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Final Report

Quality and safety assurance in marine finfish products - a pilot project

FRDC Project Number T 94/119

QUALITY AND SAFETY ASSURANCE IN MARINE FINFISH PRODUCTS - A PILOT PROJECT FRDC PROJECT T94/119

Final Report By
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FINAL REPORT

Quality and Safety Assurance in Marine Finfish Products - A Pilot Project

FRDC Project T94/119

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FUNDING SOURCES

Fisheries Research and Development Corporation
Aquatas Pty Ltd
Port Arthur Trout and Salmon Pty Ltd
Salmon Enterprises of Tasmania
University of Tasmania

NON-TECHNICAL SUMMARY

T94/119 QUALITY AND SAFETY ASSURANCE IN MARINE FINFISH PRODUCTS - A PILOT PROJECT

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BACKGROUND:

Products derived from mariculture, especially the Tasmanian Atlantic salmon industry, are perceived by the public as being prestige products of high value. As a result of this perception, there is the reasonable expectation that these commodities should be of high quality and safe to consume. The mariculture industry has striven to meet these expectations and the evidence available in 1994 indicated that, apart from a few isolated incidents, a high standard had been achieved and maintained. This project was designed as a pilot program to provide additional validation of the quality and safety of mariculture products with an emphasis on the Atlantic salmon industry.

The overall project consisted of three distinct sub-projects as under:

(1) A survey of potential bacterial hazards associated with commercial processing of fish, particularly Atlantic salmon.

(2) A laboratory study to ascertain if the important pathogen *Listeria monocytogenes* could proliferate in Atlantic salmon harvesting tanks.

(3) A survey of muscle tissues of Atlantic salmon and rainbow trout to detect and identify parasites and other causes of reduced flesh quality.

RESULTS AND DISCUSSION:

(1) Survey of potential bacterial hazards

Methods were developed to recover seven important pathogens from seafood, water and swabs. Using these methods, tissue surfaces (n=162) and flesh (n=27) of Atlantic salmon and blood water (n=9) were surveyed at three major factories using HACCP (hazard analysis and critical control points) in SE Tasmania during harvesting and initial processing. The prevalence of these potential pathogens on fish was found to be low (CP *S. aureus, A. hydrophila*) to very low (*E. coli, C. perfingens, L. monocytogenes, V. parahaemolyticus, V. vulnificus*). Wastewater produced at four major fish processing plants in SE Tasmania was also investigated. At two plants processing Atlantic salmon, some bacterial hazards present in wastewater (faecal coliforms including *E. coli,* and CP *S. aureus;* also Standard Plate Counts) were substantially lower (by >2 orders of magnitude) than in two plants processing wildcatch fish and

shellfish. Compared to fish flesh and tissue surfaces however, the levels of potential pathogens were generally much higher in wastewater, especially in effluent. At one plant studied, ultra-violet treatment of effluent reduced some bacterial hazards (faecal coliforms including *E. coli,* CP *S. aureus* and *L. monocytogenes*) to a low level.

The risks of food poisoning of the human consumer, and also of wound infections of fishing industry staff, due to the seven pathogens were provisionally assessed as minimal and low respectively. These predictions were made on the basis that HACCP systems were in operation during harvesting and initial processing. Critical control points were identified as: starvation of pre-slaughter Atlantic salmon, use of disinfected process water and equipment, temperature control (freezing or chilling at 0-2^{o}C), personal hygiene of staff, ambient water quality and staff training.

(2) Laboratory study of *Listeria monocytogenes*

A model system was designed to mimic the environment present in salmon-harvesting tanks. This system was used to determine the likelihood of *L. monocytogenes* growing in harvesting bins during normal harvesting activities.

No growth of this bacterium occurred during trial periods of 24 hour duration, but the organism did persist. Therefore, unless there is adequate cleansing and decontamination of equipment after harvesting, it is possible that *L. monocytogenes* could proliferate as temperatures increased to ambient. In this respect it was found that steam-jet cleaning was not an efficacious decontamination procedure.

(3) Survey of parasites and lesions in salmonid flesh

From September 1994 to May 1995 muscle samples were collected from 1606 Atlantic salmon and 120 rainbow trout processed at three factories in southern Tasmania.

Two instances of muscle infection were detected in Atlantic salmon. One fish had a low level of *Kudoa thyrsites* in its flesh and the other had a microsporidian infection. Both these parasites are of no health concern for humans, but are known, or suspected, to be the cause of flesh-softening of salmonid products in the northern hemisphere.

Infrequent blemishes found in fillets were found to be due to scar tissue and tumors (lymphosarcoma).

COMMENTS AND RECOMMENDATIONS

This study confirmed and reinforced the perception that Tasmanian marine salmonid products are of a high quality and can be consumed with confidence.

In order to maintain and improve on the present excellent situation the following recommendations are made:

(1) HACCP should be implemented widely in the Australian aquaculture industry to minimise possible bacterial disease outbreaks associated with contaminated, chilled, ready-to-eat foods.

(2) As initial results indicate that steam-jet cleaning is not efficacious in eliminating *L. monocytogenes* on smooth surfaces, further research on decontamination procedures for harvesting tanks is warranted with emphasis on the use of acid rinses followed by effective drying.

(3) Regular monitoring of parasites and lesions of muscle tissues from salmonids should be instituted so that any change from the present low prevalence of these blemishes can be detected and appropriate actions taken to limit them.

EXECUTIVE SUMMARY

This pilot project addressed three aspects of quality and safety of marine finfish (mainly Atlantic salmon) products, namely:

(1) Potential bacterial hazards associated with commercial harvesting and processing.

