ie aglucoside, bglucoside, xyloside, galactoside, l-fucoside, glucuronide) were generally highest for the *Nodularia* bloom. Prawn faeces had relatively high enzyme activities for sugars, particularly for a-glucoside (ie starches) and galactoside. Of the three types of prawn shell that were investigated, highest activity was for a-glucosides and the highest activities for these tissues (in descending order) were generally: prawn moult, 'tail with tail rot', and 'tail - no rot'). Tank detritus had high levels of sugar enzyme activity, particularly xyloside and l-fucoside. Interestingly, the vibrios had very low sugar enzyme activities, except for extremely high activity by pale green vibrios to glucuronide (Note: This is the MUB substrate that is used as an indicator of *E. coli* and faecal contamination of waterways).

2) Enzyme activities for esterases and lipases (ie acetate, laurate, propionate, caprylate, butyrate) were within the same order of magnitude for most materials. The *Nodularia* bloom had lowest activity for caprylate and highest activity for propionate and butyrate breakdown. Prawn faeces had moderate to high esterase activity for all substrates except for propionate. For the 3 types of prawn shells, highest activity was generally (in descending order): prawn moult, 'tail –with tail rot', and 'tail –no rot'; though there were some exceptions. Tank detritus had enzyme profiles for esterases, and sugars, that were similar to those of *Nodularia*. The esterase activity of the four types of vibrios was unexceptional.

3) Protease activity (ie leucine, guanidinobenzoate) provided interesting information. *Nodularia* bloom had relatively high levels of activity and this has previously been assumed to be due to bacteria that are attached to the gelatinous sheath (Section 6.4.1). The tank detritus, which is an accumulation of microalgae and bacteria had protease activities that were similar to that of *Nodularia*. Prawn faeces also had high levels of protease activity. The 3 types of prawn shell had distinctly different protease profiles - prawn moult had the highest activity, followed by 'tail with tail rot'. The

'tail - no tail rot' had very low protease activity. The four types of vibrios had the lowest protease activities. The findings for protease activity are consistent with our conclusions that a) leucine is usually a better indicator of protease activity than guanodinobenzoate and b) protease activity tends to be a reliable bioindicator of the activity of degrading bacteria attached to prawn tissue.

4) Chitinase activity (ie n-acetyl glucosaminide, sulfo glucopyranoside, diacety chitobioside) was substantially different for each of the three substrates used. From our experience, the most reliable substrate for measuring chitinase activity is n-acetyl glucosaminide followed by diacety chitobioside. *Nodularia* had moderately high activites for two of the chitinase substrates, while tank detritus was relatively low for all three. Prawn faeces had high rates for two chitinase substrates. For the three types of prawn shells, 'tail – with rot' and prawn moult had the highest activites for n-acetyl glucosaminide. 'Tail no tail rot' had very low chitinase activities for all substrates. As for the four types of vibrios, the chitinase activites were within the same order of magnitude (ie 2,000-4,000 nmol/L/hr) for all three substrates. These findings are consistent with the view that n-acetyl glucosaminide is generally a better substrate for determining chitinase activity. Importantly, the results reinforce the hypothesis that pathogenic bacteria, particularly those associated with shell disease (eg tail rot), have high levels of chitinase activity.

5) Phosphatase activity and sulfatase activity were highest in prawn moult, prawn faeces and *Nodularia* bloom. Once again 'Tail – with tail rot' had significantly higher levels of enzyme activity than 'Tail - no tail rot'. All vibrios had phosphatase activities of approximately 1,000 nmol/L/hr and very low sulfatase activity.

6.4.2.4 Conclusions about vibriosis

From the various aspects of the study of vibriosis a number of important conclusions and hypotheses developed. The three most important ones are summarized here.

- The histological study of shell disease demonstrated that colonies of bacteria were attached to the surface of the cuticle and it appeared that the bacteria were secreting enzymes to digest the chitin. The findings from extracellular enzyme studies supported this hypothesis. The substrates, n-acetyl glucosaminide and diacety chitobioside were reliable, key bioindicators of high levels of chitinase activity in the cuticle of prawns with tail rot as well as prawn moults and prawn faeces. In comparison, shells without any obvious signs of tail rot had levels of chitinase activity that were 100x lower than those with tail rot.

- The study of the eye provided a novel approach to understanding the signs of disease in prawns. Because the eye is of central importance in the endocrine system (ie secreting hormones and neurosecretions), it is proposed that any bacterial infections (or viral infections) that affected the eye can evoke a range of clinical signs of disease. This hypothesis is more fully developed in a paper published by Disease of Aquatic Organisms (see Appendix 5) and the discussion in Section 6.4.4.

- From the work on the bacteriology of hatcheries we concluded that wild spawners were subjected to stress from poor methods of transport and inadequate techniques in animal husbandry. The substrates, n-acetyl glucosaminide, diacety chitobioside and sulfo-glucopyranoside proved to be reliable, key bioindicators of the high levels of chitinase activity to which captive spawners are subjected. This problem leads to development of bacterial disease and possibly the triggering of latent viral diseases. The outcome being that fertility of captive spawners is significantly inhibited and survival of larvae reduced. In light of the well-known hiatus in supply of quality wild spawners and PLs, it is crucial that a bacterial study be carried out on the problems in spawner transport, handling and husbandry. The main aim of that study would be to find solutions to the problems that we have identified. The benefits to the industry would be an improved supply of healthy, fertile spawners and robust larvae.

Fig 6.4.2.4 Means Plots of extracellular enzyme activities of Vibrios and reference materials

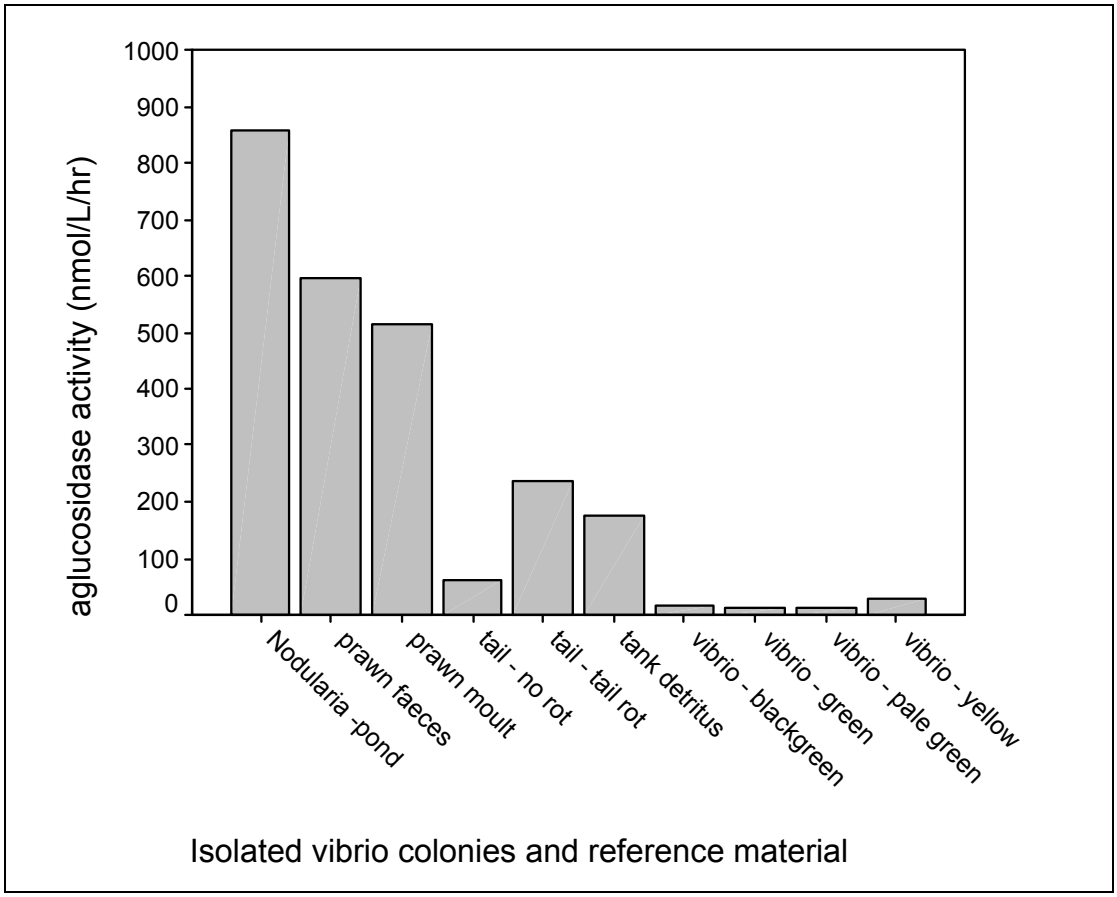
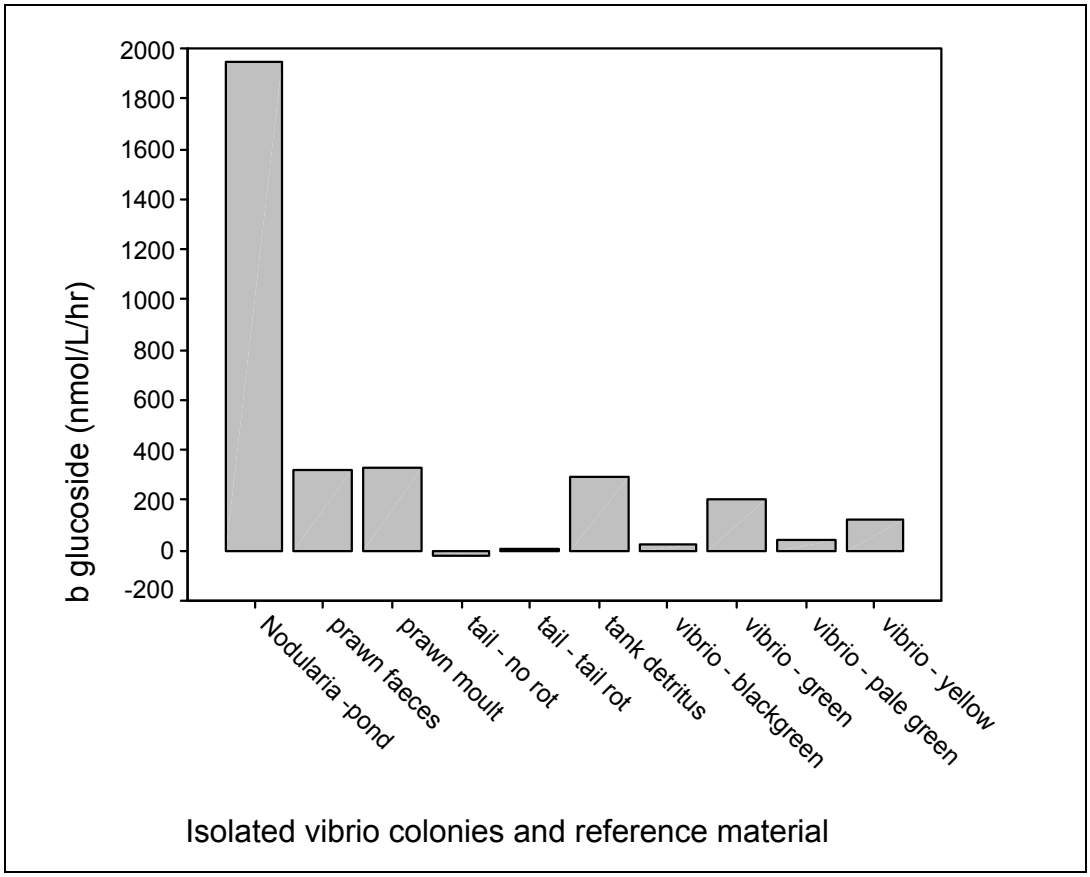


Fig 6.4.2.4 Means Plots of extracellular enzyme activities of Vibrios and reference materials (continued).



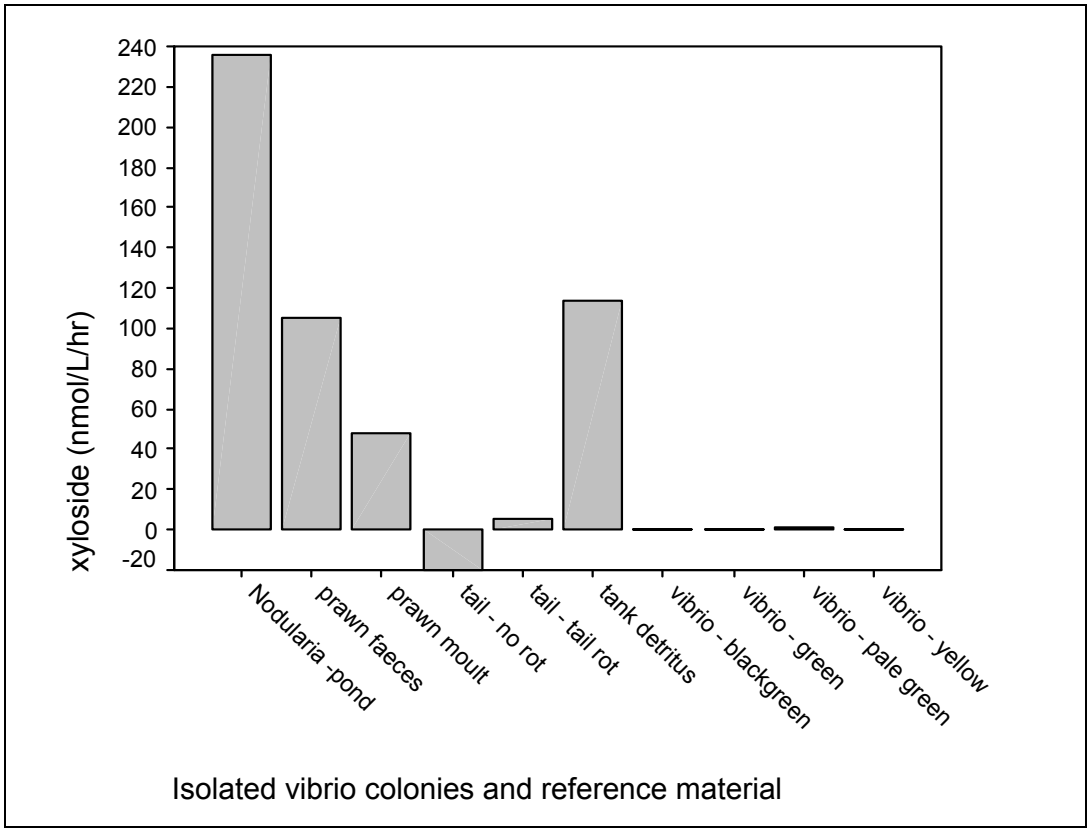
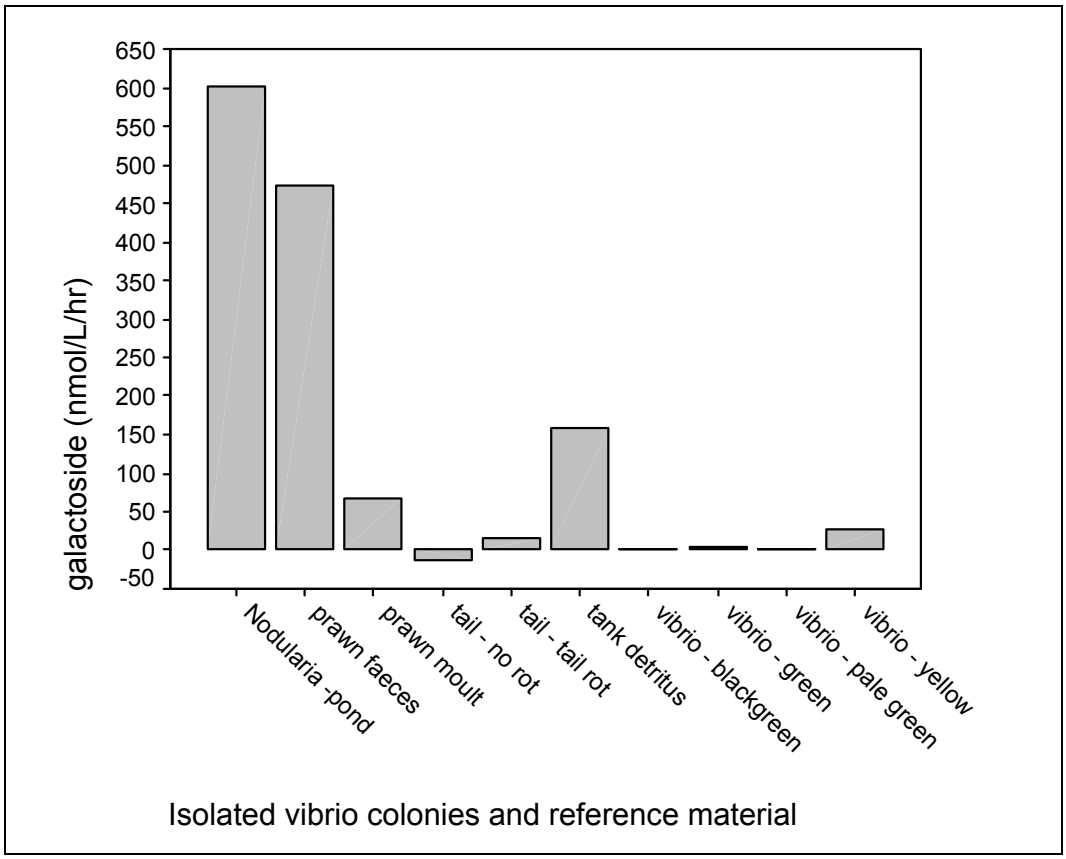


Fig 6.4.2.4 Means Plots of extracellular enzyme activities of Vibrios and reference materials (continued).