(2) Potential for growth of *Listeria monocytogenes* in harvesting tanks.

(3) Muscle parasites and lesions as a cause of reduced quality.

In essence, the study confirmed and reinforced the understanding that Tasmanian marine salmonid products are of a high quality.

The bacterial pathogen of major concern, *Listeria monocytogenes* was not recovered from Atlantic salmon during harvesting or initial processing (to the head-on, gilled and gutted stage). Also, a modelling trial indicated that this organism was not likely to proliferate in killing bins, when current management practices were followed. *L. monocytogenes* was found in wastewater at two of the four processing plants which were surveyed. This indicated that the factory itself is a potential source of this pathogen and strict hygiene within premises is mandatory.

Attempts to culture the opportunistic pathogens *Clostridium perfringens, Staphylococcus aureaus* (coagulase positive), *Escherichia coli, Aeromonas hydrophila, Vibrio parahaemolyticus* and *V. vulnificus* revealed that these organisms were present at low to moderate levels in some samples of "bloodwater" from the killing bins. These organisms were, presumably, present in the ambient water pumped into the bins. Lower levels, presumably originating from the anaesthetic bath and/or bloodwater, were detected on the fish themselves. Provided that proper hygiene is maintained during processing and storage these levels are not likely to constitute a risk to health.

Extremely low levels of muscle parasites (*Kudoa thyrsites* and microsporidians) were detected, thus emphasising the need to continue to monitor a meaningful number of fish to ensure that flesh quality is maintained. Any increase in prevalence of these parasites would require a concerted effort to identify the source and institute appropriate control procedures.

Muscle damage due to the development of scar tissue was regularly diagnosed and indicates that studies are warranted to identify the cause of this condition with a view to instituting control procedures.

A number of cases of lymphosarcoma involving the musculature of Atlantic salmon were detected and this condition warrants further research to elucidate its aetiology and method of spread.

PREFACE TO THE FINAL REPORT

This project was originally conceived as a study of flesh quality of Atlantic salmon, but on the advice of the Tasmanian Fisheries Research and Development Council it was modified to become a pilot project to study quality and safety aspects of marine finfish products. This project incorporated three discrete sub-projects as follows:

(1) A survey to assess potential bacterial hazards associated with commercial processing of fish.

(2) A modelling trial to determine if amplification of bacterial numbers could occur in killing bins used in the Atlantic salmon industry.

(3) A survey to ascertain the prevalence of flesh-softening parasites and other lesions in the muscles of sea-caged salmonids in Tasmanian waters.

Each of these sub-projects will be reported separately, but a general statement on background information, needs and objectives will precede these reports. Because the project was modified a number of times the needs and objectives evolved over a considerable period and the final, consolidated versions are incorporated in this report.

GENERAL BACKGROUND

The majority of aquaculture products produced in Australia are high value and, as a result, consumers expect them to be of high quality and safe. However, at times, these criteria may be antagonistic as fresh and mildly-processed products such as sashimi and cold-smoked fish are highly prone to carry deleterious organisms if there are lapses in hygiene and quality control during processing.

In particular, infections with *Listeria monocytogenes,* an organism capable of growing at low temperatures, has become of great concern to the food industry. Other bacteria which have been found in marine fish products include *Vibrio vulnificus* and *Vibrio parahaemolyticus*. Modern fish processing methods have substantially reduced the prevalence of such bacteria in marine finfish products, but there is a dearth of information as to where in the process contamination is likely to occur, and what conditions exacerbate the problem.

Flesh quality is affected by many, principally physiological variables, but a number of infectious agents, principally *Kudoa* species can produce profound deterioration in flesh quality. This latter parasite is prevalent in mahi mahi in Australia and Atlantic salmon in Canada, Ireland and parts of Europe, but has not as yet been recognised as a problem in the salmon industry in Tasmania. More recently microsporidian parasites have been described as causes of flesh-softening in turbot and Atlantic salmon in the northern hemisphere.

GENERAL NEEDS

In the first instance, fish processors need to be able to access fish stocks which are not likely to carry and/or be contaminated with pathogenic organisms. Also, they need assurance that these fish are unlikely to carry parasites which may affect the quality of the product or can infect man.

Secondly, they need to know where during processing contamination with pathogenic or spoilage organisms may occur and how such contamination may be obviated or reduced.

Also, processors need to understand where amplification of bacterial numbers may occur during processing and how this may be ameliorated.

GENERAL OBJECTIVES

The general objectives were:

(1) To survey fish and wastewater from fish processing factories for a range of human pathogens in order to determine, in part at least, the risk to human health.

(2) To examine in detail the killing process for Atlantic salmon to ascertain if this constituted a high risk segment of the overall harvesting and product preparation process.

(3) To survey the prevalence of flesh-softening parasites in Atlantic salmon and sea (rainbow) trout. Also, to diagnose the causes of lesions in the musculature of processed salmonids.

MUSCLE PARASITES AND LESIONS POTENTIALLY CAUSING DOWNGRADING OF SALMON PRODUCTS

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SUMMARY

From September 1994 to May 1995 muscle samples were collected from 1606 Atlantic salmon and 120 rainbow trout being processed at three factories in southern Tasmania. These samples originated from seven seafarm sites. Only two parasitic infections were detected, both in Atlantic salmon. One fish had a low-level of *Kudoa thyrsites* in its muscles and the other had focal accumulations of microsporidian spores.

Other samples collected opportunistically revealed a small number of muscle lesions caused by scar tissue, an abscess and lymphosarcoma.