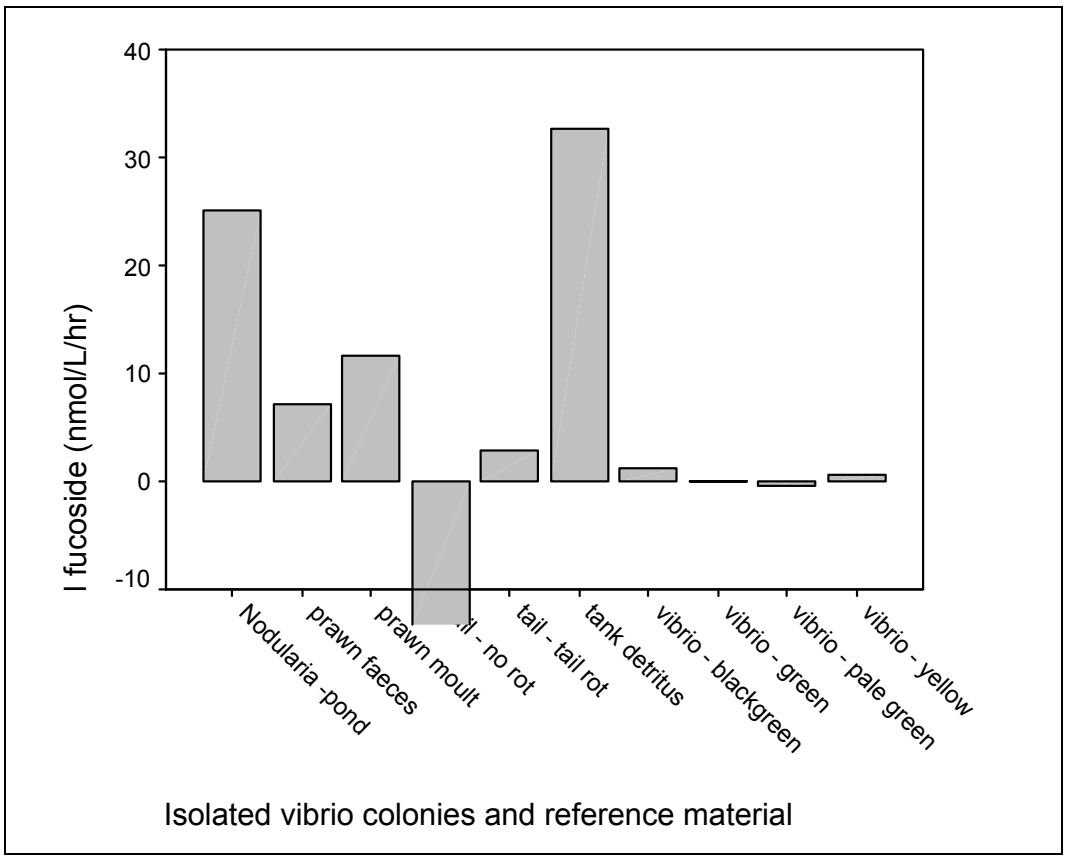
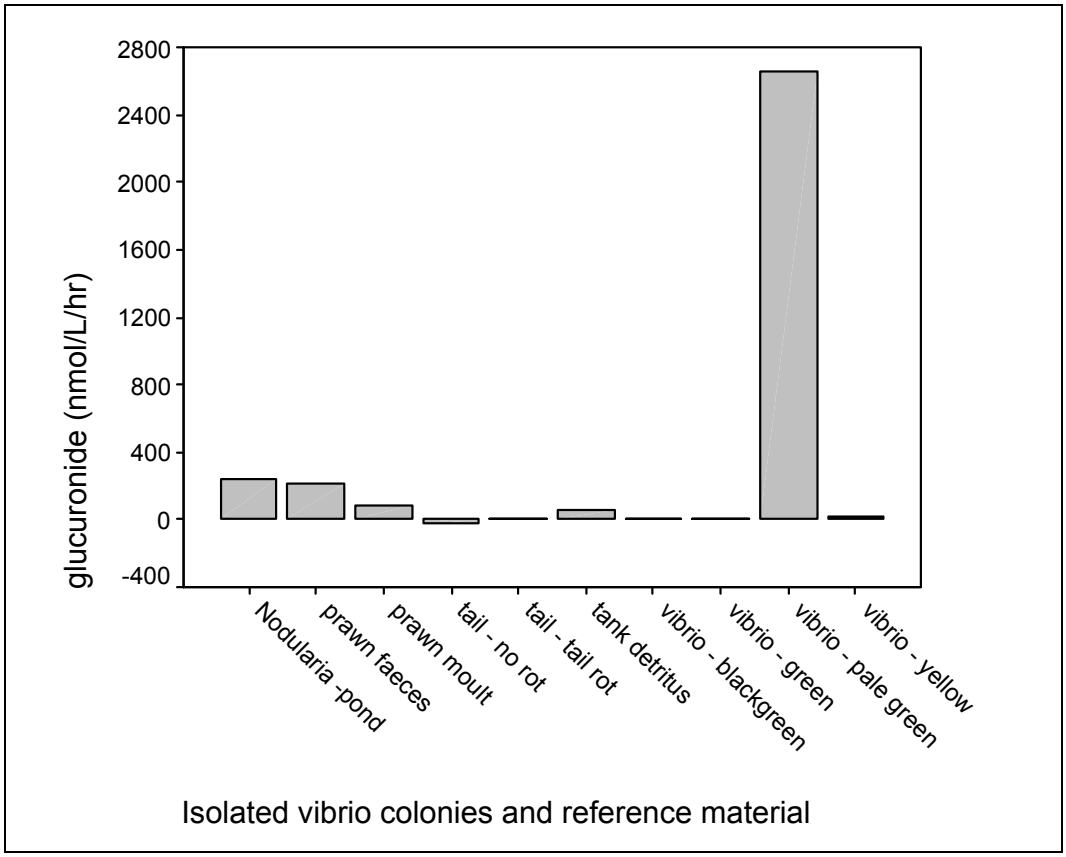


Fig 6.4.2.4 Means Plots of extracellular enzyme activities of Vibrios and reference materials (continued).



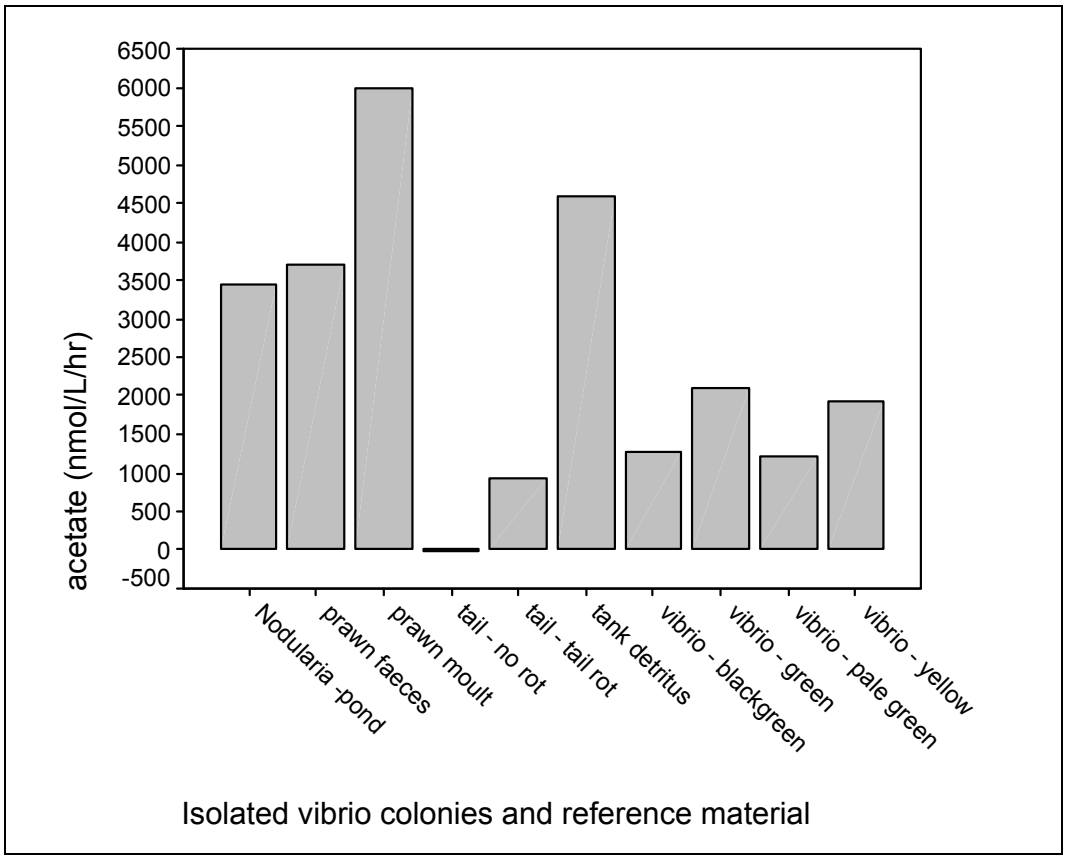
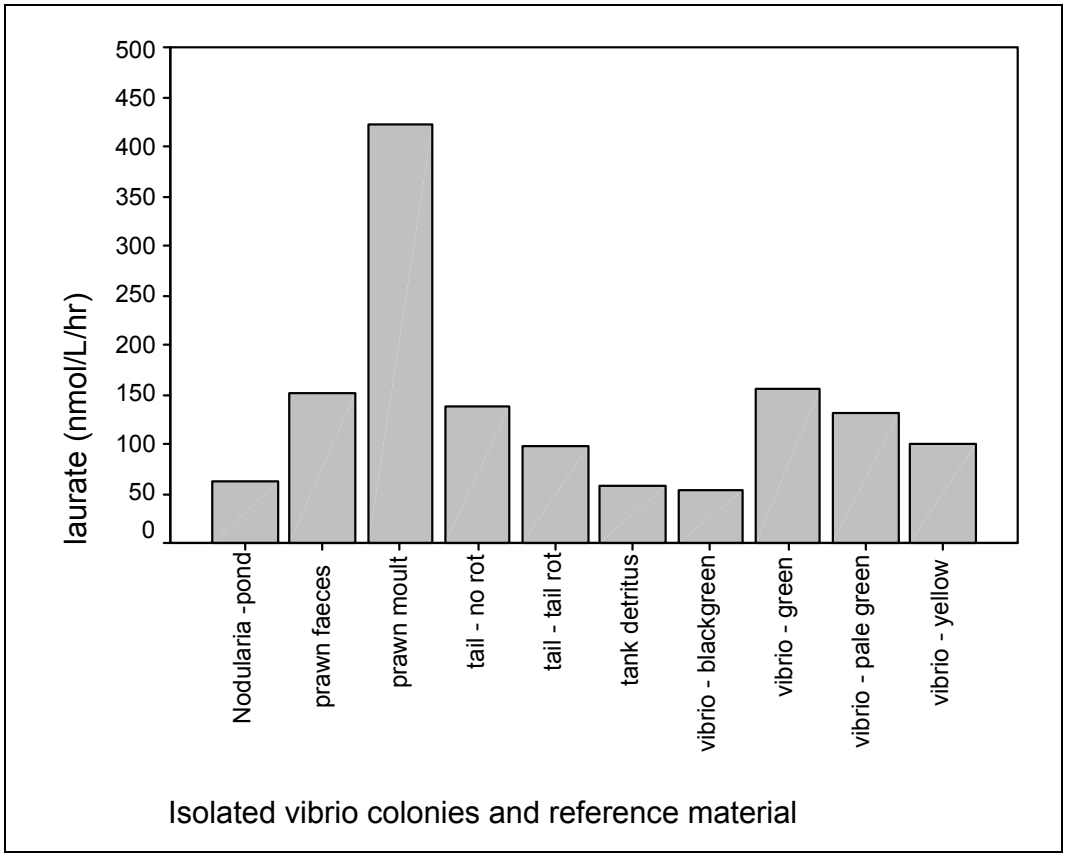


Fig 6.4.2.4 Means Plots of extracellular enzyme activities of Vibrios and reference materials (continued).



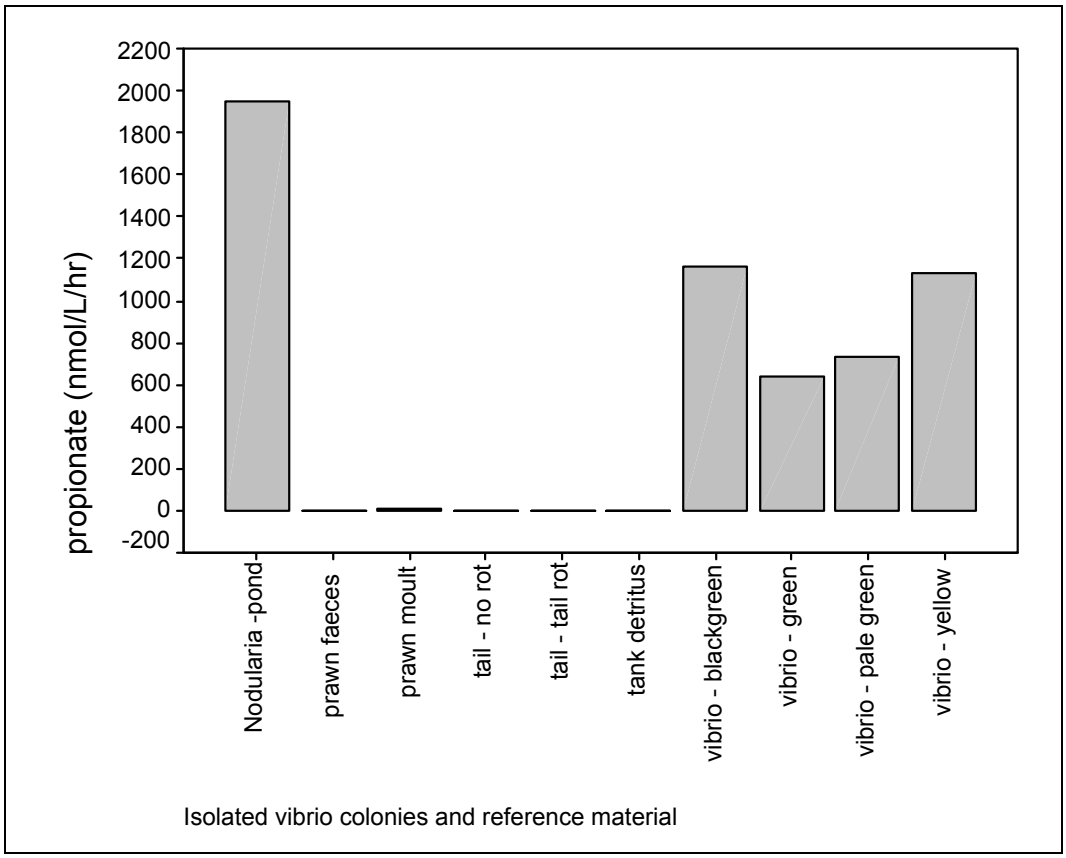
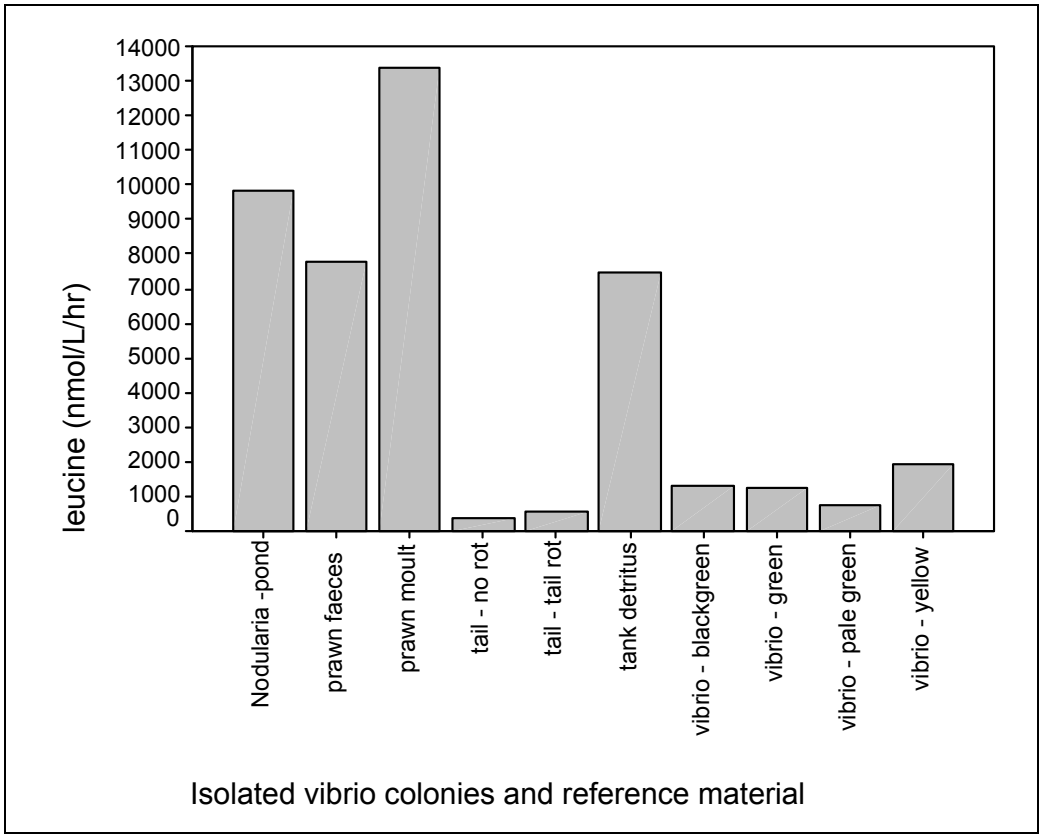


Fig 6.4.2.4 Means Plots of extracellular enzyme activities of Vibrios and reference materials (continued).



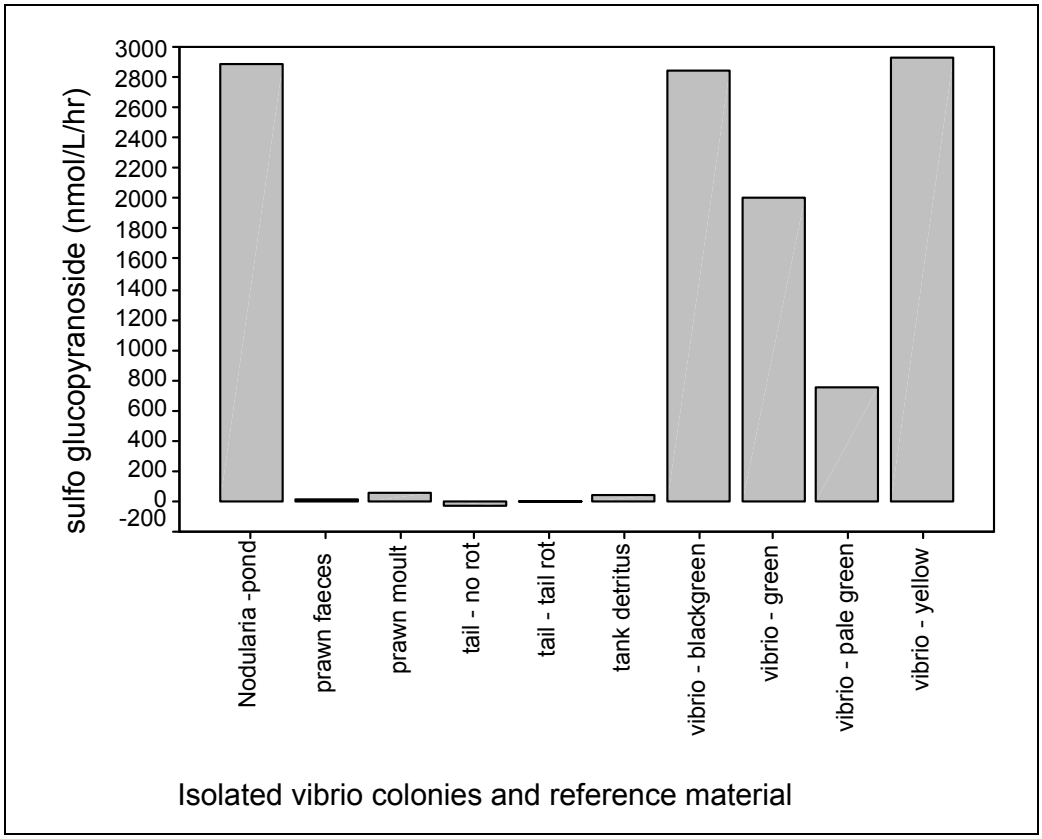
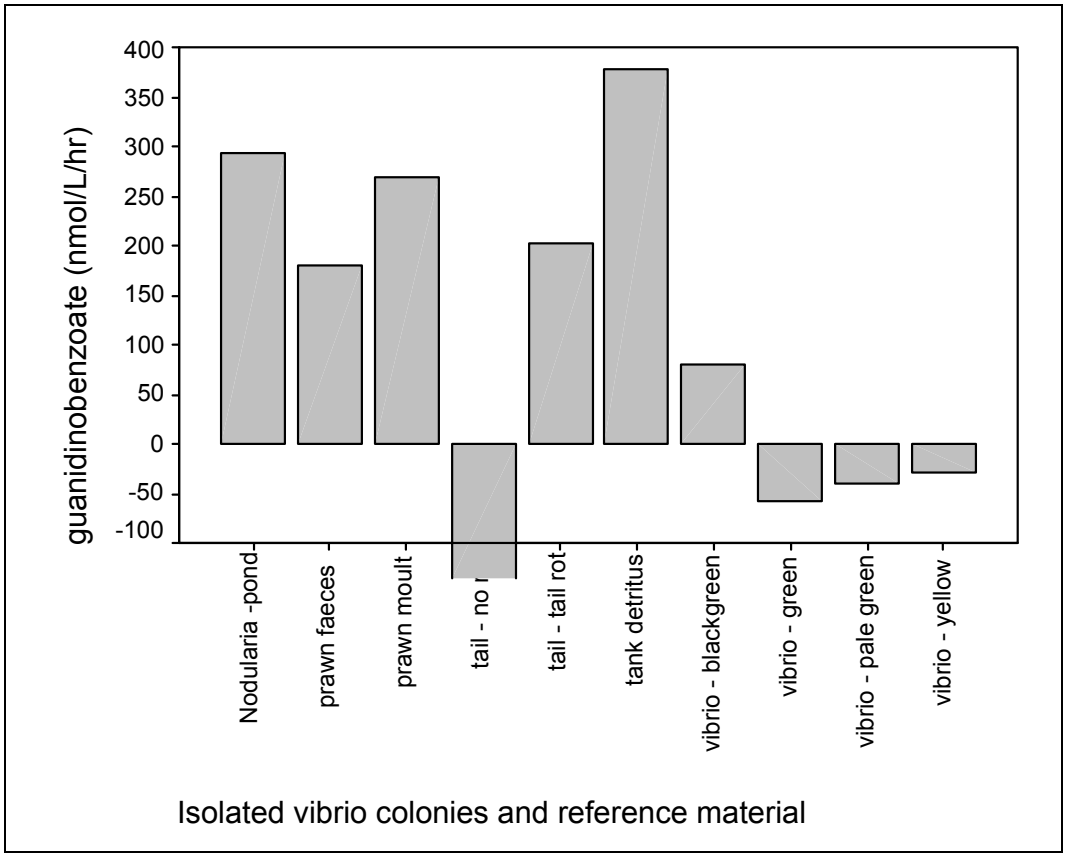


Fig 6.4.2.4 Means Plots of extracellular enzyme activities of Vibrios and reference materials (continued).



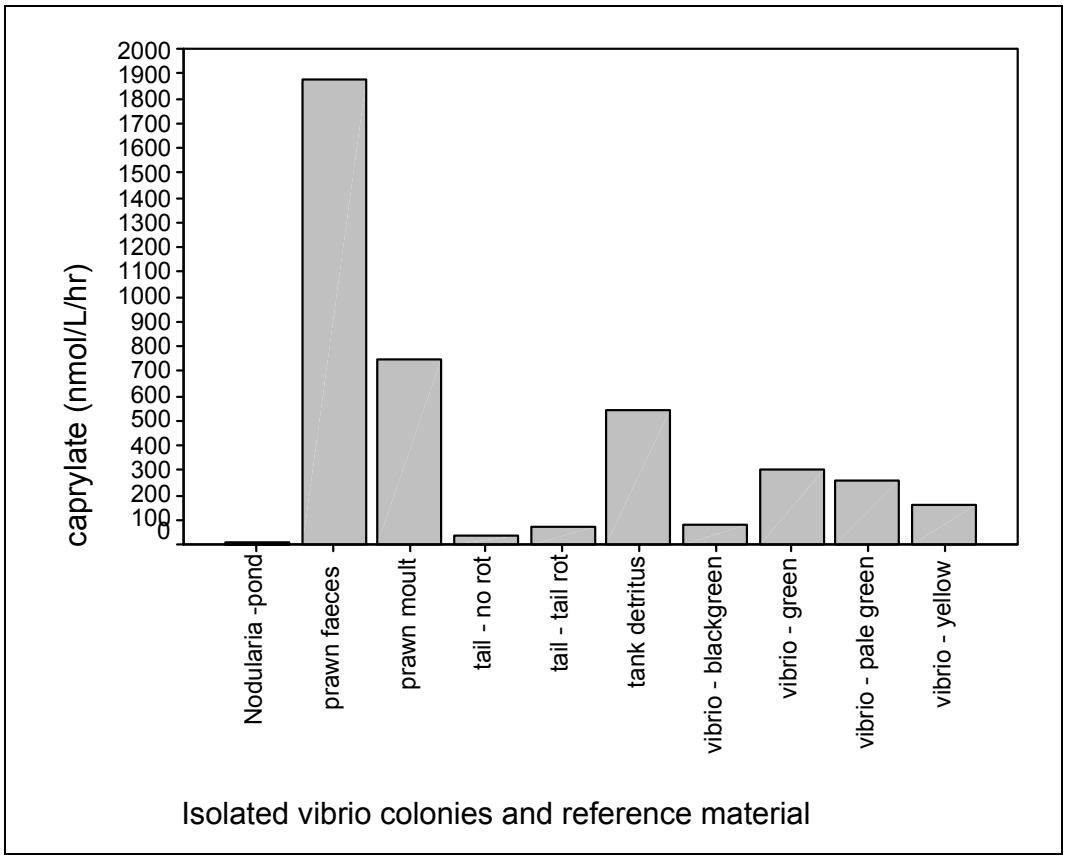
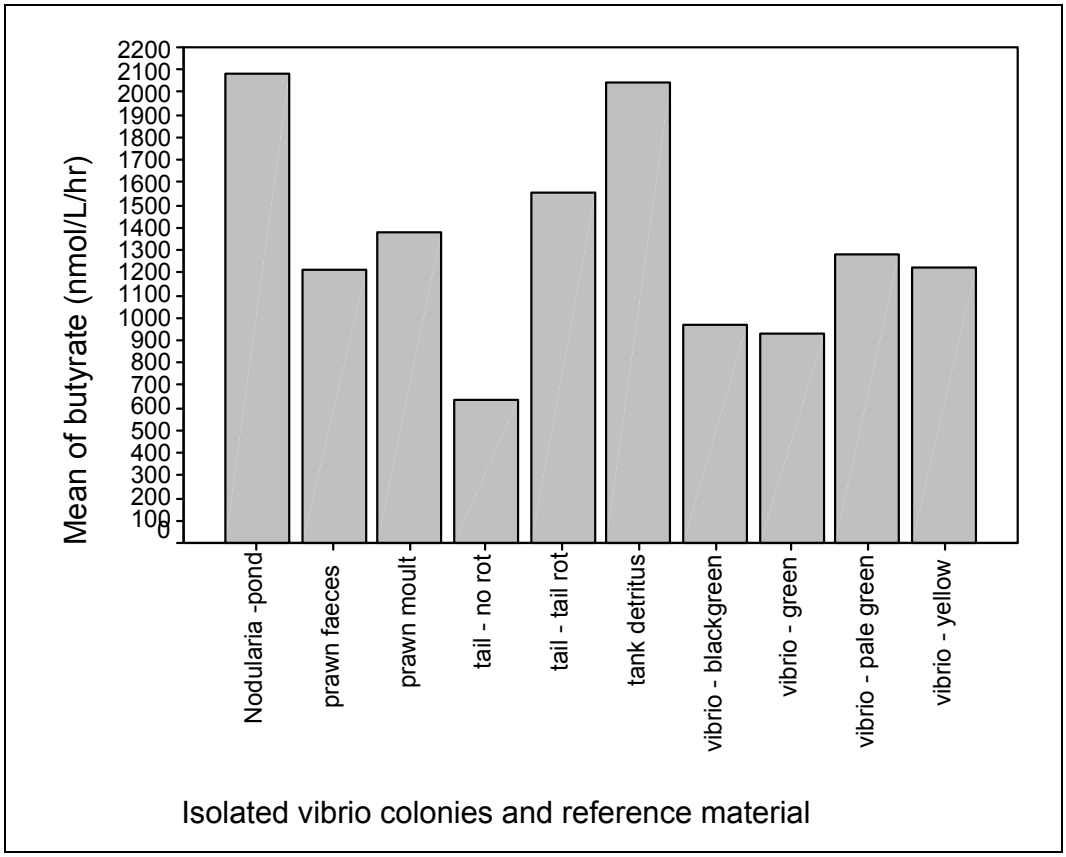


Fig 6.4.2.4 Means Plots of extracellular enzyme activities of Vibrios and reference materials (continued).



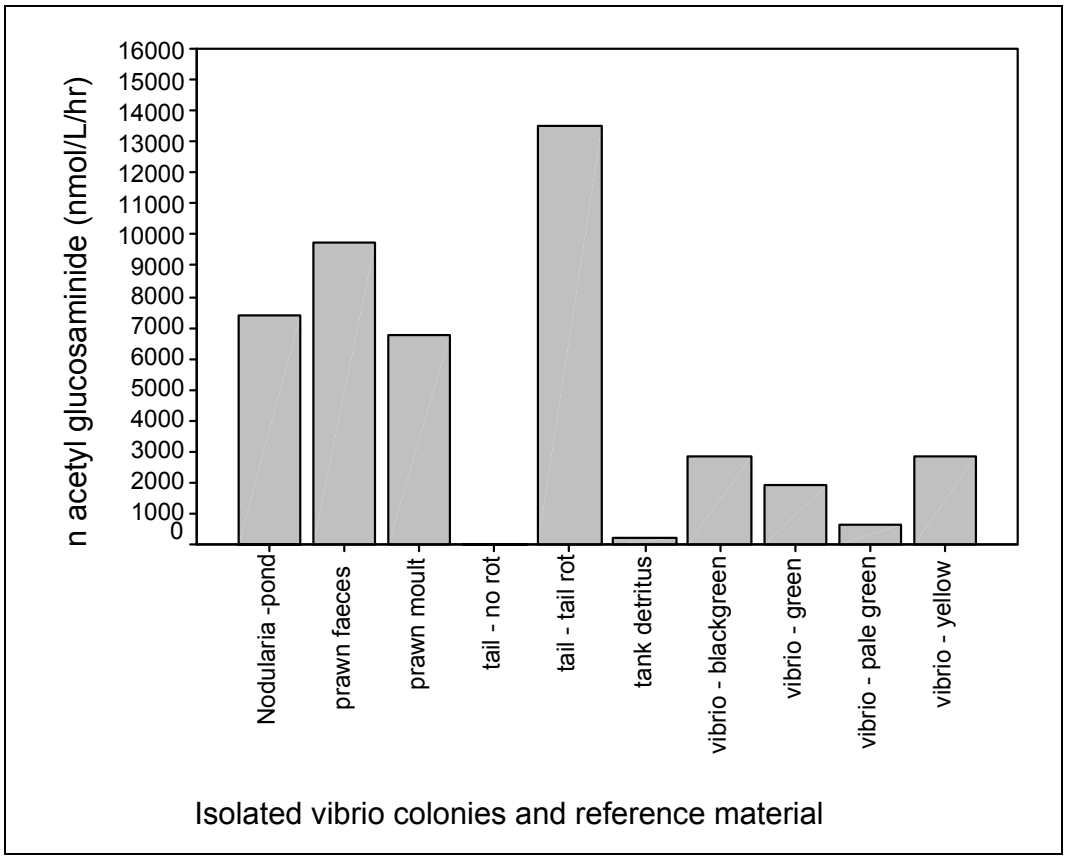
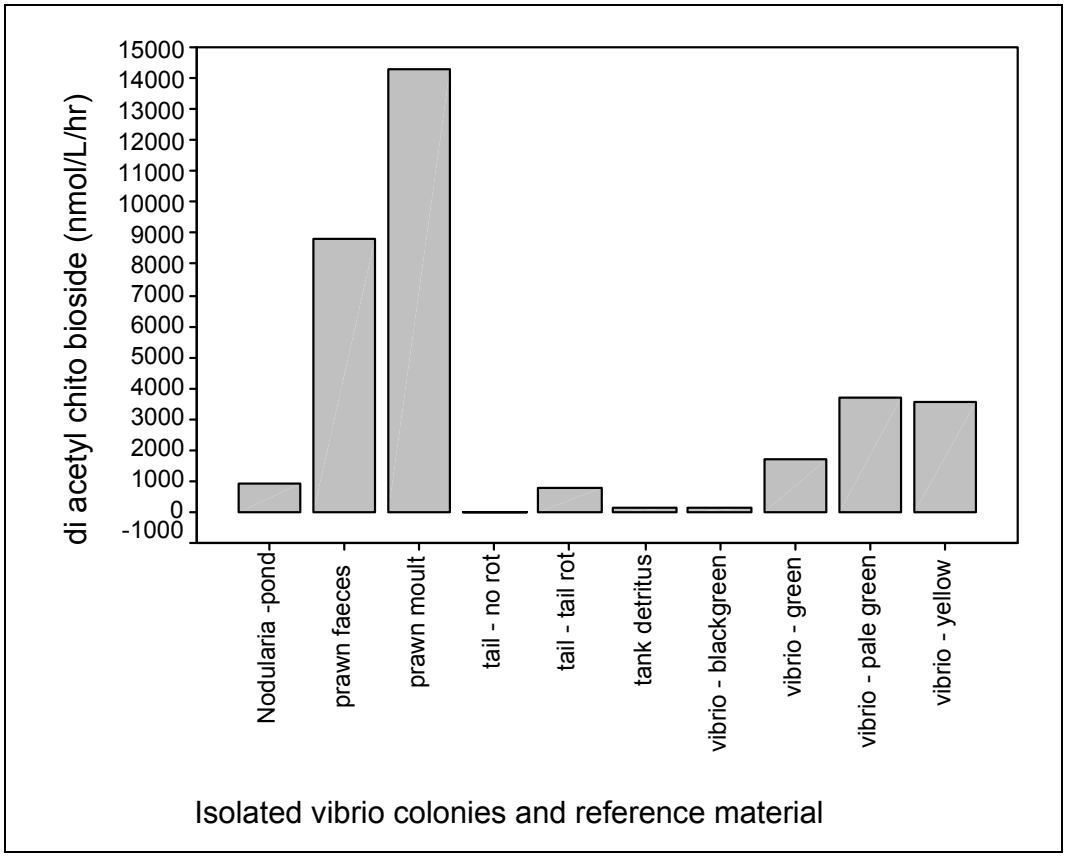


Fig 6.4.2.4 Means Plots of extracellular enzyme activities of Vibrios and reference materials (continued).



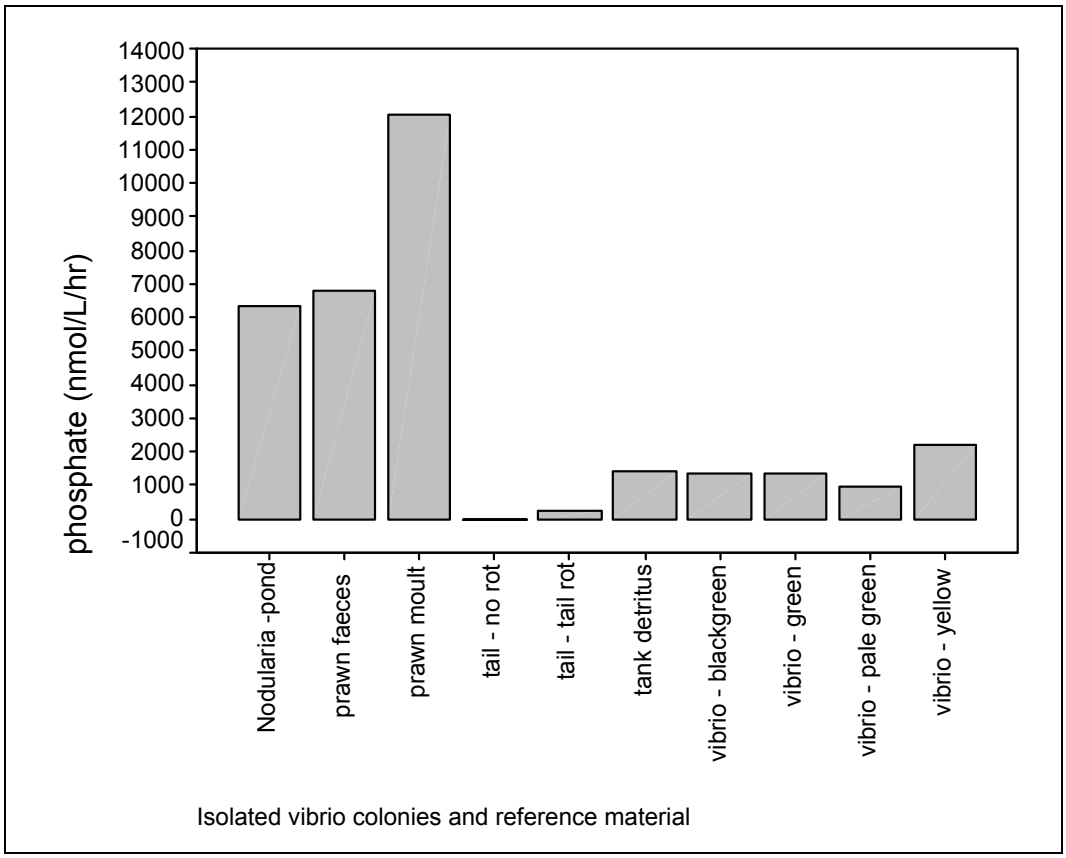
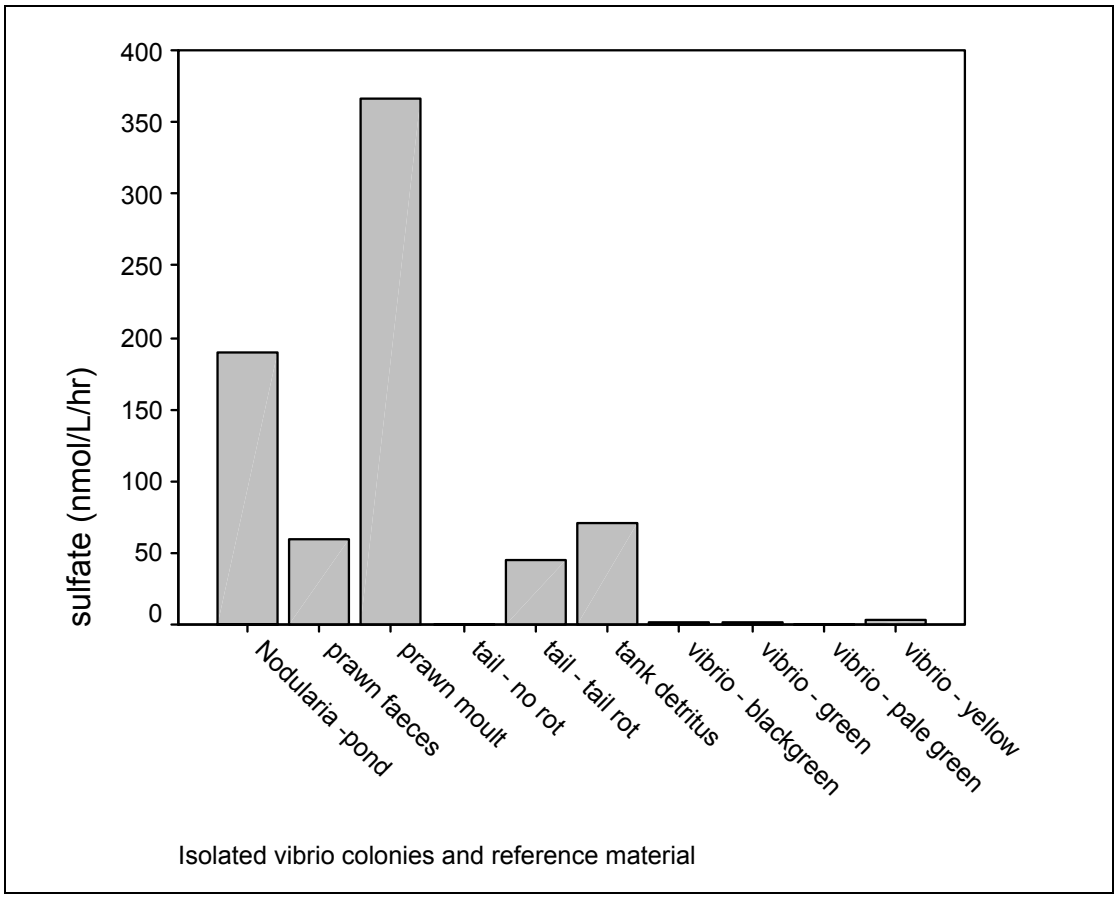


Fig 6.4.2.4 Means Plots of extracellular enzyme activities of Vibrios and reference materials (continued).