These findings further confirm the high quality of products from Tasmanian sea-caged salmonids.

BACKGROUND

The quality of products prepared from Atlantic salmon and rainbow trout can be adversely affected by a number of parasitic infections and pathological processes. The work reported here was related entirely to those agents/processes which affect the quality of meat products derived from salmonids.

One of the major concerns worldwide in regard to fish meat quality is flesh-softening due to the myxosporean parasite *Kudoa thyrsites.* This parasite causes post-mortem liquefaction of muscle in a number of fish species including Atlantic salmon (Gillchrist 1924, Davies and Beyers 1947, Harrell and Scott 1985, Kabata and Whitaker 1989, Whitaker and Kent 1991, Barja and Toranzo 1993 and Baudin Laurencin and Bennassr 1993). In salmonids flesh-softening may not be detected until considerable expense has been incurred in smoking thus resulting in a significant economic penalty. In recent years *Kudoa thyrsites* has emerged as a demonstrated or perceived commercial problem in salmon farming in western Canada (Needham 1994) and Ireland (Palmer 1994). Although muscle softening has only been a minor problem to date in Tasmanian salmonids, *K. thyrsites* has been detected associated with softening in smoked product (Handlinger pers. comm.), and as a result the industry was concerned that a potential problem may exist.

The microsporidian parasite *Tetramicra* sp. described from turbot Esterez et al. (1992) has also been implicated in flesh-softening of Atlantic salmon in Ireland (Palmer, pers. comm.) and could be a particular cause of concern because of its direct life cycle.

A wide variety of pathological changes have been reported in the muscle tissues of fish (Ferguson, 1989), however the intention in the survey as undertaken was to opportunistically collect lesions which made the product unsaleable.

NEED

Tasmanian Atlantic salmon and sea (rainbow) trout have international reputations for being safe, "clean and green" products and it is imperative that this situation continues in order that market-share can be retained. In this regard it is not adequate to adopt a laissez-faire attitude, but the industry, and its scientific support, must be pro-active in identifying and quantifying potential threats and, where appropriate, taking action to minimise these threats. Consequently, research to identify and quantify causes of muscle deterioration and/or defects is a high priority.

OBJECTIVES

1 To determine prevalences of *Kudoa thyrsites* and other muscle parasites in sea-caged salmonids kept at different sites in Tasmania.

2 To opportunistically collect samples of lesions detected during processing and to identify the pathological processes leading to the presence of the lesion.

INTRODUCTORY TECHNICAL INFORMATION

Kudoa thyrsites is a microscopic, myxosporean, protozoan parasite which is found as multinucleate plasmodia or mature spores with four valves and four polar capsules (Fig. 1).