6.4.3 Viral diseases

a) Background

Gill-associated virus (GAV) is an enveloped, rod-shaped RNA virus that was first detected in *Penaeus monodon* in Australia (Spann, 1997) in 1995-1996. Lymphoid Organ Virus (LOV), first observed in 1994 (Cowley, 1999), appears to be the non-lethal form of GAV (Cowley, 2000a). GAV causes mortalities of up to 100% within 17 days of infection in controlled tests (Spann, 2000) and is now one of the most serious pathogens in Australian farms. It causes a pink to red colouration of the body and appendages and yellowing of the gills. Tail rot, fouling lethargy and loss of appetite are also signs of the diseases (Spann, 2000). In the middle stages of this study, electron microscopic examination revealed for the first time that cytoplasmic vesicles containing GAV-like virions were present in neurones in the eye of morbid *P. monodon*. Smith (2000) hypothesised that non-specific signs of disease are caused by damage to the eye and this new hypothesis is discussed further in the next section.

White Spot Syndrome Virus (WSSV) on the other hand, is a constant threat to the shrimp farming industry (Flegel, 1997). First reported in Japan in 1993-1994 (Inouye, 1994, OIE Diagnostic Manual for Aquatic Animal Diseases, 2000), WSSV causes lethargy, anorexia, white spots on the cuticle and a general pink to red discolouration (Hossain, 2001). WSSV is an enveloped, rod-shaped virus, which contains double-stranded DNA (dsDNA) (Tang, 2000). It is extremely virulent with up to 100% mortality within 2-7 days of the onset of symptoms (Hsu, 1999).

Polymerase chain reaction (PCR) produces a product (amplicon) based on a target sequence of genetic material (RNA or DNA) that is specific for the organism that is under investigation. PCR is a sensitive method that is routinely used to detect the presence of prawn viruses (Dhar, 2001). Real-time PCR is a more recent development that can use fluorogenic dyes, such as SYBR Green, to quantitatively determine viral loads in shrimp. SYBR Green intercalates with dsDNA in the minor groove and emits enhanced fluorescence when bound to dsDNA (Dhar, 2001). At the end of each PCR cycle, the fluorescence is measured. SYBR Green dye is excited and the emission is measured at 510nm wavelength. The specificity of the amplicon is monitored by its melting curve as the temperature is steadily increased to almost 100^{o}C. The melt curve is affected by GC content, length of the amplicon and sequence composition of the product thereby making it possible to distinguish non-specific amplification (Ririe, 1997). One of the main advantages of Real-time PCR is that it is a much more rapid method than conventional PCR, taking around 6 hrs to complete instead of 24 to 36 hrs.

Currently, the Australian prawn farming industry does not routinely use PCR techniques to test spawners, post larvae (PLs) or juveniles for viruses. Although recent research has indicated WSSV is not present in *P. monodon* stocks in Australia, the industry needs to be vigilant. Experiences in other countries has demonstrated that PCR testing is an important diagnostic tool for detecting the virus and minimising its spread. As far as we know, Real-time PCR had not been applied to detecting GAV.

The aim of this study was to use Real-time PCR procedures to investigate the occurrence of GAV and WSSV in *P. monodon* at farms in Far North Queensland and the Clarence River. In the final year of the project (2001), whenever ponds had prawns showing signs of disease, field workers collected 5 to 10 morbid prawns and sent them chilled to UWS. In order to validate the effectiveness of Real-time PCR, conventional PCR procedures were also carried out on representative samples. The outcomes of this study provide the industry with a rapid, efficient new procedure to test for GAV virus.

b) Findings

Supplement #18 shows an example of the result for a test on various tissues from moribund prawns collected from ponds 22 & 26 from Searanch on 31st May 2001. The hepatopancreas, antennule, eye and gills were tested with GAV PCR primers. After approximately 18 cycles of the Real-time PCR (ie approximately 23 minutes), the fluorescence commenced increasing for the 4 tissues and the curves reached a peak after 50 to 60 cycles. The melt curves for the products (amplicons) had peaks at 80.9 to 81.0^{o}C for all tissues. Also, electrophoresis of the product from Real-time PCR is shown in Supplement #18. These results are consistent with positive GAV controls and indicate GAV was present in all 4 tissues.

Table 6.4.1 summarises the results for Real-time PCR tests on moribund prawns from farms in Far North Queensland and the Clarence River in 2001. Prawns were tested from a total of 10 field trips and no positive results were obtained for WSSV. However, 12 of the 12 collections were positive for GAV as determined from the melt curve or by electrophoresis of the PCR product. Melt curves from the Real-time PCR method indicated 10 of the collections were positive for GAV in at least two of four tissues. Melt curves for the PCR products were very consistent with results in Supplement #18. Electrophoresis of PCR products showed that almost all tissues from all collections were positive for GAV.

c) Conclusion.

Real-time PCR proved to be a rapid, cheap method for determining the viral status of moribund prawns. The results show that without exception, GAV was present in moribund prawns at the farms we studied while WSSV was not detected.

Table 6.4.1 Results of Real-time PCR tests for GAV (Gill associated virus) and WSSV (White spot virus) on tissues from moribund prawns.

		GAV Tests			WSSV Test		
Sample Date	Tissue Sample	Date of test	Ave. Melt peak (°C)	Electro-phoresis	Date of test	Ave. Melt peak (°C)	Electro-phoresis
31/05/01	HP	4/10/01	80.9	+	22/01/02		–
	Antennule		80.9	+			–
	Eye		81.0	+			–
	Gills		81.0	+			–
18/05/01	HP	17/01/02	80.4	+	22/01/02		?
	Antennule		79.9	+			–
	Eye		80.1	+			–
	Gills		79.9	+			–
12/04/01	HP	19/12/01	80.0	–	22/01/02		–
	Antennule	18/01/02		+			–
	Eye		80.0	–			–
	Gills		80.0	+			–
24/05/01	HP	19/12/01		–	22/01/02		?
	Antennule			+			–
	Eye			–			–
	Gills			+			–
23/03/01	HP	17/01/02	80.1	+	22/01/02		–
	Antennule		80.1	+			–
	Eye		79.1	+			
	Gills		79.8	+			–
19/04/01	HP	21/01/02		+	23/01/02		
(Ca)	Antennule		80.0	+			–
	Eye		80.0	+			–
	Gills		79.9	+			–
27/04/01	HP	21/01/02	79.6	+	23/01/02		–
(CR)	Antennule		79.2	+			–
	Eye		79.6	–			–
	Gills		80.1	+			–
19/04/01	HP	21/01/02	79.6	+	22/01/02		–
(CR)	Antennule		79.9	+			–
	Eye		79.7	+			–
	Gills		79.6	+			–
27/04/01	HP	21/01/02	80.0	+	23/01/02		–
(Ca)	Antennule		80.0	+			–
	Eye		80.0	+			–
	Gills		80.0	+			–
4/05/01	HP	18/01/02	80.0	+	22/01/02		–
(CR)	Antennule			+			–
	Eye			+			–
	Gills		80.0	–			–

		GAV Tests			WSSV Test		
Sample Date	Tissue Sample	Date of test	Ave. Melt peak (°C)	Electro-phoresis	Date of test	Ave. Melt peak (°C)	Electro-phoresis
4/05/01	HP	18/01/02	79.0	+	22/01/02		–
(Ca)	Antennule		–	+			–
	Eye		79.0	–			–
	Gills		79.0	+			–
6/07/01	HP	24/01/02		+	24/01/02		–
	Antennule			+			–
	Eye			+			–
	Gills			+			–

6.4.4 Eye diseases and pre-mature sexual development

During the project a study of eye disease in Australian prawns was completed (Smith 2000). The work brought together a number of observations as well as studies carried out by the author using histopathology and electron microscopy. The paper concluded that bacterial and viral infections of the eye could result in impaired neuroendocrine functions, which could cause a range of clinical signs of disease. Importantly for the prawn farming industry, the paper reported that in at least one disease event, eye lesions were associated with pre-mature sexual development in *P. monodon*.

A similar event of premature sexual maturation in moribund *P. monodon* was found in ponds at a prawn farm on the Clarence River in May 2002. Fig 6.4.4.1 shows an example of a moribund prawn with early stages of premature sexual maturation. The prawn was 28 g and its signs of disease and maturation were similar to those of other moribund male and female prawns in the pond. As with the incident reported in Smith (2000), only the morbid prawns around the edge of the pond were sexually mature, while prawns collected by cast net from deeper areas of the pond were obviously healthy and not sexually mature. In some prawns the ovaries were further developed and were green – indicating late stages of sexual maturity.

Fig 6.4.4.2 shows examples of prawn tissue that were not included in Smith (2000). The photomicrographs illustrate important aspects of eye disease and the associated sexual maturation.

In conclusion, the publication of results describing lesions in prawn eyes and relating that damage to changes in neurosecretory substances, is a new way at looking at causes of clinical signs of disease. It may explain, for example “red body disease”, lethargy, etc since the neurosecretions from the eye control most of the endocrine (neurosecretions) in crustaceans – ie body pigmentation, blood sugar level, inhibition of sexual development, moulting. The new hypothesis is very important because it may provide researchers with an exciting new type of method for investigating methods for controlling sexual maturation in farmed prawns. This aspect is reported in Smith (2000) but it requires considerable investigation.

Fig 6.4.4.1: Moribund prawn with early stages of sexual maturation.

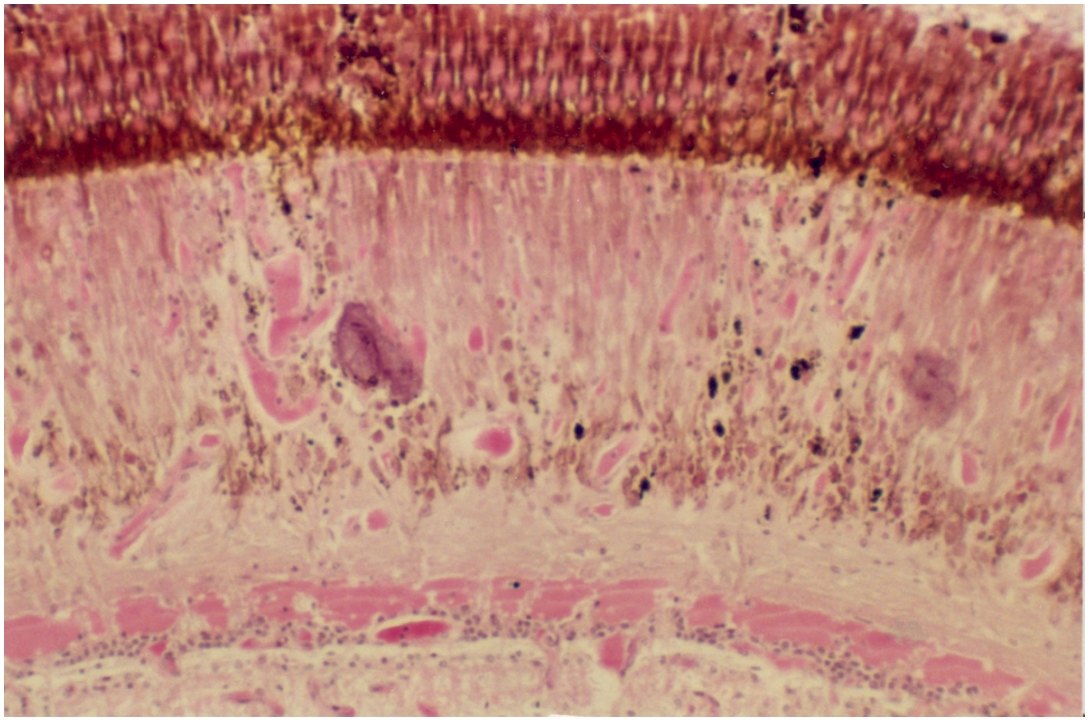
a) The prawn at rest at the edge of the pond.

b) The early stages of sexual maturation can be seen through the cuticle on the dorsal surface of the tail.

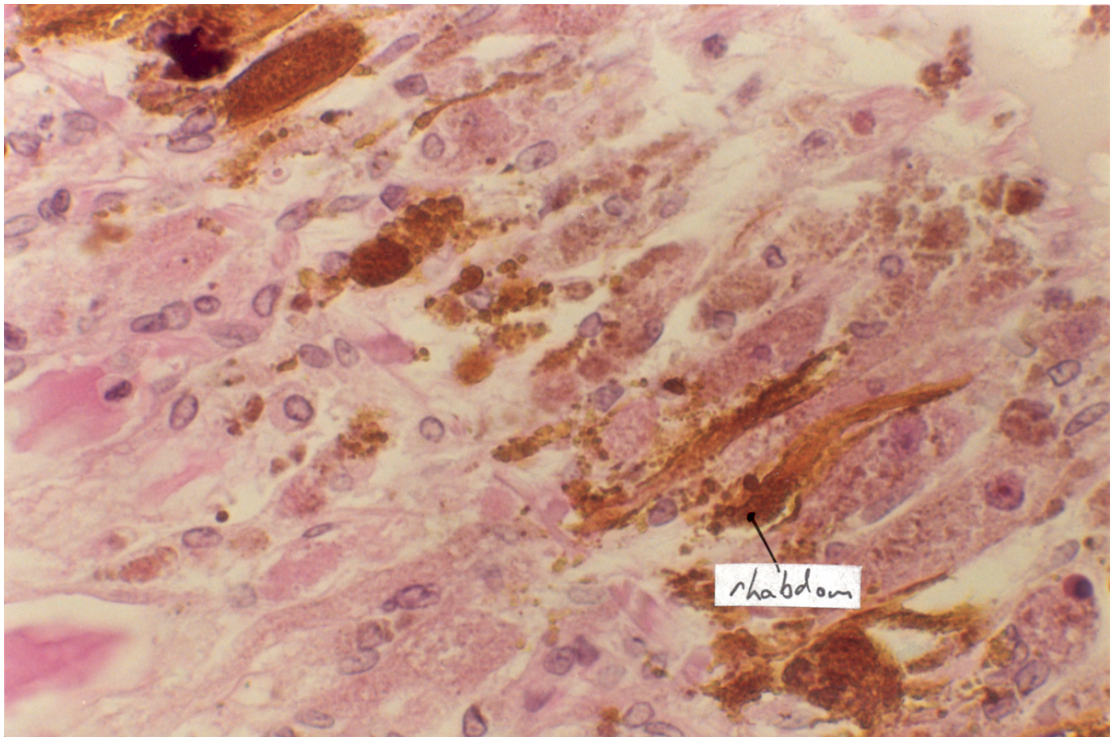
c) The ovary has been dissected – the yellowish colour indicates that it is in the early stages of development.

Fig 6.4.4.2 Micrographs showing lesions in the eye and sexual development of eggs in the ovary of a moribund prawn.

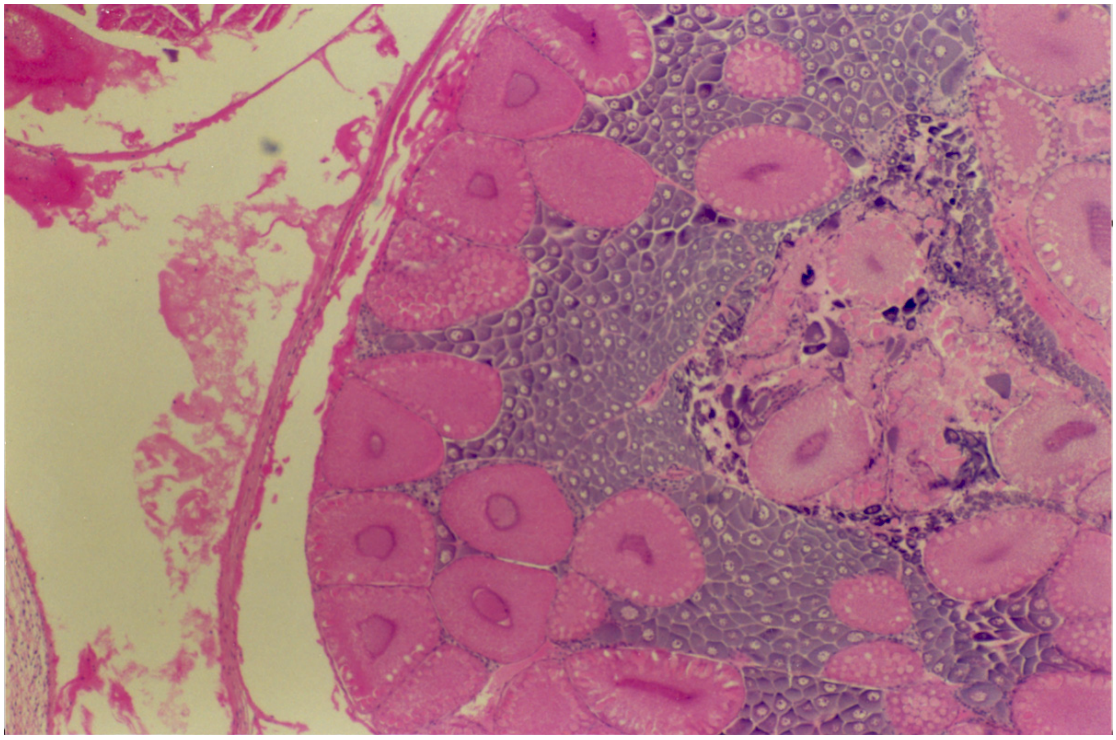
a) The retinal layer of the eye (brown layer) and the underlying nervous tissue of the Fasciculated zone both show lesions (brown and black melanized cells) – H&E stain, 100x.



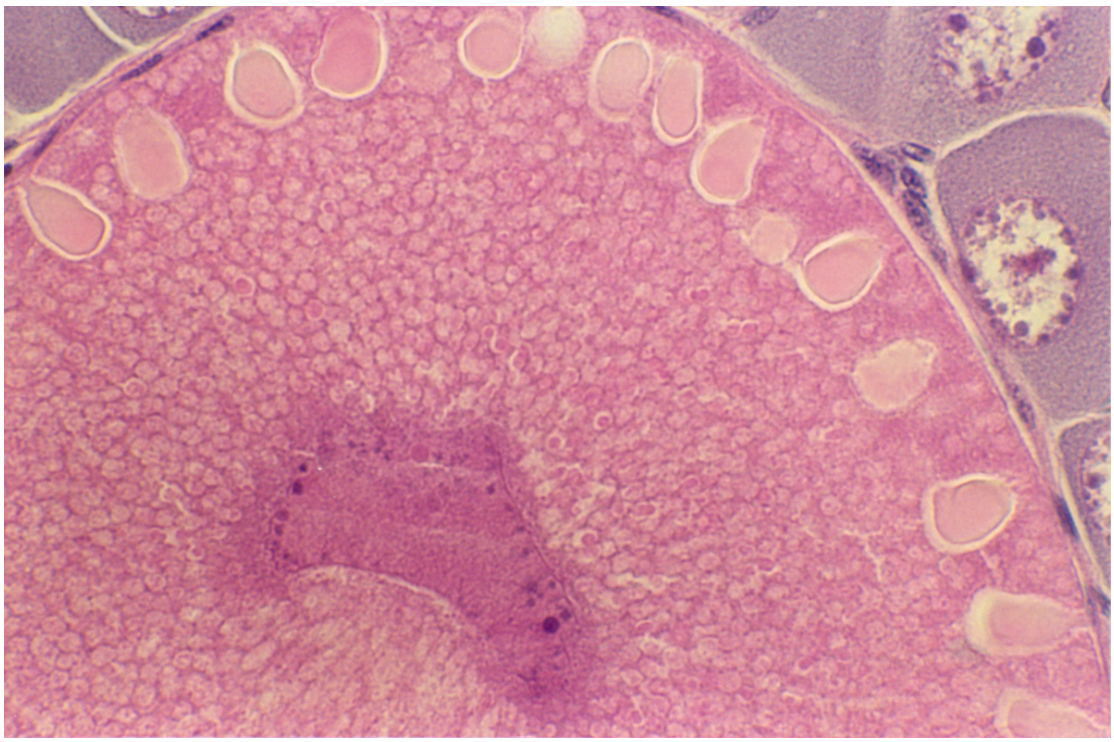
b) A closer view of the junction between the retinal layer (individual rhabdom which absorbs light) is indicated. Nuclei are basophilic (purple) and swollen – H&E stain 400x.



c) A portion of the ovary of a female prawn showing numerous mature ova (large purple bodies) – H&E stain 100x.



d) A mature ovum egg with well-developed central nucleus, surrounded by yolk (in the form of globules or vacuoles), and lightly coloured rod-like peripheral bodies around the outer membrane. Younger ova are purple in colour and they surround the mature ovum (x400).