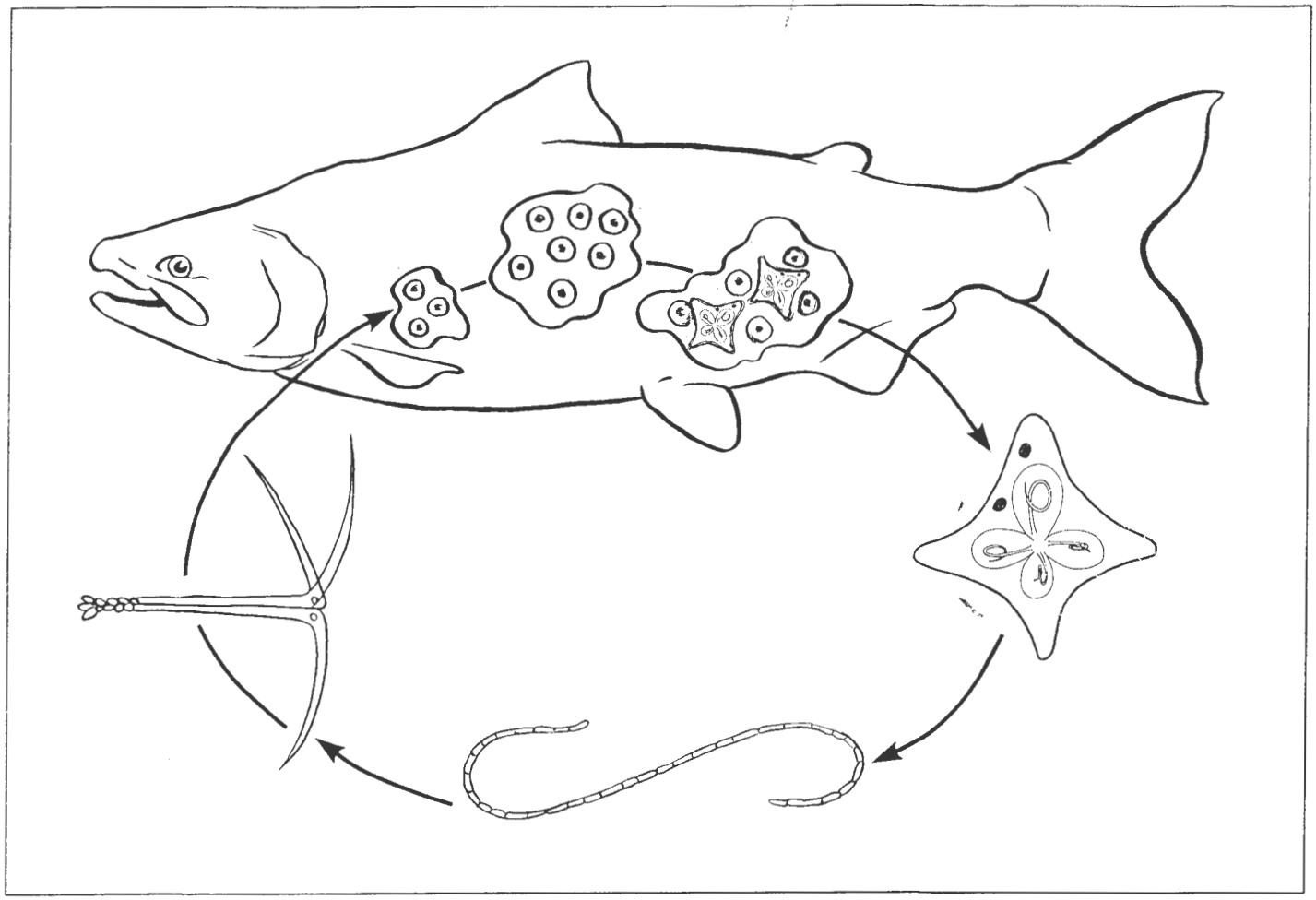

Figure 1. Possible life cycle of *Kudoa thyrsites* (after Kent 1992).

Consequently, detection of the parasite is by light microscopy using conventional tissue section or digests of the muscle.

Although the alternative form and host of *K. thyrsites* is not known it is probably an actinosporean stage in an oligochaete worm such as those described by Marques (1984) and Hallet et al. (1995).

METHODS

Survey of Muscle Samples for Parasites

1 Source of samples.

Samples were collected monthly (September 1994 - May 1995) from three processing plants in southern Tasmania servicing seven farms in south eastern Tasmania.

2 Numbers and types of fish sampled.

Usually 60 fish were sampled at each plant in each month. On six occasions 100-120 fish were sampled from the largest plant.

Presuming a 100% sensitivity for the test procedure, this sampling intensity should have detected a 5% prevalence with 95% confidence at any one sampling (Simon and Schill 1984). As many of the sequential samples were from the same populations the level of detection would have been at a lower level of prevalence in some instances - this will be discussed later.

There were 1606 samples from Atlantic salmon varying in age from 12-36 months of age (most 25-31 months of age) were examined.

3 Methodology

All examinations were undertaken on fish "frames" left after removing fillets for smoking. Some fish were fresh and some had been frozen.

Methods of examination were:-

(i) Visual for liquefaction.
(ii) Histology.

Muscle tissue from four different sites was taken, fixed in 10% formal saline, and processed by routine means to produce sections 5-6 um thick which were stained with haematoxylin and eosin and examined by light microscopy. The total area of muscle section examined was approximately 400 squmm per fish.

(iii) Formalin-ethyl acetate sedimentation technique.

This modification of the formalin-ether technique (Palmer 1994) was used on bulk samples from 60 fish.

Survey of incidental lesions

Abnormal tissues detected during processing by plant supervisors were fixed in 10% formal saline and processed by routine methods for preparation of stained (H x E) slides which were examined by light microscopy.

DETAILED RESULTS

1. Survey for muscle parasites (see Table 1)

Of the 1606 Atlantic salmon examined light infections with *Kudoa thyrsites* and a microsporidian (*Pleistophora* or *Tetramicra* sp.) were detected in one fish each from different farms by histology only. There was no evidence of muscle liquefaction.

In all, six individual farms were surveyed with the following levels of presumptive sensitivity (95% confidence, presuming test 100% sensitive).

Farm	No. fish	Parasites detected	Detectable % prevalence (i.e. 95% confidence in ability to detect at least one infected specimen when infected % equals that given).
1	483	1 x *Kudoa*	approx. 0.75%
2	400	1 x *Pleistophora* or *Tetramicra*	approx. 0.75%
3	280	nil	approx. 1%
4	303	nil	approx. 1%
5	80	nil	approx. 3.5%
6	60	nil	approx. 5%

Only one rainbow trout farm was surveyed with 120 samples being examined (detectable % prevalence 2.5%) and no parasites being detected.

2. Lesions submitted by processors.

Thirteen accessions were received. The diagnoses were;

Lymphosarcoma - three Atlantic salmon.
Scar tissue (some with melanosis) - six Atlantic salmon, three rainbow trout.
Abscess - one Atlantic salmon.

All of these results were presented and discussed at a meeting with industry representatives on 28 June 1995.

DISCUSSION INCLUDING BENEFITS

As *K. thyrsites* infection occurs at high prevalences in some species of fish in the wild in Australia (Langdon et al. 1992), there is no reason to believe that a similar situation could not prevail in Tasmanian salmonids, especially as they are grown under environmental conditions not too disimilar from those in Ireland and France where myoliquefaction due to this parasite is common. Without full knowledge concerning the parasite's life cycle it is difficult to determine why this difference exists, but two factors could be involved, namely;

1 Degree of net fouling.

If the alternative host is an oligochaete worm it is possible that this host could establish itself in the biofouling on the nets used to constrain the fish. As net changes are frequent under Tasmanian conditions it would be unlikely that the life cycle could be established within the netpen environment.

2 Absence of pancreas disease from Australian salmonid aquaculture.

Pancreas disease is common in Atlantic salmon cultured in European and, to a lesser extent, North American waters. This disease does not necessarily product high mortalities, but the fish become unthrifty and seek out "wild" feed rather than taking pellets. Under these circumstances the fish could well ingest substantial numbers of alternative hosts of *K. thyrsites* .

The finding of a *Pleistophora* or a *Tetramicra* sp. in an Atlantic salmoni is of concern because the latter organism is believed to cause flesh-softening in Atlantic salmon in Ireland. As these parasites have direct life cycles, the most likely source is wild marine fish. Indeed, a very similar organism was detected in wild barracouta (*Thyrsites atun).* (Su and Munday unpublished). Consequently, it would be prudent to exclude wild fish from netpens.