6.5 Associations between extracellular enzyme activities, productivity indices and pond management

6.5.1 Extracellular enzyme activities and productivity indices

Various statistical methods were used to identify the variables that were associated with high pond productivity. Results from those methods are summarised here.

Scatterplots and statistical techniques (using SPSS) were used to find the extent of correlations between the 9 productivity indices and 22 enzyme substrates for water and sediment. General trends indicate the following.

- **Harvest age vs extracellular enzyme activity**: 15 positive correlations, 2 negative and 5 not significant. Trend is generally a positive correlation (ie older crop age correlates with higher enzyme activities).
- **Survival (%) vs extracellular enzyme activity**: 4 positive correlations, 9 negative and 9 not significant. Trend is generally for a negative correlation between survival and extracellular enzyme activity (ie highest survival correlates with lower enzyme activities).
- **Survival index vs extracellular enzyme activity**: 4 positive correlations, 12 negative correlations. Trend is generally for a negative correlation between survival index and enzyme activity (ie highest survival correlates with lower enzyme activities).
- **Average weight at harvest vs extracellular enzyme activity**: 4 positive correlations, 2 negative correlations. Trend is for no significant correlation between average weight at harvest and enzyme activity.
- **Production (kg/ha) vs extracellular enzyme activity**: 12 positive correlations, 3 negative correlations. Trend is for a positive correlation between production and enzyme activity (ie high production correlates with high enzyme activity).
- **Production index vs extracellular enzyme activity**: 4 positive correlations, 9 negative correlations. General trend is for negative correlation between productivity index and enzyme activity. Note that this contradicts the finding in previous paragraph.
- **Total feed (kg/ha/crop) vs extracellular enzyme activity**: 13 positive correlations and 4 negative correlations. General trend is for a positive correlation between total feed and enzyme activity (ie high feed input correlates with high enzyme activity).
- **FCR vs extracellular enzyme activity**: 5 positive correlations and 3 negative correlations. (ie suggesting a high FCR correlates with enzyme activity).
- **FCR index vs extracellular enzyme activity**: 3 positive correlations and 14 negative correlations. Trend is for negative correlation between good FCR index and enzyme activity (ie good FCR correlates with low enzyme activity).

There are at least two difficulties in attempting to interpret the statistical analyses, such as the example described above. Firstly, scatterplots in Section 6.1 showed that enzyme activity varied with factors such as feed input and the age of the crop. Hence there are time-dependent changes in enzyme activity which are not considered in simple statistical analyses. Secondly, the use of 9 productivity indices is cumbersome. Hence an improved method for investigating the data was attempted. Enzyme activity was plotted against age of crop and the cases were grouped according to a

combination of the productivity indices. A Combination Productivity Index was made by combining the three most significant indices: survival index, production index and FRC index, as follows:

Combination Productivity Index = 20 x (survival index + production index + FRC index) / 3

The Combination Productivity Index has a maximum value of 200 for optimum productivity, and a minimum of 0 for lowest productivity. Fig 6.5.1 to 6.5.12 are plots of the Combination index for pond productivity for 12 of the key enzyme substrates. There is an important common trend in these plots. In almost every plot, the enzyme activities for ponds with low and below average productivity are the first to rise to a peak of enzyme activity, at approximately 50 days. In comparison, ponds with high and above average productivity usually have lower enzyme activities throughout the crop. This trend can be seen for the key bioindicators of bacterial activity - proteases (Fig 6.5.1, 6.5.9) and chitinases (Fig 6.5.10, 6.5.11) – as well as sugar and ester enzymes. This is an interesting result because it indicates that the ponds with the highest productivity have the highest biomass and highest input of feed. However, the key bioindicators of bacterial enzyme activity, particularly protease and chitinase, are at lower levels in those ponds. This is contrary to what could be expected. That is, the project has shown that enzyme activity is driven by input of feed, and so it would seem logical that ponds with greater biomass and higher feed inputs should have higher enzyme profiles. It is tempting to believe that when high productivity was obtained in ponds it was in part because those ponds had a less stressful bacteriology – ie lower levels of bacteria that produced extracellular proteases and chitinases.

In summary, low bacterial enzyme profiles are associated with ponds with high productivity. This finding indicates the value of the extracellular enzyme technique for ascertaining the bacteriology of prawn ponds. It also provides a strong case for investigating management practices and factors that lead to low extracellular enzyme profiles.

6.5.2 Pond management and productivity indices

Since prawn ponds are complex, dynamic units there will not be a single factor that determines productivity in a well-run farm. With all of the data that was available, statistical analysis was used to determine the factors (ie pond management) associated with pond productivity. Supplement #19 contains some plots which show the Combined Productivity Index vs factors such ‘region’, ‘year of the project’, ‘month stocked’, ‘type of aeration system’, ‘hatchery source of PLs’, ‘water colour’, and ‘type of sediment’. These plots illustrate the spread of data. Also, curve fits for key variables are shown.

General linear regression was used to identify the key factors (variables) for the Combination Productivity Index and the results are shown in Table 6.5.1 The most significant factor was ‘depth of pond’ (R^2 = 0.447). The most significant enzyme substrate was ‘leucine’ (R^2 = 0.115).

Table 6.5.1 General Linear Model

Multivariate Tests(c)

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	1.000	65312717 2.083(a)	9.000	3466.000	.000
	Wilks' Lambda	.000	65312717 2.040(a)	9.000	3466.000	.000
	Hotelling's Trace	1695944.7 63	65312717 2.040(a)	9.000	3466.000	.000
	Roy's Largest Root	1695944.7 63	65312717 2.040(a)	9.000	3466.000	.000
PPINDEX	Pillai's Trace	3.032	38.375	414.000	31266.000	.000
	Wilks' Lambda	.018	42.673	414.000	30672.005	.000
	Hotelling's Trace	5.561	46.532	414.000	31178.000	.000
	Roy's Largest Root	1.244	93.953(b)	46.000	3474.000	.000

a Exact statistic
b The statistic is an upper bound on F that yields a lower bound on the significance level.
c Design: Intercept+PPINDEX

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	geographic region	280.137(a)	46	6.090	61.013	.000
	month stocked	488732907159 43500000000.0 00(a)	46	1062462841650 947000000.000	61.035	.000
	pond size (ha)	134.177(b)	46	2.917	30.838	.000
	PLs per square metre	122134.938(c)	46	2655.107	52.316	.000
	average depth (m)	134.438(d)	46	2.923	71.945	.000
	leucine	866195320.973 (e)	46	18830333.065	9.818	.000
	n acetyl glucosaminide	7184453.466(f)	46	156183.771	5.810	.000
	hatchery source of PLs	7913.326(g)	46	172.029	53.646	.000
	type of aeration	112.282(h)	46	2.441	75.562	.000
Intercept	geographic region	1792.084	1	1792.084	17954.251	.000
	month stocked	300291268453 24810000000.0 00	1	3002912684532 4810000000.00 0	1725.085	.000
	pond size (ha)	783.928	1	783.928	8287.937	.000
	PLs per square metre	1207299.902	1	1207299.902	23788.751	.000
	average depth (m)	1311.627	1	1311.627	32288.553	.000
	leucine	4025402824.88 8	1	4025402824.88 8	2098.867	.000
	n acetyl glucosaminide	4391394.053	1	4391394.053	163.355	.000
	hatchery source of PLs	43312.345	1	43312.345	13506.687	.000
	type of aeration	6436.488	1	6436.488	199250.633	.000
PPINDEX	geographic region	280.137	46	6.090	61.013	.000
	month stocked	488732177075 22600000000.0 00	46	1062461254511 361000000.000	61.035	.000
	pond size (ha)	134.177	46	2.917	30.838	.000
	PLs per square metre	122134.938	46	2655.107	52.316	.000
	average depth (m)	134.438	46	2.923	71.945	.000
	leucine	866195320.973	46	18830333.065	9.818	.000

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
	n acetyl glucosaminide	7184453.466	46	156183.771	5.810	.000
	hatchery source of PLs	7913.326	46	172.029	53.646	.000
	type of aeration	112.282	46	2.441	75.562	.000
Error	geographic region	346.753	3474	.100		
	month stocked	604730837936 55100000000.0 00	3474	1740733557675 7370000.000		
	pond size (ha)	328.594	3474	.095		
	PLs per square metre	176308.538	3474	50.751		
	average depth (m)	141.121	3474	.041		
	leucine	6662761990.38 1	3474	1917893.492		
	n acetyl glucosaminide	93389718.187	3474	26882.475		
	hatchery source of PLs	11140.192	3474	3.207		
	type of aeration	112.222	3474	.032		
Total	geographic region	11636.000	3521			
	month stocked	142331986430 232200000000. 000	3521			
	pond size (ha)	5241.851	3521			
	PLs per square metre	5285358.160	3521			
	average depth (m)	7399.122	3521			
	leucine	24161767198.4 72	3521			
	n acetyl glucosaminide	117313934.335	3521			
	hatchery source of PLs	259920.000	3521			
	type of aeration	30484.000	3521			
Corrected Total	geographic region	626.890	3520			
	month stocked	109346374509 598700000000. 000	3520			
	pond size (ha)	462.771	3520			
	PLs per square metre	298443.476	3520			
	average depth (m)	275.559	3520			
	leucine	7528957311.35 4	3520			
	n acetyl glucosaminide	100574171.653	3520			
	hatchery source of PLs	19053.518	3520			
	type of aeration	224.504	3520			

a R Squared = .447 (Adjusted R Squared = .440)
b R Squared = .290 (Adjusted R Squared = .281)
c R Squared = .409 (Adjusted R Squared = .401)
d R Squared = .488 (Adjusted R Squared = .481)
e R Squared = .115 (Adjusted R Squared = .103)
f R Squared = .071 (Adjusted R Squared = .059)
g R Squared = .415 (Adjusted R Squared = .408)
h R Squared = .500 (Adjusted R Squared = .494)

Fig 6.5.1 Leucine protease in water vs age of crop

by productivity index

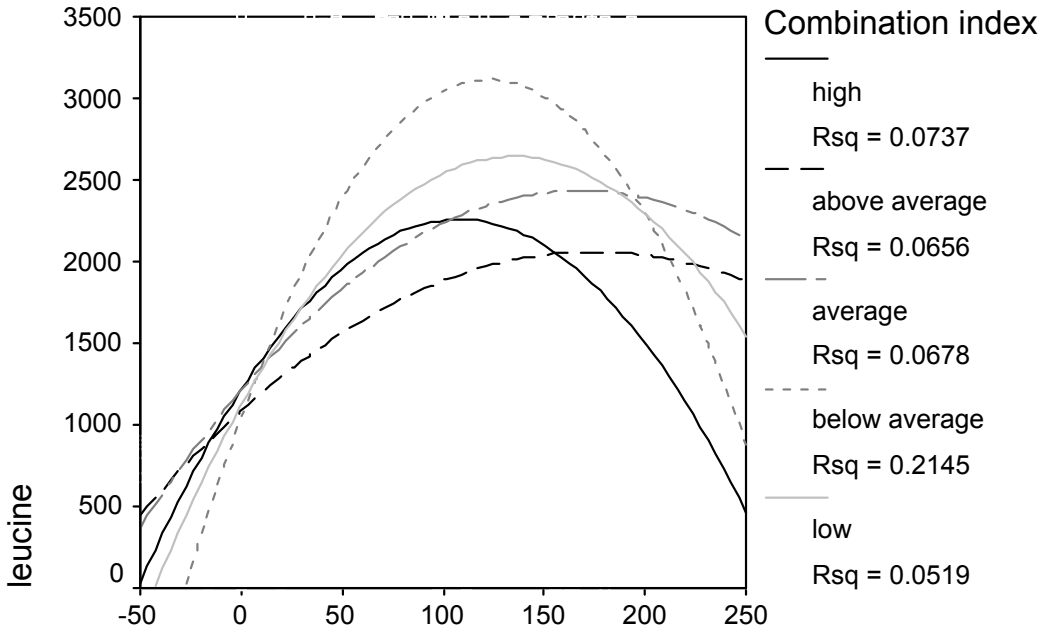
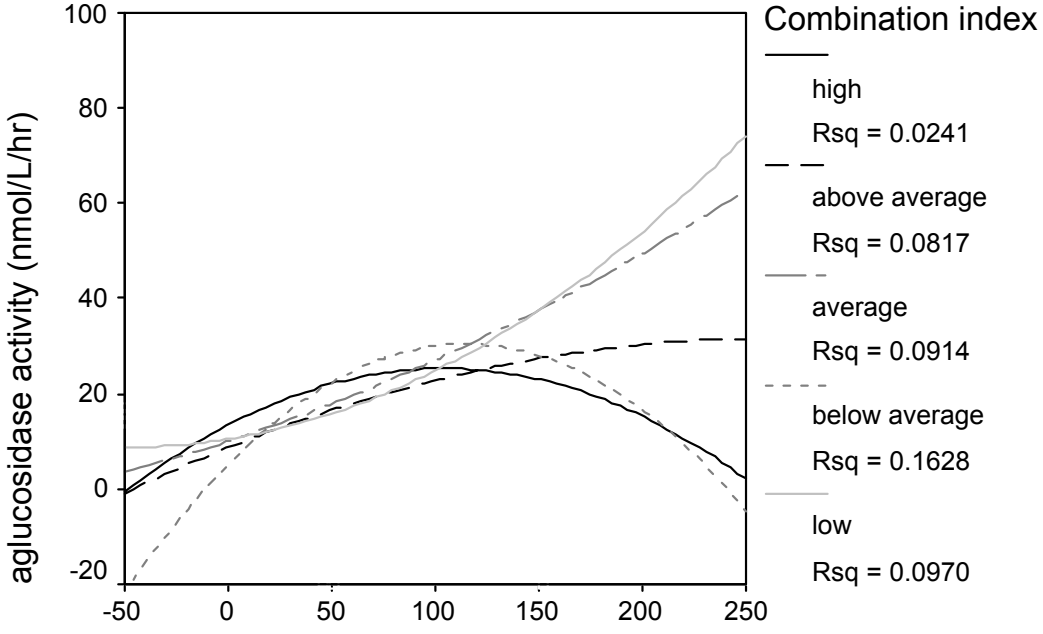


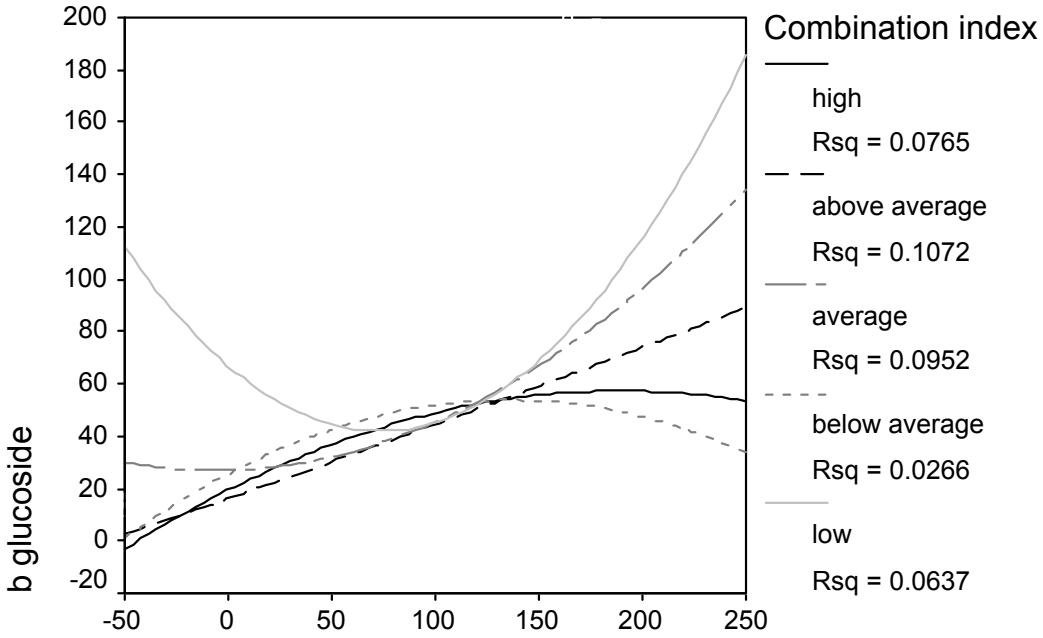
Fig 6.5.2 a-glucosidase activity in water vs age of crop

by productivity index



6.5.3 b-glucosidase in water vs age of crop

by productivity index



6.5.4 xylosidase for water vs age of crop

by productivity index

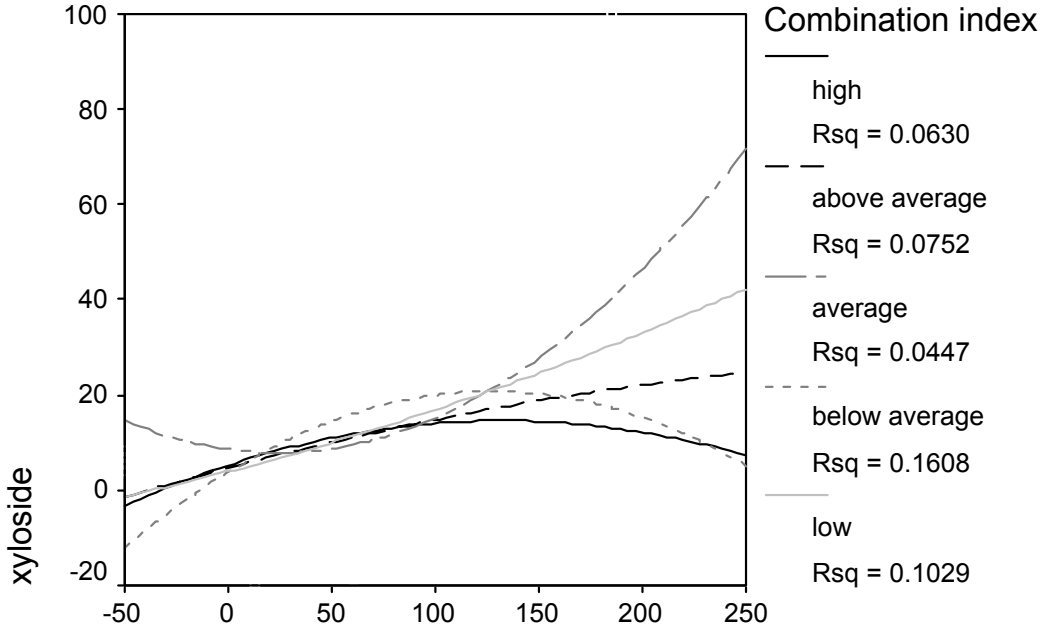


Fig 6.5.5 galactosidase activity in water vs age of crop by productivity index

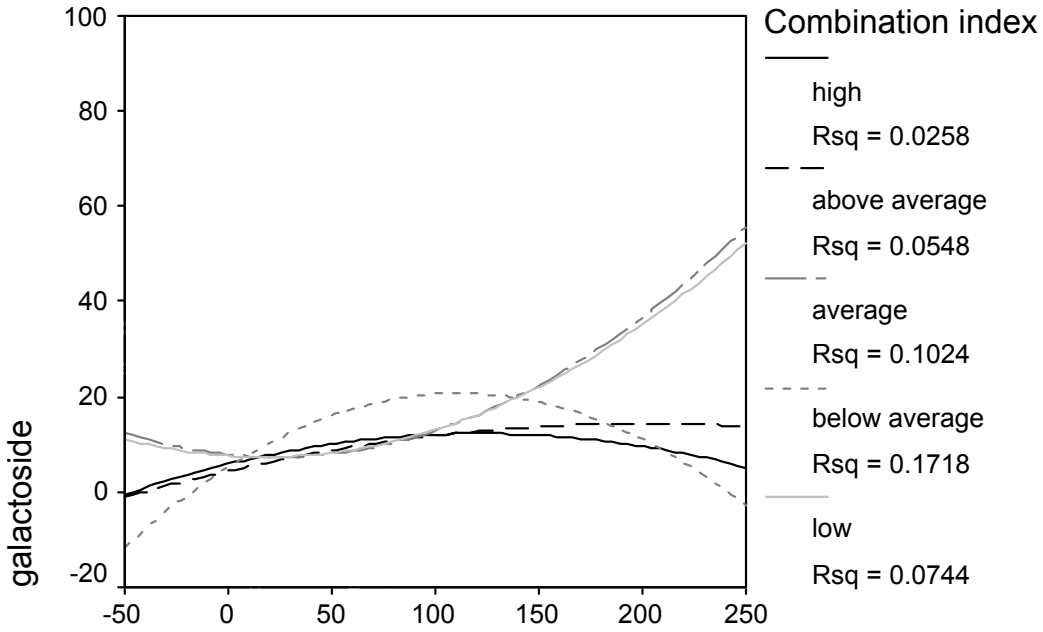


Fig 6.5.6 Glucuronidase activity in water vs age of crop by productivity index

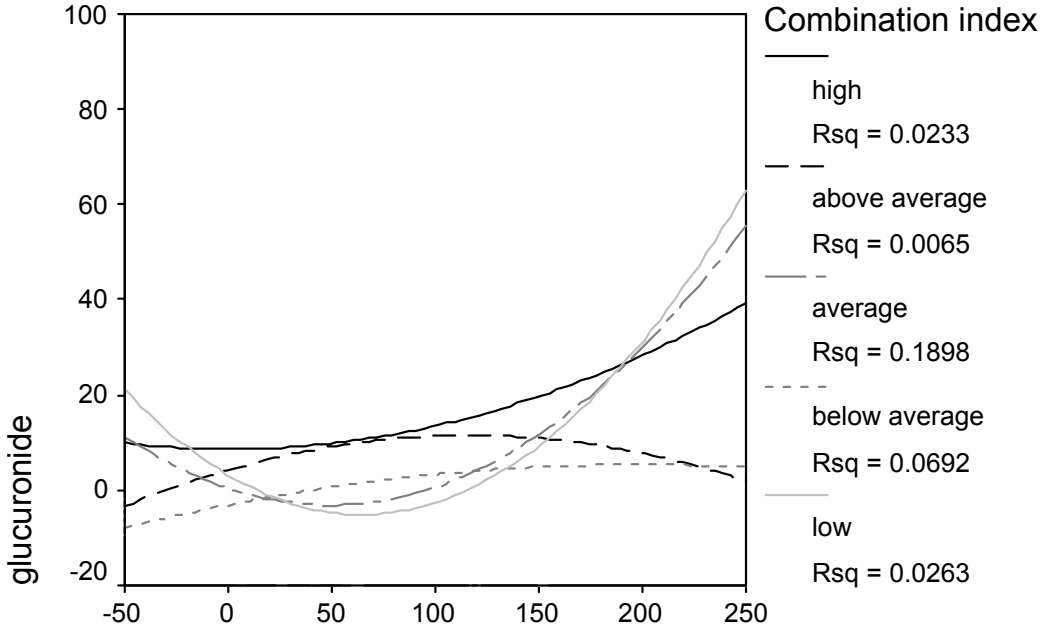


Fig 6.5.7 acetatase activity in water vs age of crop

by productivity index

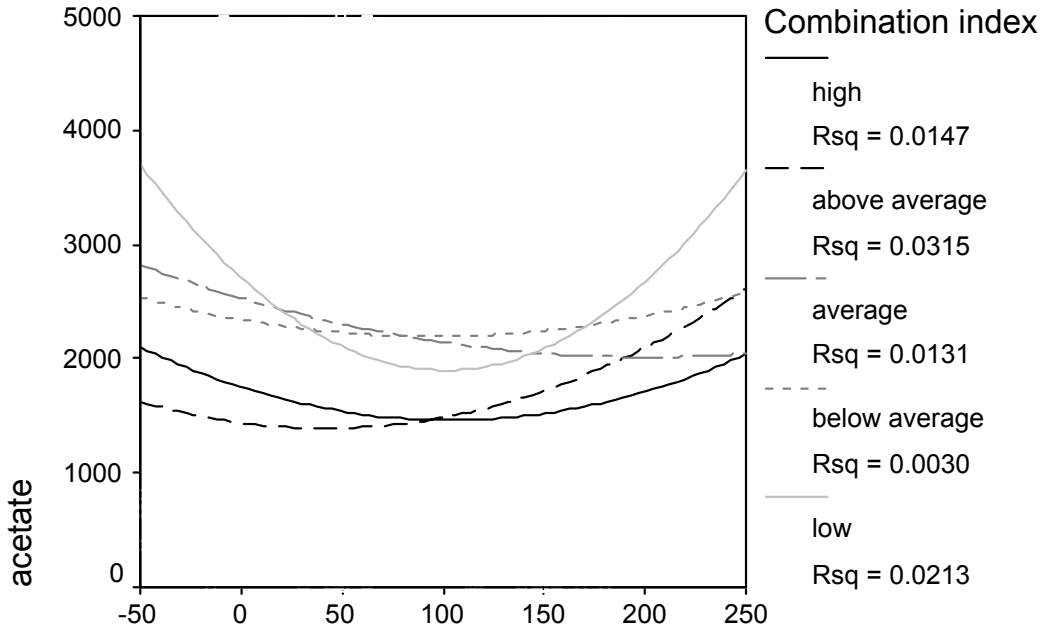


Fig 6.5.8 lauate esterase activity in water vs age of crop

by productivity index

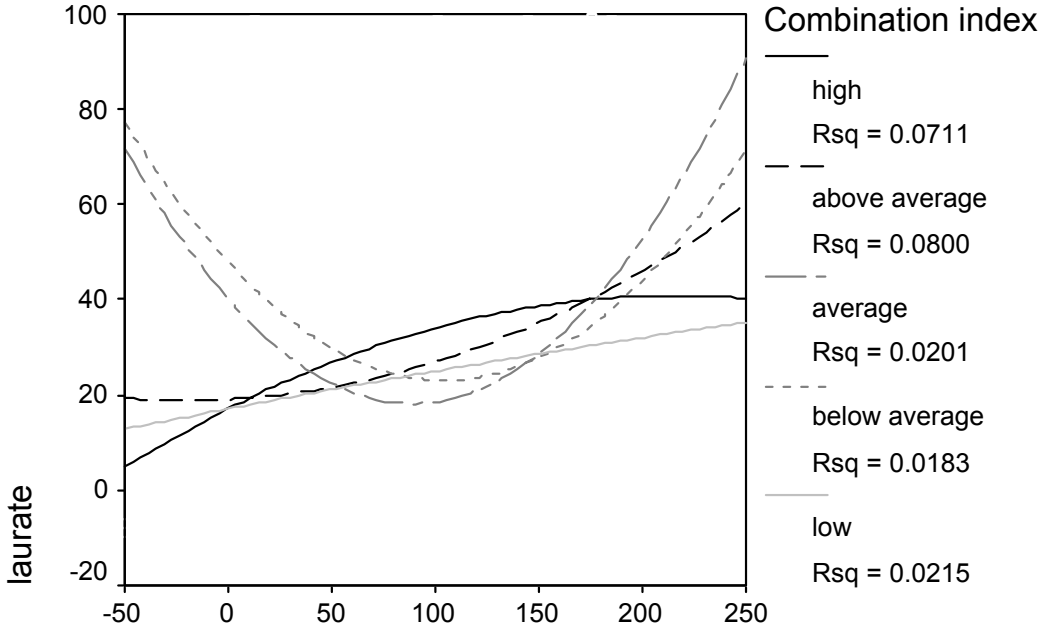


Fig 6.5.9 protease activity in water vs age of crop

by productivity index

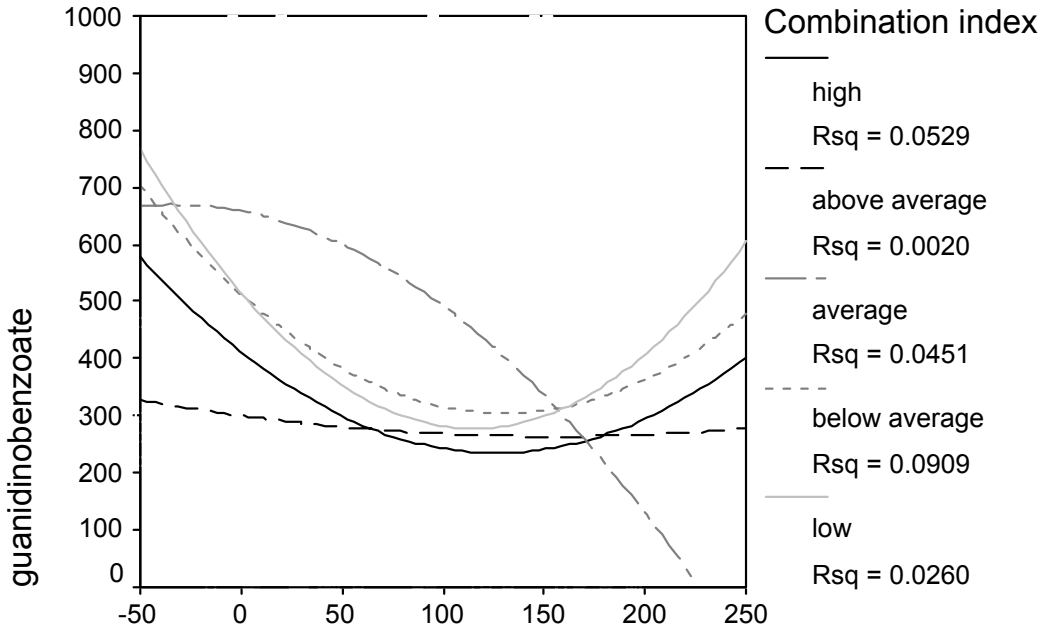


Fig 6.5.10 chitinase activity in water vs age of crop

by productivity index

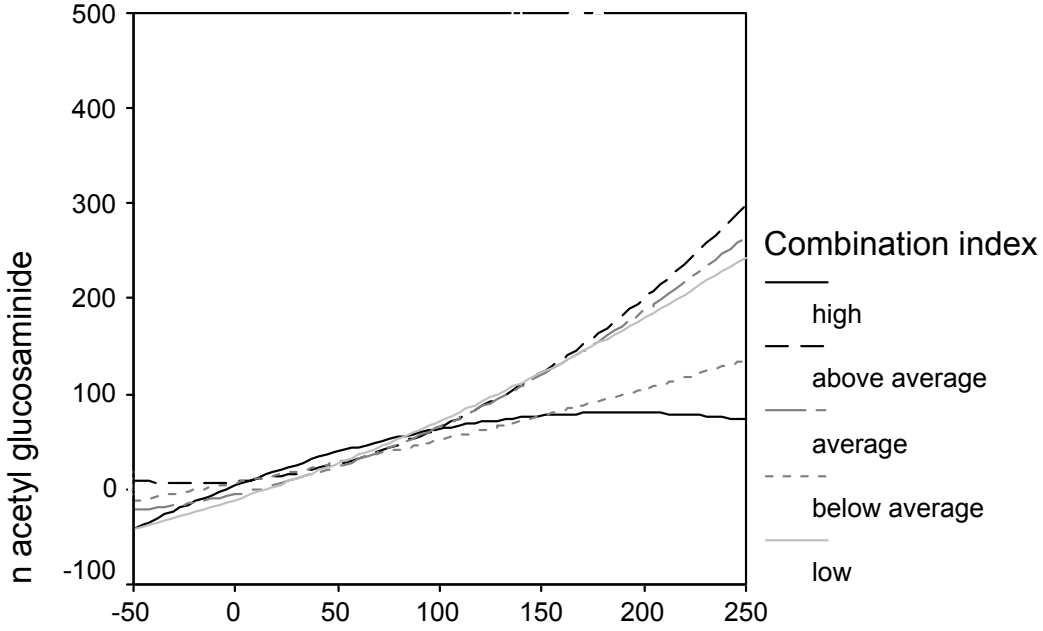


Fig 6.5.11 chitinase in water vs age of crop

by productivity indes

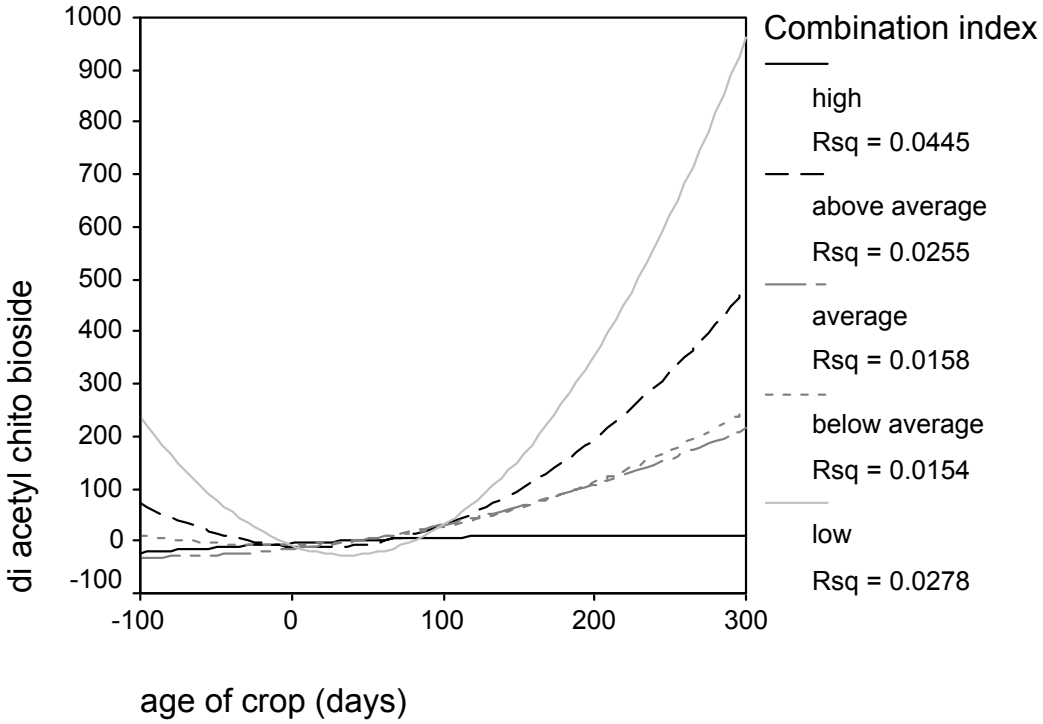
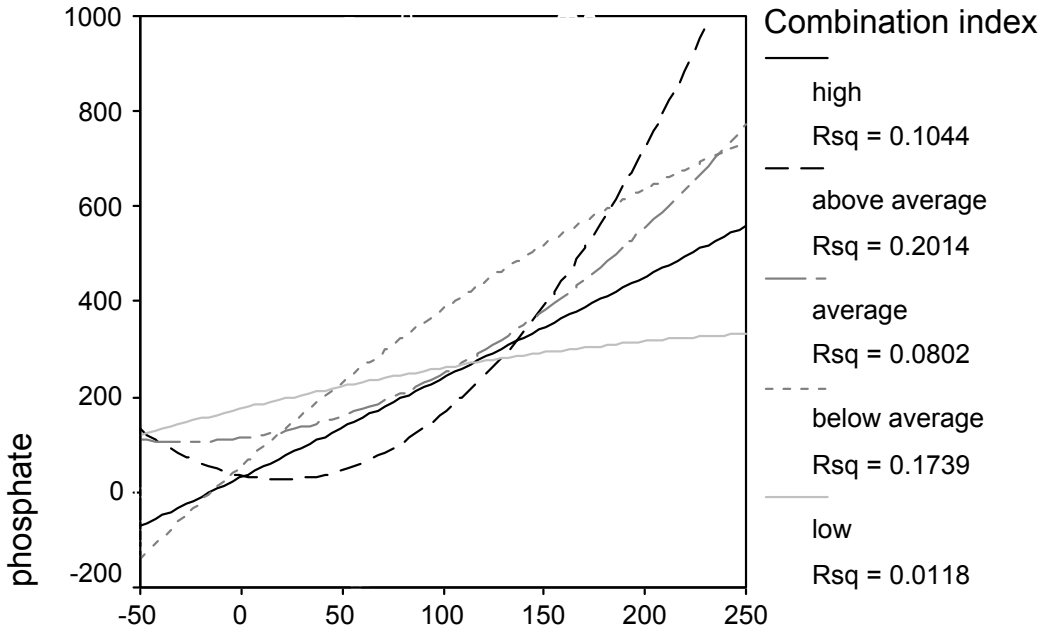


Fig 6.5.12 phosphatase actvity in water vs age of crop

by productivity index



6.6 A HACCP-based Support Program for Good Management of bacteria in culturing *Penaeus monodon*

Today most seafood businesses have a Seafood Safety Plan based on Hazard Analysis and Critical Control Points (HACCP) to ensure that consumers have safe seafood. The HACCP approach is a systematic method for dealing with any hazards that could occur from harvest to final food product. Critical Control Points (CCPs) are identified in order to prevent, eliminate or reduce hazards to acceptable levels. Seafood operations also have supporting programs, including Good Manufacturing Practices (GMP) and Good Hygenic Practices (GHP), for controlling food safety. These programs are developed by individual producers and tailored to their individual operation.

In this section the principals of HACCP have been used to produce a Support Program for Good Management Practices for controlling and reducing hazards from bacteria in the culturing of farm prawns. This section considers all stages in prawn culture - from spawning in hatcheries to harvest of prawns at farms. It is based on the findings of this study on the bacteriology of *Penaeus monodon* at Australian prawn farms and hatcheries. This section aims to provide the industry with a generic support program which could be used by individual businesses to develop their own programs. An attempt has been made to pinpoint steps in the process that appear to have deficiencies and require improved techniques.

The study did not investigate the effectiveness of application of **chemicals**, such as antibiotics, algicides (eg simerzine) and disinfectants (eg BKC, formalin). And during the study there were no instances when farms were known by us to use any of these types of chemicals, except for formalin to disinfect PLs when they arrived at the farm. From personal observations over a number of years, the use of these chemicals is not satisfactory because:

a) the application of chemicals usually causes more problems that it solves (eg residues of antibiotics can be detected in harvested prawns resulting in rejection of the product or simerzine produces a bitter taste that persists for along time after its application);
b) the purchase of chemicals usually adds a large financial cost to production (eg in Thailand some 20-40% of production costs were commonly attributed to purchase of chemicals (Smith 1999);
c) the application of these chemicals does not treat the cause of a problem, so that if a benefit is thought to occur, it will be short-lived because the cause of the problem has not been rectified;
d) many of these chemicals are illegal for aquaculture and farmers and hatchery operators should not use any chemicals until they have checked with the relevant authorities;
e) antibiotics in feeds (ie medicated feeds) are expensive and generally do not work because when a pond suffers from a bacterial disease, the sick prawns considerably reduce their food intake or stop eating altogether, while the healthy prawns cannibalise the dead prawns;
f) the use of formalin to disinfect PLs when they arrive at a farm is a very dubious practice because it further stresses the animal after an extremely

stressful period of transport (discussed earlier) and delays the acclimatization process.

Table 6.6.1 HACCP-based audit table of a Support Program for Good Management Practices for Bacteria in culturing *Penaeus monodon*. Hazards that do not have appropriate control measures are indicated. The use of chemicals is not advocated in this program for the reasons listed in Section 6.6.