The origin of the scar tissue lesions is not known but is presumed to be the end result of trauma, either anthropogenic or inflicted by predators.

Lymphosarcoma is a relatively common neoplasm in Atlantic salmon and it's aetiology needs to be investigated to ascertain if it has any relationship to plasmacytoid leukaemia of salmonids in North America.

FURTHER DEVELOPMENT

The low level of *K. thyrsites* and microsporidian infections in Tasmanian Atlantic salmon is gratifying and needs to be maintained if possible. Recommendations in relation to these infections/diseases are:-

1 Regular surveys of at least one processing plant should be conducted to confirm that there are ongoing, low prevalences of *K. thyrsites* and *Pleistophora* or *Tetramicra* sp. infections.

2 If management practices change so that more biofouling establishes on nets then this surveillance should be intensified.

3 Every effort should be made to keep pancreas disease out of Australia.

Efforts should be made to identify the circumstances under which traumatic lesions leading to significant scar formation occur.

Appropriate action should be taken to ascertain whether or not lymphosarcoma in Tasmanian Atlantic salmon is the same as plasmacytoid leukaemia in Canada.

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ACKNOWLEDGMENTS

The authors acknowledge the considerable assistance provided by the management and staff of Aquahealth, Aquatas, Marine Research Laboratory, Nortas and Tassal.

TABLE 1

RECORD OF *KUDOA THYRSITES* SURVEY - ATLANTIC SALMON

DATE	NO. SAMPLES	FISH AGE(M)	FISH TYPE	*KUDOA* (NO. FISH)		INFECTION	NO. LIQUEFIED	OTHER PARASITES
				HS	ST			
7/9/94	60	12	Diploid	1 (No. 54)	0		0	0
8/9/94	60	13	Triploid	0	0		0	0
3/10/94	60	27	Diploid	0	0		0	0
5/10/94	100	28	Diploid	0	0		0	0
		29	Diploid	0	0		0	0
11/10/94	63	26	Diploid	0	0		0	0
2/11/94	100	27	Diploid	0	0		0	0
		27	Diploid	0	0		0	0
4/11/94	60	27	Diploid	0	0		0	0
4/119/94	33	26	Diploid	0	0		0	0
7/11/94	30	26	Diploid	0	0		0	0
TOTAL	566			1	0		0	0

RECORD OF *KUDOA THYRSITES* SURVEY - RAINBOW TROUT

DATE	NO. SAMPLES	FISH AGE(M)	FISH TYPE	*KUDOA* (NO. FISH)		INFECTION	NO. LIQUEFIED	OTHER PARASITES
				HS	ST			
5/9/94	60	22	Diploid	0	0		0	0
TOTAL	60			0	0		0	0

HS = Histological survey
ST = Sedimentation technique

MODELLING GROWTH OF *Listeria monocytogenes* IN FISH HARVESTING TANKS

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SUMMARY

A model system designed to mimic the environment present in salmon-harvesting tanks was used to determine the capacity of *Listeria monocytogenes* to grow during the harvesting process. The two environments thus examined contained sea water at 1 °C, pH = 5.6, bubbled with CO_2 or sea water at 9.6°C, pH = 7.6, bubbled with air. Over 24 hours no growth of *L. monocytogenes* was observed in either of these environments. The fish-harvesting process typically is completed within 5 h. However, it is likely that *L. monocytogenes* will survive in these environments. Therefore, efficient cleaning of tanks is essential to prevent persistance and slow buildup of bacterial populations. In this regard steamjet cleaning is ineffective. The temperatures that can be generated on flat surfaces are not lethal to *L. monocytogenes* and the pressure of the jet will likely disperse these cells.

Background

Concern over foodborne illness due to *Listeria monocytogenes* has greatly increased over the last two decades. While outbreaks of listeriosis are not common, they do have high case fatality rates (Benenson, 1990). *L. monocytogenes* has been commonly identified in environmental samples, fish and fish products (reviewed by Dillon and Patel, 1992, Fuchs and Reilly, 1992, Gibson, 1992). Colburn *et al.* (1990) reported the presence of *L. monocytogenes* in both fresh and marine waters, albeit with lower numbers in marine waters. Animals such as fish are known to carry *L.monocytogenes* as part of their normal microbiota (reviewed by Sutherland, 1989). Furthermore, there is ample evidence demonstrating the ability of *L. monocytogenes* to survive or even grow at refrigeration temperatures, in the presence of salt and at values of pH greater than 5 (e.g. Buchanan *et al.*, 1989, Buchanan and Phillips, 1990, Connor *et al.*, 1986, El-Shenawy and Marth, 1988, Parish and Higgins, 1989, Walker *et al.*, 1989). Because of this, concern has been raised about foods which are not subjected to strong heating prior to consumption (Dillon *et al.*, 1992). For example; smoking of salmon is usually achieved by a procedure that does not involve heating above 30°C at which temperature *L. monocytogenes* could comfortably survive and quite possibly grow. Sources of *L. monocytogenes* can either be the processing plant if it is not properly cleaned, or the fish itself during slaughtering and gutting. As the initial processing of salmon involves bleeding the fish in tanks, it is conceivable that *L. monocytogenes* derived from the animals, or their associated water, may grow in these tanks and thus contaminate other fish.

Need

Tasmanian Atlantic salmon is a prestige product with a reputation for quality. In order to maintain this reputation microbiological contamination must be kept to a minimum. As, prior to this study, it was not known if bacterial multiplication occurred in the high nutrient, "blood-water" generated during harvesting there was an urgent need to investigate this aspect of the processing procedure.