Process step	*Hazard*	*Control measures*	*It this a CCP?*	*Critical limit*	*Monitoring procedure*	*Corrective action*	*Records*
Stage 1: The hatchery							
1. Transport of wild spawners to hatchery by fisher	High bacterial load in spawner bag	**Improved method for transporting live animals is needed.**	Yes	Visual signs of vibriosis (eg red body, lethargy), TCBS plate counts of 5000 cfu/mL, protease activity of 2000 nmol/L/hr	**What**: pathogenic bacteria **How:** written record; hatchery & lab testing **When:** upon arrival of spawners at hatchery **Who**: hatchery micro-biologist	Investigate failure of transport procedure. Reject spawners if vibriosis is severe.	Spawner records
2. Spawners in hatcheries	High bacterial load in spawner tank	Spawner tank has appropriate biofiltration system (various other techniques may be appropriate eg UV treatment)	No			Improve tank disinfection and biofiltration system and monitoring system for bacteria.	
3. Hatching of atremia in hatchery	High bacteria load in atremia hatching tank	Thorough rinsing of nauplii with clean seawater prior to use.	No			Be aware that atremia can be a source of crustacean viruses and other prawn pathogens that **cannot** be removed.	
4. Culture of microalgae in hatchery	Algae contamina t-ed by *Vibrio* species	Sterilise and filter seawater for algal tanks (verify by testing water).	No			Temporarily replace algae with other feeds.	
5. Culture of larvae and post larvae in hatchery	High bacterial load in larval rearing	Sterilise and filter seawater for larval tanks. Prevent cross-contamination of	Yes	Visual signs of vibriosis (eg dead larvae, weak & whitish	**What**: pathogenic bacteria **How**: written record;	Investigate source of bacterial contaminat-ion.	Larval records.

Process step	*Hazard*	*Control measures*	*It this a CCP?*	*Critical limit*	*Monitoring procedure*	*Corrective action*	*Records*
	tanks	tanks. Try probiotics as a means of controlling pathogenic bacteria.		larvae), TCBS plate counts of 5000 cfu/mL, protease activity of 2000 nmol/L/hr	hatchery & lab testing **When**: regularly during larval culture **Who**: hatchery micro-biologist	Disinfect and destroy larval is mortalities due to vibriosis exceeds 70%.	
5. Culture of larvae and post larvae in hatchery	Cyano-bacteria contamina te larval rearing tanks	Filter and sterilise larval tank water, Prevent cross-contamination of tanks.	No			Trial the use of commercial probiotics or generate activated microbes with feed supplement.	
6. Transport of PLs from hatchery to farm	High bacterial load in PL transport bags	**Improved method for transporting live animals is needed.**	Yes	Dead PLs, lethargy and visual signs of vibriosis (eg red & dead PLs, lethargy), TCBS plate counts of 5000 cfu/mL, protease activity of 2000 nmol/L/hr	**What**: pathogenic bacteria **How**: written record; farm & lab testing **When**: upon arrival of PLs at farm **Who**: farm micro-biologist	Investigate failure of transport procedure. Reject PLs if vibriosis is severe and there are high mortalities.	Farm records for ponds

Process step	*Hazard*	*Control measures*	*It this a CCP?*	*Critical limit*	*Monitoring procedure*	*Corrective action*	*Records*
Stage 2. The farm							
7. Process of acclim-atization of PLs, carried out upon arrival of PLs at farm	High levels of pathogenic bacteria build up during acclimatization.	Monitor levels of carbon dioxide, oxygen and temperature. Also transfer into acclimatiz-ation tank as soon as the transport bag is open.	Yes	Dead PLs and visual signs of vibriosis (eg red & dead PLs, lethargy), TCBS plate counts of 5000 cfu/mL, protease activity of 2000 nmol/L/hr	**What**: pathogenic bacteria **How**: written record; farm & lab testing **When**: prior to adding PLs to ponds **Who**: farm micro-biologist	Investigate failure of acclimatization procedure. Reject PLs if vibriosis is severe and there are very high mortalities.	Farm records for ponds
8. Develop-ment of algal blooms	Inappropriate fertilisation causing bloom crashes or cyanobacterial blooms	Ongoing process throughout crop to monitor Secchi depth, nutrients, bloom character-isitics, pH, alkalinity, and DO.	No			Use a) feed supplements to generate activated microbes; b) green/blue dyes to reduce benthic cyanobact-erial blooms, c) recycle water or pump water from ponds with best blooms.	

Process step	*Hazard*	*Control measures*	*It this a CCP?*	*Critical limit*	*Monitoring procedure*	*Corrective action*	*Records*
9. Feeding of prawns with commerc-ial pellets	1. Over-feeding causing blooms of pathogeni c bacteria. 2. Use of inappropri ate feed – especially feed with excessive levels of protein.	Carefully monitor feed trays and adjust feed rates accordingly (take into account the need to reduce feed during moulting or low temperatures). Use diver to examine pond bottoms, prawn behaviour and feed uptake. Reduce turbulents during feeding (turn off aerators).	Yes.	Dead prawns and visual signs of vibriosis (eg red body, lethargy & dead prawns at edge of ponds, seagulls diving), TCBS plate counts of 5000 cfu/mL, protease activity of 2000 nmol/L/hr	**What**: pathogenic bacteria **How**: written record; farm & lab testing **When**: ongoing tests and observations throughout crop **Who**: farm micro-biologist	Always underfeed, never over-feed. Experiment with high carbon low nitrogen feed supple-ments. Encourage growth of zooplankton bloom (ie natural feed) prior to stocking and try to maintain for as long as possible.	Farm records for ponds.
10. Water exchange	Stress caused by sudden changes in bacteriolo gy caused by introducti on of "uncondi-tioned" water.	Monitor rate of water exchange and effect on prawns (eg stress causes moulting).	No			Preferably introduce intake water after a few days of conditioning in intake canal. Recycle or pump water from ponds with best blooms.	
11. Use of probiotics or feed supplement	Overfeedi ng with commerci al feed, or not fermentin g suppleme nt feeds before applicatio n.	Carefully monitor feed trays and adjust feed rates accordingly because the supplement should reduce the need for commercial feed.	No			Still in the experiment-al stage so have control ponds and test ponds. If the supplement has an undesirable effect, stop using it.	
12. Vibriosis and bacterial disease	Disease outbreak.	Carefully monitor feed trays, growth rates and survivals in each pond.	Yes	Dead prawns and visual signs of vibriosis (eg red body, lethargy & dead prawns at edge of	**What**: pathogenic bacteria **How**: written record; farm & lab testing **When**: ongoing tests and	In severe (ie acute) cases with rapid increases in mortalities, harvest live prawns. In mild cases,	Farm records for ponds.

Process step	*Hazard*	*Control measures*	*It this a CCP?*	*Critical limit*	*Monitoring procedure*	*Corrective action*	*Records*
				ponds, seagulls diving)), TCBS plate counts of 5000 cfu/mL, protease activity in pond water of 2000 nmol/L/hr	observations throughout crop **Who**: farm micro-biologist	reduce commercial feed, reduce stresses (ie **no** rapid water exchange), and partial harvest to reduce biomass.	
13. Viral disease outbreak	Disease outbreak.	Carefully monitor feed trays, growth rates and survivals in each pond.	Yes	Dead prawns and visual signs of disease (eg dead prawns at edge of ponds or on pond bottoms, seagulls diving)), Positive PCR tests for virus (eg GAV)	**What**: prawn virus **How**: written record; farm & lab testing **When**: test diseased animals and ongoing observations throughout crop **Who**: farm micro-biologist	In severe (ie acute) cases with rapid increases in mortalities, harvest live prawns. In mild cases, reduce commercial feed, reduce stresses (ie **no** rapid water exchange), and partial harvest to reduce biomass.	Farm records for ponds.
14. Treatment of pond effluent	Unnecess-ary impacts on the immediate environme nt of the farm through cyanobact erial blooms, increased nutrient levels and increased pathogeni c bacteria levels.	Monitor water quality of discharges according to the licence conditions set by regulators. Adopt world's best practices for effluent treatment and verify with an appropriate monitoring system.	No			The water should be treated by passing it through an appropriate settlement pond, a biofiltration area for nutrient stripping and wherever possible recycled.	Farm records for ponds.

Process step	*Hazard*	*Control measures*	*It this a CCP?*	*Critical limit*	*Monitoring procedure*	*Corrective action*	*Records*
15. Resuspens-ion of sediments in water column	High levels of pathogeni c bacteria in water, increased fertilisatio n of algae, reduced water quality in effluent and high total suspended solids in water and effluent.	Ongoing process throughout crop to monitor bloom character-isitics and sediment condition.	No			Take action to stabilise walls from wave erosion, carefully adjust aerators to reduce resuspend-ing bottom sediments, minimise resuspend-ing pond sediments during drain-down, use appropriate harvesting methods to avoid disturbing sediments.	

One of the reasons for having a Support Program for Good Management Practices for bacteria is to reduce stress on *Penaeus monodon* at every stage in its culture. Many good practices are carried out by farmers and it is not necessary to reinforce them here. However, there are some key issues that were clearly identified in the study and it is recommended that some changes in approach be adopted. The reasons are summarised as follows.

1) **Testing by PCR techniques**. Spawners, larvae, PLs and juvenile prawns are routinely tested by PCR methods in every overseas country in order to detect and reduce the occurrence of prawn viruses. This study found that whenever moribund prawns were found at farms, PCR testing revealed they were suffering from GAV (see Section 6.4.3). This finding is consistent with the viral work by Spann and others at the CSIRO as well Owens at James Cook University. However, PCR testing for GAV or any other virus is **not** routinely carried out in Australia in the hatchery stage. In fact, important groups, such as the CSIRO (P. Walker APFA 2001) recommend that PCR testing should be limited to surveying wild prawns, not in hatcheries. Their argument is that false positives would cause a hatchery to unnecessarily close down. In my opinion, this approach is at the very least naïve and dangerous. False positives are becoming rare events in PCR work and positives can be rapidly re-checked by alternative approaches before a hatchery is required to shut. The industry needs to adopt best practices in this area of disease control and have a clear, transparent, proactive viral testing program.

2) **Quality Assurance and best practices**. Hatcheries should have a clear and transparent Quality Assurance (QA) program regarding the health and history of spawners they use as well as the PLs they produce. The records from the QA program should be provided to farmers when they purchase PLs. The records should mainly consist of a simple check-sheet with appropriate brief descriptions that convey to the farmers the date that tests and observations were made and the results of all tests and observations. This QA program should be controlled and regulated by the industry (ie APFA) rather than by government agencies.

3) **Live transport of animals**. One of the most important, but poorly performed operations, is the transport of live spawners and PLs. This is a key, critical, urgent issue for research. This study found that bacterial loads and pathogenic conditions in transport bags is a major cause of stress to spawners and to PLs. I believe that these conditions cause expression of latent (ie dormant viruses), poor fecundity (ie reproductive output) and introduction of high loads of pathogenic bacteria to hatcheries and farms.

4) **Acclimatization of PLs at farms**. Farmers need to have an improved system for acclimatizing newly arrived PLs. It is not unusual for PLs to arrive at farms after travelling for 12 to 24 hrs, then be held in formalin-treatment tanks for a few hours prior to release into ponds. The whole process usually causes stress to PLs through an increase in pathogenic bacteria and build up of carbon dioxide. (Carbon dioxide is a silent killer because it is more soluble in water than oxygen and causes anaesthesia and death, even though oxygen levels may be very high.) In order to monitor any immediate effects of the transport and acclimatization process, it is recommended that farmers should routinely hold subsamples of PLs in pond enclosures or tanks for about one week after arrival. These PLs should be observed daily so that any mortalities can be easily determined and sampled for testing.

5) **The application of supplementary feed with activated microbes** is one of the most significant advances in prawn farming in recent times and this study has shown that it may be a very powerful tool. It is a means by which a farmer can culture beneficial bacteria (ie probiotic) bacteria and a supplementary food for prawns.

CHAPTER 7. BENEFITS

The benefits of the project can be summarized as follows.

a) Overall, the outputs for farmers are i) a new tool to determine the bacteriology of ponds, ii) a sensitive set of bioindicators, namely bacterial extracellular enzymes, and iii) new knowledge from the results of project. These outputs are capable of providing benefits to farmers through improved pond productivity and better price/kg. To explain, the results of the project could be applied to add value to the farmer's operations and hatchery operations by improving survival rates, improving growth rates, lowering feed conversion ratios, and increasing productivity. Financial benefit should follow, since an improved appearance of farm product should add value to the sale price of prawns and better productivity should increase the income of prawn farmers.

The project clearly identified problems in the bacteriology of current practices in transport of spawners and postlarvae. It is proposed that these hostile, stressful conditions trigger outbreaks of viral and bacterial disease. This is an area that requires further research and substantial improvements are achievable and essential. Further, the project provides a better understanding of the cause and effect of bacterial diseases such as: black marks on shells, tail rot and toxicity of blue-green algae (cyanobacteria). Also, the project provided an extra benefit in describing the effects of infectious agents, such as *Vibrio* bacteria and Gill Associated Virus (GAV), on the nervous system and endocrine system of prawns. This provides farmers and researchers with a new way to interpret signs of disease and effects of pathogens.

b) Since pathogenic bacteria represent the most significant obstacle to re-using effluent from ponds, the new technology and results of the study provide both knowledge and techniques to improve the quality of farm effluent. This is a critical issue because effluent can be a significant hazard and concern for prawn farms (i.e. self-pollution, compliance with permit conditions, load based-licencing fees) and fisheries habitats (release of pathogenic bacteria and deterioration of estuarine habitats). In the project, the new technology was used to investigate the efficiency of various types of effluent treatment systems. Also various methods of aeration and biofiltration were examined in mesocosms and some of these methods were also tested on farms. The findings can be used by farmers to reduce impacts of farm effluent on fisheries habitats. Existing farms and applicants for new farms could benefit by using the knowledge from the project to design improved treatment systems as well as use the new technology to test the effectiveness of treatment systems on the bacteriology of farm effluent.

c) Other forms of aquaculture that use earthen ponds (such as barramundi farming, silver perch farming, trout farming and eel farming) should be able to benefit by applying the new technology to their situations.

d) Finally, estuarine habitats are often used as spawning areas and fish nurseries for many commercial species of fish. The study has provided a new technique, new bioindicators and knowledge for understanding the bacteriology of these important fisheries habitats. This is capable of providing benefit to Australia's environmental resources and resources of the commercial fishing industry.

CHAPTER 8. FURTHER DEVELOPMENT

From 1993 to 2001, there has been a marginal increase in the number of prawn farms in Australia (from 35 to 43) while other forms of aquaculture have boomed. For example, in South Australia the rise of tuna farming and oyster farming has been spectacular. The prawn farming industry has received approximately $20 million in funds from FRDC, CRC Aquaculture, ACIAR and various other agencies over the last 15 years (Dundas-Smith, 2000). It is a common complaint of the prawn farming industry that they have not received value for this expenditure. I have my own views on whether this comment is justified; however I am determined to ensure that this comment cannot be applied to this project.

It is essential that the results be communicated to the individual prawn farmers, hatchery operators and other stakeholders. To this end, the full results of the project will be made available through a variety of means. Most importantly, once this final report has been distributed in late-2003, manuscripts will be sent to a variety of publishers. Articles will be written for "*Global Aquaculture Advocate*"; a well-read magazine that it is circulated among Australian farmers and contains the concise findings of projects in layperson's language. The manuscripts will contain details of the key results from the project that are relevant to sustainable prawn farming and treatment of farm effluent for re-cycling.

The other means of communicating results to the industry and stakeholders will be through publication of scientific papers in refereed journals (see Appendix 5: Tangible outputs of the project).

The next question is: "Where to from here for future research regarding farm bacteriology"? The Principal Investigator will endeavour to follow up important observations that were made during the project. Two examples of potential projects that should be further developed are as follows.

1) The project identified key extracellular enzymes that appeared to be reliable signatures for prawn farm effluent. Further, these key bioindicators should be used in trials to investigate improved methods for treating farm effluent. The aim of that study would be to develop cheap, reliable, low maintenance methods for treating and polishing farm effluent so that it would be suitable for re-use, thus minimising impacts to fisheries habitats. Also, these vital bioindicators could be used to determine the carrying capacity of coastal estuarine habitats.

2) The project used mesocosms to identify cheap organic supplements that appeared to improve prawn growth. In 2002, after the FRDC project finished, P Smith used supplementary funds to carry out a small study on a farm on the Clarence River using one of those organic additives (i.e. rice pollard). It appeared to improve microalgal bloom quality, reduce the incidence of blue-green algae in ponds, improve prawn growth and improve the appearance of prawns (by reducing incidence of black marks on the shell from bacterial disease). This type of farm-based research needs to continue with a more extensive range of cheap organic supplements.

CHAPTER 9. PLANNED OUTCOMES

The impacts and outcomes for the industry that were originally planned have not occurred to any substantial degree. So far communication of findings has been through preliminary articles (see Appendix 5: Tangible outputs of the project). Unfortunately, since the first report of preliminary results of the project to the Australian Prawn Farmers Association at its Annual General Meeting in July 2000, attempts to speak at meetings of the APFA in 2001 and 2002 have not been accepted by the executive. Without an opportunity to explain the findings of the project at this forum, it has been difficult to communicate effectively with the industry. As a consequence, it seems that the most appropriate method of conveying the results and achieving the planned outcomes will be through the publication of the findings in well-read magazines and journals.

CHAPTER 10. CONCLUSION

The objectives of this study focused on developing a new technique for investigating the bacteriology of prawn farms, using the technique to identify hazards posed by bacteria and identifying methods for better management of pond bacteriology. The project was able to successfully carry out its objectives and the outputs were as follows.

a) Development of a new technology based on bacterial extracellular enzymes for determining bacteriology of prawn ponds and hatcheries.
b) Verification that bacterial extracellular enzymes can be used as sensitive bioindicators of prawn ponds and hatcheries.
c) Identification of evidence that bacterial extracellular enzymes can provide a new level of information than conventional tools for monitoring conditions in water and sediment.
d) Identification of key extracellular enzymes (proteases, chitinases) that are associated with critical stages in pond deterioration and that can be used to forecast development of unfavourable pond conditions for prawn growth.
e) Identification of levels of key extracellular enzymes that are associated with high productivity and favourable conditions for prawn growth.
f) Identification of hostile, stressful conditions in the transport of spawners and post larvae.
g) Identification of hazards that bacteria pose to the prawn farming industry and critical control points for farmers to be aware of in the HACCP process.
h) Identification of management practices that can be used by farmers to improve pond conditions and effluent quality. This is particularly important for the industry as it aims to develop effluent treatment and re-cycle systems.

The project's outputs provide a strong basis for producing the outcomes that were planned for the prawn farming industry and fisheries habitats. With regards the prawn farm industry, the results of the project are capable of adding value to farm and hatchery operations by improving survival rates, improving growth rates, lowering feed conversion ratios and increasing productivity. The communication of the results of the study will occur through the publication of articles for farmers. By this means the project aims to deliver outcomes to the prawn farming industry.

The outcomes for fisheries habitats should occur in estuaries, which are well-known as spawning areas and fish nurseries for many commercial species of fish. The study has provided a technique with new bioindicators and new knowledge for understanding the bacteriology of these important fisheries habitats. This is capable of providing benefit to Australia's environmental resources and resources of the commercial fishing industry.

Further research work would be of great benefit to the industry and fisheries habitat. At least three topics for study have been identified. Key extracellular enzymes could be used to assist in investigating improved methods for treating farm effluent so that it could either be re-used. Also, improvements in transport of spawners and postlarvae should be researched. Finally, use of bacteria in the preparation and application of inexpensive, low-value organic feeds (eg bran) should be investigated.

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Appendix 1:
Intellectual Property

Much of the data obtained in this project has not been published and the author plans to write-up the results with the assistance of the active researchers in the project. These manuscripts will be submitted to international journals and magazines that are circulated among prawn farmers. Our aim is to submit one manuscript every 4-6 weeks following the printing of the Final Project Report.

Appendix 2:
Staff

A total of 44 people were involved in the project. The following eight academic staff carried out the main research activities for the project.