Objectives

To determine whether *Listeria monocytogenes* will grow in salmon-harvesting tanks during the processing of the fish.

To determine the effectiveness of the current tank steam-cleaning protocol to kill *Listeria monocytogenes*.

METHODS

Introductory technical information

Protocol for harvesting seacage salmon

The fish are starved for 24 hours prior to harvesting to empty their gut contents. Once removed from the sea cage, salmon are passed through a sequence of 4 stainless steel tanks:

- Tank 1 contains sea water with ice and is continually bubbled with CO_2. This environment is designed to anaesthetise the fish which are then individually removed and the aorta cut.
- Tanks 2 and 3 contain fresh water and are continually bubbled with O_2. In these tanks the fish are allowed to bleed for about 20 minutes.
- Tank 4 contains recirculated sea water to rinse the fish.

The anaesthetising and bleeding treatment lasts about 30 minutes for each fish, and the water in the tanks is changed every 3 to 4 hours if harvesting is to last for longer than this. After harvesting the tanks are drained, rinsed and steam-jet cleaned. During the bleeding process Tanks 2 and 3 become heavily contaminated with blood and mucus and probably to a small extent with faecal material.

Materials and methods

Field monitoring

The temperature, pH, dissolved oxygen and concentration of blood (as percentage packed cell volume) in the 4 tanks at Farm A were measured throughout a harvesting run in August 1995. On the basis of these data, a model system was established to mimic the fish harvesting environment. Temperatures achieved during steam cleaning of the processing tanks at Farm A were monitored on 21 December 1995.

Model system for growth studies

The model harvesting system was constructed to mimic tanks 1 and 2/3. That is, model tank 1 had sea water bubbled with CO_2 and was incubated at 1°C. Model tank 2/3 had sea water with 3% sheep's blood bubbled with air and incubated at 9°C. While tank 2/3 is initially filled with fresh water, it will be contaminated with sea water as fish are passed through during a harvesting run. A constant salinity should impart less stress than variable salinity and so the model system is likely to overestimate the ability of *L. monocytogenes* to grow *in situ*.

The model system consisted of five hundred millilitre flasks containing 300 mL of 0.2 µm-filtered sea water were incubated at different temperatures and types of (0.2 µm-filtered) gas bubbling, with or without added blood. All flasks were shaken during incubation on either an Oxoid Signal incubator rotary shaker or a Ratek Instruments orbital shaker.

Growth experiment 1

A preliminary experiment to give an indication of the order of magnitude of change in numbers of *L. monocytogenes* in the model harvesting system over three hours, which approximated the normal time taken for harvesting.

An overnight blood agar culture of *L. monocytogenes* was suspended in saline to give an absorbance of 0.2. This suspension was then inoculated to give final dilutions of 10^{-4} and 10^{-5} in sea water:

1. at pH = 5 bubbled with CO_2 and incubated at 1°C. One flask for each dilution.
2. with 3% sheep's blood at pH = 7.5 bubbled with air and incubated at 9°C. Two replicate flasks for each dilution.

One hundred microlitre samples were drawn from each flask at 30 minute intervals for 3 hours, spread plated onto blood agar and incubated at 37°C for 48 hours.

Growth experiment 2

To determine if any growth of *L. monocytogenes* occurred in the model harvesting system within the time that fish would be present in the processing system.

An overnight blood agar culture of *L. monocytogenes* was suspended in saline to give a count of 1.8×10^8 cells mL^{-1}. Flasks containing filtered sea water were inoculated and incubated with:

1. 1.8×10^6 cells mL^{-1}, bubbled with CO_2, pH = 5 at 1°C. Two replicate flasks.
2. 1.8×10^6 cells mL^{-1}, with 3% sheep's blood, bubbled with air, pH = 7.5 at 9°C. Two replicate flasks.

Both sets of flasks were incubated for 6 hours and 5 mL samples taken every 30 minutes for 2 hours and then hourly. The samples were diluted to 10^{-6}, spread plated on blood agar and incubated at 37°C.

Growth experiment 3

The second experiment indicated the possibility that some growth occurred during the first 30 minutes of incubation. To check this, experiment 2 was partially repeated and the blood-containing treatment at 9°C was performed in triplicate. To acclimate the cells they were diluted in saline at room temperature and 3 mL of this dilution (containing 1.08 X 10^6 cells mL^{-1}) added to 290 mL of cooled (to 9°C) sea water containing 3% blood. The flasks were intermittently bubbled (continuous bubbling caused foaming) with filtered air and were sampled at half hourly intervals for 2 hours and then at 4, 6 and 24 hours. Samples were immediately diluted to 10^{-6}, spread-plated in duplicate onto blood agar and incubated at 37°C.

Persistence experiment

To examine the survival of *L. monocytogenes* during steam-cleaning of the tanks after harvesting.