Dr Paul T Smith (Principal Investigator)
Daniel Ivanoff (Full-time field-based Research Assistant in Cairns 1999-2001)
Chantelle Agha-Hamilton (Full-time lab-based Research Assistant 1999-2001)
Mario Godoy (Full-time lab-based Research Assistant 2001)
Michael Cassaniti (Full-time lab-based Research Assistant 2001)
Juliana Tasevska (Full-time lab-based Research Assistant 2001)
Dr Stuart Findlay (Advisor on limnology)
Dr Than Pe (Advisor on statistics)

The following table lists 72 project-related activities that were carried out by a further 36 people and the source of funding.

Name	*Period*	*Occupation*	*Source of funds*	*Topic of activity*
Research Record Book #1				
Dr Jes Sammut	20-25 July 1998	Academic	FRDC	Launch of Project
Stephen Masters	20-25 July 1998	Academic	FRDC	Launch of Project
Tai Le	20-25 July 1998	Academic	FRDC	Launch of Project
Professor Jane Marceau	20-25 July 1998	Academic	UWS	Launch of Project
James Walsh	20-25 July 1998	Academic Administration	UWS	Launch of Project
Rita Maher	Spring 1998	Casual Employment	P Smith's Quantum funds	Electron Microscopy of eye virus
Mariam Fares	Spring 1998	Casual Employment	P Smith's Quantum funds	Electron Microscopy of eye virus
Raney Elahmadien	Spring 1998	Casual Employment	P Smith's Quantum funds	Isolation of bacteria from samples from Jangalade Hatchery
Ernest Bajzek	Spring 1998	Casual Employment	P Smith's Quantum funds	Isolation of bacteria from samples from Jangalade Hatchery
Craig Downes	Spring 1998	Casual Employment	P Smith's Quantum funds	Heavy metal analysis and histology
Grace Lee	Spring 1998	Casual Employment	P Smith's Quantum funds	Enzyme studies (chitinase)
Veronica Mendez	Spring 1998	Casual Employment	P Smith's Quantum funds	Enzyme studies (chitinase)
Vivian Chen	Spring 1998	Casual Employment	P Smith's Quantum funds	Cyanobacteria toxins

Name	*Period*	*Occupation*	*Source of funds*	*Topic of activity*
Sajid Mahmood	Spring 1998	Research student	UWS – Ph D student	MUB protocol development
Research Record Book #2				
Madj El Haj	Spring 1998	Casual Employment	P Smith's Quantum funds	Electron Microscopy of eye virus
Walaa Nasr	Spring 1998	Casual Employment	P Smith's Quantum funds	Electron Microscopy of eye virus
Naz Al-Hafid	Spring 1998	Casual Employment	P Smith's Quantum funds	Electron Microscopy of eye virus
Dory Sadek	Spring 1998	Casual Employment	P Smith's Quantum funds	Electron Microscopy of eye virus
Wade McKechnie	Spring 1998	Casual Employment	P Smith's Quantum funds	Jangalade Hatchery: bacteria culture
Vickie Page	Spring 1998	Casual Employment	P Smith's Quantum funds	Jangalade Hatchery: bacteria culture
Leo Bascur	Spring 1998	Casual Employment	P Smith's Quantum funds	MUB protocol with microplates
Hien Tran	Spring 1998	Casual Employment	P Smith's Quantum funds	MUB protocol with microplates
Ernest Bajzek	Spring 1998	Casual Employment	P Smith's Quantum funds	Isolated bacteria from Jangalade samples
Raney Elahmadien	Spring 1998	Casual Employment	P Smith's Quantum funds	Isolated bacteria from Jangalade samples
Dalia Khanangah	Spring 1998	Casual Employment	P Smith's Quantum funds	Photography of blue-green algae
Christeen Nasralla	Spring 1998	Research student	UWS – 111 yr project	Use HPLC to isolate toxin
Nina Woodcock	Spring 1998	Research student	UWS – 111 yr project	Use HPLC to isolate toxin
Juliana Tasevska	Spring 1998	Casual Employment	P Smith's Quantum funds	Blue-green algae culture
Ben Thompson	Spring 1998	Casual Employment	P Smith's Quantum funds	Blue-green algae culture
Glenn Brown	Spring 1998	Casual Employment	P Smith's Quantum funds	Toxicity tests
Graig Downes	Spring 1998	Casual Employment	P Smith's Quantum funds	Histology of eye virus, B/G toxicity tests
Kellie Watts	Spring 1998	Casual Employment	P Smith's Quantum funds	Electron Microscopy of eye virus
Laura Mahmood	Spring 1998	Casual Employment	P Smith's Quantum funds	Maintenance of equipment
Maria Tembo	Spring 1998	Casual Employment	P Smith's Quantum funds	Maintenance of equipment
Research Record Book #3				
Barry O'Rourke	September 1998	Academic Administration	UWS	Designed "Prawn farming downunder"

Name	*Period*	*Occupation*	*Source of funds*	*Topic of activity*
Dr Clem Kuek	September 1998	Academic	UWS	Design of Website for project
Research Record Book #4				
Leo Bascur	Spring 1999	Casual Employment	UWS – student scholarship	Soil sampling at farms in Cairns
Craig Downes	Spring 1999	Casual Employment	UWS – student scholarship	Soil sampling at farms in Cairns
Research Record Book #5				
Nadine Fenerty	Spring 1999	Casual Employment	FRDC	Set up ACCESS software for data entry
Research Record Book #6				
Stuart Pearce	Nov to Dec 1999	Casual employment	FRDC	Culture of microalgae and cyanobacteria.
Vickie Page	Nov to Dec 1999	Casual employment	FRDC	Culture of microalgae and cyanobacteria.
Chaza Ferdaws	Spring 1999	BSc project	UWS	Culture bacteria and measure MUB profiles with various foods
Lena Rayan	Spring 1999	BSc project	UWS	Culture bacteria and measure MUB profiles with various foods
Kathrine Morris	Spring 1999	BSc project	UWS	Culture bacteria and measure MUB profiles with various foods
Ben Thompson	Spring 1999	Casual Employment	P Smith's Quantum funds	Soil analysis
Brad Foreman	Spring 1999	Casual Employment	P Smith's Quantum funds	Soil analysis
Stephen Masters	Spring 1999	Casual Employment	P Smith's Quantum funds	Soil analysis
Gabriella Correa	Spring 1999	Casual Employment	P Smith's Quantum funds	Soil analysis
Dalia Khanangah	Spring 1999	Casual Employment	P Smith's Quantum funds	Soil analysis
Mariam Fares	Spring 1999	Casual Employment	P Smith's Quantum funds	TEM of eyes
Rita Maher	Spring 1999	Casual Employment	P Smith's Quantum funds	TEM of eyes
Ernie Bazek	Spring 1999	Casual Employment	P Smith's Quantum funds	Soil analysis
Raney Elamadieh	Spring 1999	Casual Employment	P Smith's Quantum funds	Soil analysis
Walla Nasr	Spring 1999	Casual Employment	P Smith's Quantum funds	Soil analysis
Research Record Book #7				
Zero Crawford	March to December 2000	Casual employment	FRDC	Collection of samples from farms in FNQ

Name	*Period*	*Occupation*	*Source of funds*	*Topic of activity*
Stuart Pearce	March to December, 2000	Casual employment	FRDC	MUB of bacteria, plankton counts and TCBS plating
Vickie Page	March to December, 2000	Casual employment	FRDC	MUB of bacteria, plankton counts and TCBS plating
Research Record Book #8				
James Walsh	24/7/00 to 28/7/00	Academic Admin	UWS	Mid-Project Meeting in Cairns (results presented to farmers)
Min Ah Yoon	Spring 2000	Casual Employment	P Smith's Quantum funds	Prepare samples of prawns for TEM
Tim Goddard	Spring 2000	BSc research project student	UWS	MUB measurements in tanks tests
Mario Godoy	Spring 2000	BSc research project student	UWS	MUB measurements in tanks tests
John Huynh	Spring 2000	BSc research project student	UWS	MUB measurements in tanks tests
Research Record Book #9				
Mario Godoy	Autumn 2000	Casual employment	P Smith's Quantum funds	Process samples from Tomei Hatchery
Pamela Garcia	Spring 2000	Casual employment	P Smith's Quantum funds	*Nodularia* sub-culture and freeze-dry
Khan Ngyuen	Spring 2000	Casual employment	P Smith's Quantum funds	*Nodularia* sub-culture and freeze-dry
Grace Hey	Spring 2000	Casual employment	ACIAR	Acid sulfate tests on soils from farms
Madj El Haj	Spring 2000	Casual employment	ACIAR	Acid sulfate tests on soils from farms
Tai Le	Feb 2001	Academic	FRDC	Project meeting & planning workshop
Research Record Book #10				
Richard Von Fister	1st March to 31st July 2001	Casual employment	FRDC	Collected farm samples from Clarence River
John Huynh	2000	Casual employment	P Smith's Quantum funds	Processed sediment samples from Cairns
Tim Goddard	2000	Casual employment	P Smith's Quantum funds	Cultured of prawns in tanks for MUB tests
Research Record Book #11				
Alan Sluter	December 2001	Casual employment	P Smith's Quantum funds	Histology work
Research Record Book #12				
Richard Von Fister	1st October 2001 to 31st May 2002	Casual employment	P Smith's Quantum funds	Collected farm samples from Clarence River

Appendix 3:
Formal training and in-service for staff

During the project the staff were able to improve their level knowledge and skills in electron microscopy, HPLC (High Pressure Liquid Chromatography) and molecular genetics. The following table lists the training that was undertaken.

Date	*Location*	*Participants*	*Objectives*	*Source of funds*
8/3/99 to 12/3/99	Sydney University Electron Microscopy Centre	P Smith and C Agha-Hamilton	Electron microscopy training	P Smith's Quantum funds
2/11/00	UWS laboratory	P Smith, C Agha-Hamilton, M Godoy, M Cassaniti, J Tasevska	HPLC training: cyanobacteria toxin identification	P Smith's Quantum funds
20/2/01 to 24/2/01	UWS Laboratory	J Munro, C Agha-Hamilton, D Ivanoff and P Smith	PCR training for WSSV identification	P Smith's Quantum funds
18/6/01 to 22/6/01	UNSW	P Smith, C Agha-Hamilton, J Tasevska	Recombinant DNA Techniques: Theory and Practice	P Smith's Quantum funds

Appendix 4:
Field trips and farm visits

Daniel Ivanoff carried out the bulk of sample collection in the farms in Far North Queensland with a total of 102 field trips. Also Richard von Fister carried out 15 field trips to farms on the Clarence River during 2001-2. A further 24 field trips to farms and 1 trip to a workshop in Brisbane were undertaken by P Smith and other people. These included 12 field trips to farms in Far North Queensland, 11 field trips to farms on the Clarence River and one field trip to Darwin on a related ACIAR project.

The following table lists the field trips and travel by team members (other than weekly field collections in Far North Queensland by Daniel Ivanoff and the Clarence River by Richard von Fister).

Date	*Location*	*Paricipants*	*Purpose*	*Data gathered*
2/10/98 to 11/10/98	Clarence River	P Smith	MUB tests on PLs from hatchery in QLD (Fortune) and ponds 22-26 at Tru Blu; Toxicity test with cyanobacteria (blue microcystis 8/1/96) and PLs	MUB, TPS, B/G killed PLs –checked high ammonia level (on 6/10/98: 100% mortalities at 0.4ml/10ml with boiled, unheated and filtered in 4hrs; on 7/10/98, 50% mortalities after 6 hrs with 0.016ml/10ml).
2/11/98 to 5/11/98	Cairns	P Smith	Coordinate project for farms around Cairns, MUB tests on 3 farms (Ponderosa, Searanch, Coco's farm)	MUB tests, collect diseased prawns and B/G algae
27/11/98 to 30/11/98	Cairns	P Smith, Dr S Findlay	MUB protocol, select sample sites, investigate morbid prawns, blood tests	MUB, diseases prawns, chilled at 4.5^{o}C, see 8/12/98 for results of blood tests.
1/12/98 to 3/12/98	Clarence River	P Smith, Dr S Findlay	MUB protocol, select sample sites, Hatchery work with MUB	MUB, pH of autoclaved sediment was 9.3, live sediment was 8.3 = increased pH caused increased fluorescence
17/12/98 to 21/12/98	Clarence River	P Smith	MUB protocol - filter centrifuge, fresh samples	MUB of many samples from each pond (ie statistical reproducibility)
15/2/99 to 18/2/99	Cairns	P Smith and D Ivanoff	Relocate D Ivanoff to Cairns, introduce him to farmers and QDPI, train him in his field duties	Selection of sites and methods for sample collection and processing and sending samples to UWS
15/3/99 to 18/3/99	Cairns	P Smith and D Ivanoff	Review MUB work so far, plan next steps, develop program for entering data into database	Identified equipment required for field work, format for database, confirm sample sites for 1999 at 3 farms near Cairns

Date	*Location*	*Paricipants*	*Purpose*	*Data gathered*
2/4/99 to 11/4/99	Clarence River	P Smith	MUB work, eye pathology, blood samples for bacteria, B/G toxicity	Checked results of histopathology slides from EMAI (see 4/4/99), collected sick prawns and B/G for histopathology and TEM (see 5 & 6/4/99& 10/4/99), collected blood from healthy and sick prawns (see 5,6,7/4/99), MUB samples (see 8/4/99), challenge with *Vibrio* isolates (i.m. injection see 9&10/4/99)
14/6/99 to 18/6/99	Cairns	P Smith, C Agha-Hamilton	Team meeting to discuss results	Developed ACCESS software program for entry of data into database (see list of variables for SPSS analysis 18/6/99).
10/7/99 to 17/7/99	Cairns	P Smith	Team meeting, construct walkways at Searanch for sample collection, further work on database	Walkways constructed and bore holes marked, database: variables clearly defined
6/9/99 to 10/9/99	Darwin	P Smith, Sajid Mahmood	ACIAR funded project	Soil sampling
24/9/99 to 3/10/99	Clarence River	P Smith	MUB work on hatcheries	MUB data on various waters from hatcheries, TCBS data (see 27/9/99, MUB results for 10 bacterial isolates given saline and peptone (see 9 & 10/9/99)
8/11/99 to 11/11/99	Cairns	P Smith	Disease outbreak at Searanch	Fixed prawns for histopathology and TEM (see 8/11/99), B/G toxicity challenge test with filtered and boiled extracts– prepared for histopathology and TEM (see 10/11/99 & 14/11/99)
14/12/99 to 22/12/99	Clarence River	P Smith	MUB of filtered and unfiltered samples, B/G work	Fixed prawns for B/G histopathology, MUB for filtered vs unfiltered.(See 17-22/12/99)

Date	*Location*	*Paricipants*	*Purpose*	*Data gathered*
20/3/00 to 23/3/00	Cairns	P Smith, C Agha-Hamilton	Meeting with Chris Stafford and Chris Robertson (QDPI) regarding their NHT project at Ponderosa, challenge test with heavy metals, B/G challenge, progress with data collection and entry	Challenge tests completed, samples taken for histopathology
20/4/00 to 30/4/00	Clarence River	P Smith	MUB over 6 days of measurements and treatment ponds, B/G tests, metal tests, check disease in farms.	*Nodularia spumegina* & *Microcystis* sp in Tru Blu pond 6, MUB collected vs time and treatment systems, TCBS counts
24/5/00 to 25/5/00	Brisbane	P Smith	Attend Prawn farm environmental workshop	Provided information on NSW treatment ponds and environmental monitoring
20/7/00 to 24/7/00	Cairns	P Smith, C Agha-Hamilton, D Ivanoff, J Walsh	Mid-term project report to Cairns farmers. (ie MUB results for Pondersoa, Searanch and Melivan farms). Training on SPSS for Daniel and Charlie on Daniel's computer.	Feed back from farmers on data.
20/9/00 to 10/10/00	Cairns	P Smith	Soil collection and Cairns sample collection while Daniel got married.	Soil samples collected and evidence of acid sulfate soils and the impacts on farming.
1/12/00 to 5/12/00	Clarence River	P Smith	MUB work on 3 farms, including treatment systems	MUB, feed, nutrients, biophysical data, management. Note: PLs died in test bucket in Pond 2 at Tru Blu – becoming red and had a pulsating excretory reflex (see 3/12/00) – preserved in 50% ethanol. Measured blood sugar level of 2 gram prawns ca 2-4 mg/dL (see 4/12/00)

Date	*Location*	*Paricipants*	*Purpose*	*Data gathered*
14/1/01 to 21/1/01	Clarence River	P Smith	MUB work on 3 farms, including treatment systems	Treatment systems & ponds, Blood sugar level of prawns (ca 1 for *P monodon* and king after 14hrs starvation, 4-16 mmol/L 45min after feeding) – see 17 &18/1/01), Seagull disease (in ponds 14,23,24,25,26) (see Sunday 13/1/01 & Thursday 18/1/01) and B/G toxicity tests (including *Nodularia*) (see 20/1/01)
12/2/01 to 16/2/01	Cairns	D Ivanoff, S Findlay, C Agha-Hamilton, T Pe, T Le & P Smith	MUB workshop, farm visits, data analysis and planning.	Visits to Ponderosa, Seafarm and Melivans, Seminars, workshop and discussions.
26/6/01 to 3/7/01	Clarence River	P Smith	Collect soils at end of season and attend NSW Fisheries meeting (3/7/01)	Soil samples brought back for chemical analysis.
31/7/01 to 4/8/01	Cairns	P.Smith	B/G toxicity, data discussions with Daniel and farmers, sample farm discharge	Toxin challenge see 3/8/01;
10/12/01 to 14/12/01	Clarence River	P Smith	Final productivity results for 2001, implementation of adding rice pollard. B/G toxicity and survival tests	Histopathology and survivals from B/G tests, observation of lack of B/G blooms with the use of rice pollard (see 14/12/01).

Appendix 5:
Tangible outputs of the project

A. Published work

a) Publications in international refereed journals

Smith PT 2000. Diseases of the eye of farmed shrimp *Penaeus monodon*. *Diseases of Aquatic Organisms*, **43**: 159-173.

b) Publication of a book chapter.

Smith PT 2000. In "Seafood toxicity" Editor L. Botana. Elseveir

c) Edited publications

Smith PT 1999. Bacteriology of shrimp ponds. In "World Shrimp Farming 1999" No. 12. Editor B. Rosenberry. Shrimp News International: San Diego. pp 75-6.

Smith PT 2000. Broodstock quality. In "World Shrimp Farming 2000" No. 13. Editor B. Rosenberry. Shrimp News International: San Diego. pp 116-7.

Smith PT 2001. Pond bacteria. In "World Shrimp Farming 2001" No. 14 Editor B. Rosenberry. Shrimp News International: San Diego. pp 198-199.

d) Conference presentations

Smith et al 2000. Australian Prawn Farmers Association, Brisbane.

e) Conference posters

Smith PT 2000 HAB 2000 Hobart
Le T and Smith PT 2000. HAB 2000 Hobart
Smith PT et al 2001. Australian Prawn Farmers Association
Smith PT and Kankaanpaa H 2001 Noosa

f) Industry reports

Smith PT 1999. Melivans prawn farm.
Smith PT 2000. Ponderosa prawn farm.
Smith PT 2000 Symons prawn farm.
Smith PT 2000 Tomei hatchery.
Smith PT 2001 Reefarm Hatchery.

B. Planned publications

A number of publications will occur following the printing of the Final Project Report. Below is a list of the first set of manuscripts being prepared for submission to refereed journals and magazines.

Smith PT, Agha-Hamilton C, Ivanoff D, Pe T, Findlay S Microbial extracellular enzymes in ponds at *Penaeus monodon* farms and nearby estuaries.

Agha-Hamilton, C, Smith PT, Tasevska J, Ivanoff D, Klimpel KR Survey of the Occurrence of GAV and WSSV at *Penaeus monodon* Farms in Australia.

Godoy M Cassaniti M, Smith PT Effect of biofiltration and supplementary foods on the growth of *Penaus monodon* and bacterial extracellular enzymes of mesocosoms.