Low numbers of cells (0.1 mL aliquots of 10^3 cells mL^{-1}) were placed onto 20 sterile coverslips, 10 sterile petri dishes and into 10 sterile 30 mL bottles. Half of these treatments were allowed to dry overnight at ambient temperature (≈ 20°C) prior to steaming and the other half were immediately subjected to steam jet treatments in a class 2 biohazard chamber. The steam treatments were as follows:

a) Coverslips. Distance = 2 mm, time = 30 s, temperature = 62 - 64°C, 5 replicates. Individual coverslips were then placed into 10 mL of Oxoid UVM1 broth and incubated at 37°C for up to 20 days.

b) Coverslips. Distance = 5 cm, time = 10 s, temperature = 48 - 55°C, 5 replicates. Individual coverslips were then placed into 10 mL of Oxoid UVM1 broth and incubated at 37°C for up to 20 days.

c) Coverslips placed into individual sterile petri dishes prior to steaming. Distance = 5 cm, time = 10 s, temperature = 48 - 55°C followed by distance = 2 mm, time = 30 s, temperature = 62 - 64°C, 5 replicates. Individual coverslips were then placed into 10 mL of Oxoid UVM1 broth and incubated at 37°C for up to 20 days. In addition 20 mL of Plate Count Agar was added to the petri dish which was then incubated at 37°C for up to 2 weeks.

d) 30 mL bottles. Distance = 2 mm, time = 10 s, temperature = 96 - 97°C, 5 replicates. After steaming 10 mL of Oxoid UVM1 broth was added to the bottle which was then incubated at 37°C for up to 2 weeks.

e) Petri dishes. Distance = 2 mm, time = 30 s, temperature = 48 - 55°C, steam jet moved back and forth across petri dish, 5 replicates. After the steaming 20 mL of molten Plate Count Agar was added to the dish which was then incubated at 37°C for up to 2 weeks.

DETAILED RESULTS

Environmental monitoring

The data obtained from monitoring temperature, pH and dissolved oxygen during a harvesting period are given in table 1.

Table 1: Temperature (°C), pH and dissolved oxygen (mg L^{-1}) in salmon-harvesting tanks at Farm A during a harvesting run in August 1995.

Time	Temperature °C	pH	Dissolved oxygen mg L^{-1}
Tank 1: Sea water bubbled with CO_2 and cooled with ice.			
05.00	1.2	5.1	8.7
06.00	0.8	4.2	5.3
07.00	1.3	4.3	4.8
08.00	1.2	4.6	4.9
Tank 2/3: Fresh water bubbled with O_2 and containing fish blood.			
05.00	8.9	7.3	8.3
06.00	8.4	7.0	8.0
07.00	9.3	7.3	8.8
08.00	9.6	7.2	8.1
Tank 4: Recirculated sea water.			
05.00	7.9	8.0	9.2
06.00	9.9	7.9	8.1
07.00	9.5	7.7	7.9
08.00	9.1	8.1	8.5

The packed cell volume of blood in tanks 2/3 was found to be 3%. During a harvesting run in April 1995 the temperatures of the tanks were reported to be:

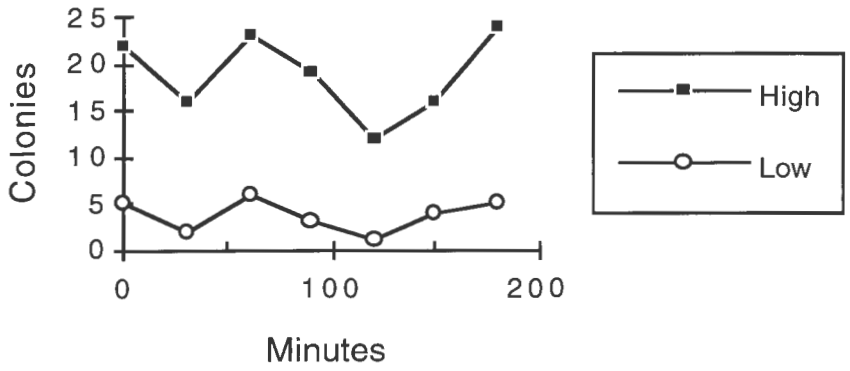
- tank 2: 14.8 - 6.6°C (decreasing over the two hour period).
- tank 3: 15.1 - 10.7°C (decreasing over the two hour period).
- tank 4: 15.0 - 15.2°C (constant over the two hour period).

Growth experiment 1

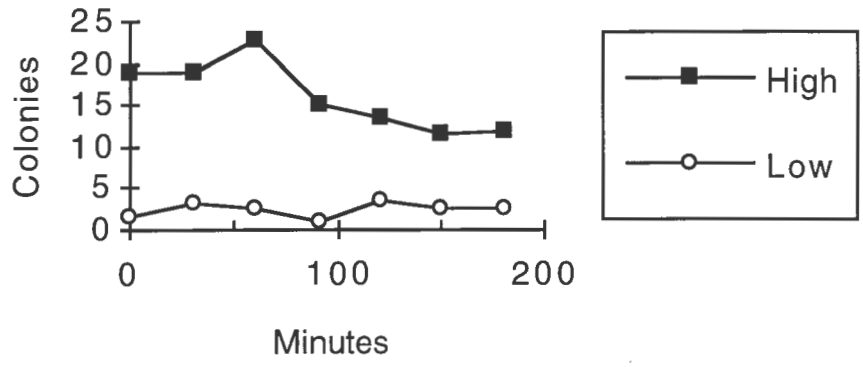
During this experiment no growth of *L. monocytogenes* occurred at either 1 or 9°C with either the high or low inocula. Colony counts demonstrating this are given in figure 1.

Figure 1: Numbers of colonies in 0.1 mL subsamples taken from the model harvesting system inoculated with a suspension of *L. monocytogenes* diluted to 10^{-4} or 10^{-5}. a) Model tank 1 at 1°C and b) model tank 2/3 at 9°C, mean of duplicate counts.

a)



(b



Growth experiment 2

From the results of the first experiment the inoculum was increased to 1.8 X 10^6 cells mL^{-1} and the number of cells monitored at 30 minute intervals for 2 hours and then hourly for another 4 hours. Results are demonstrated in figure 2. The numbers in the model tank with blood incubated at 9°C were higher than those in the CO_2 tank at 1°C. One-way ANOVA for each treatment indicated no significant difference in cell numbers at any time (P = 0.291 for 1°C treatment, P = 0.339 for 9°C treatment). Some growth may have occurred in the first 30 minutes of the incubation. To assess this the 9°C experiment was repeated.

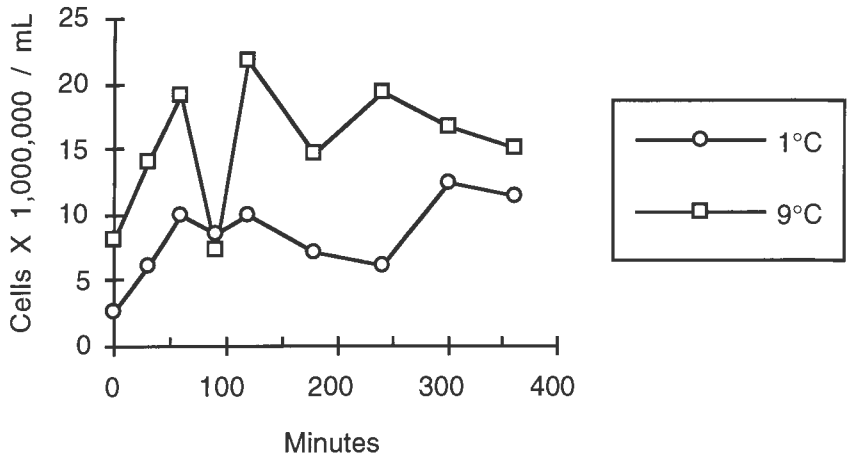


Figure 2: Mean cell concentrations (n=3) per mL of *L. monocytogenes* incubated in sea water with CO_2 bubbling at 1°C or with 3% sheep's blood, air bubbling and at 9°C.

Growth experiment 3

The model system was inoculated to give 1.1 X 10^4 cells mL^{-1} and the t = 0 samples contained 1.16, 1.02 and 1.08 X 10^4 cells mL^{-1}, indicating that no significant damage affecting cell viability occurred during inoculation. The mean and standard deviation for the replicate counts (n = 3) are illustrated in figure 3. No increase in the cell numbers is apparent throughout the incubation, indicating that no growth occurred. The mean cell counts in the model system are listed in table 2. One-way ANOVA indicated no significant difference in the counts at any time (P = 0.23).

Figure 3: Mean and standard deviation of cell numbers of *L. monocytogenes* in sea water containing 3% blood, shaken and bubbled with air at 9°C.

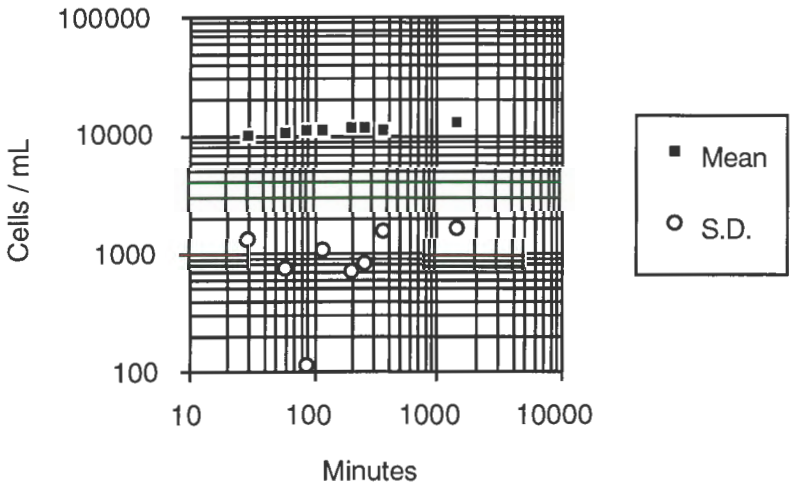


Table 2: Mean numbers per mL and standard deviation (n = 3) of *L. monocytogenes* in sea water containing 3% blood, bubbled with air and held at 9°C.

Time (minutes)	(Cells mL^{-1}) X 1000 Mean (n=3)	s.d.
0	10.9	0.70
30	10.0	1.30
60	10.6	0.72
90	11.1	0.12
120	10.6	1.04
200	11.3	0.70
260	11.7	0.83
360	11.2	1.53
1440	12.6	1.56

Persistence experiment

Survival (culturability) of *L. monocytogenes* after 5 different steam treatments is given in table 3 below. Essentially, the cells were not killed unless they and the steam were trapped in a confined space (treatment D, the 30 mL bottle). This enabled lethal temperatures above 90°C to be speedily obtained. In open spaces the temperatures were much lower and the cells were rapidly dispersed away from the steam to cooler areas of the surface. This was particularly noted when the *L. monocytogenes* was present as a wet sample at the time of steaming. On the petri dishes the colonies were all too numerous to count after incubation, indicating a high proportion of cells survived these steam treatments. For comparison, the temperatures generated during steam cleaning of the harvesting tanks at Farm A were always less than 69°C, generally in the range 40 - 55°C and sometimes as low as 35°C.

Table 3: Culturability of wet and dried *L. monocytogenes* in 0.1 mL aliquots at 10^3 cells mL^{-1} after different steam treatments. a) coverslip in a petri dish, 60 - 64°C for 30 s. b) coverslip in petri dish, 48 - 55°C for 10 s. c) coverslip in petri dish, 48 - 55°C for 10 s and then 60 - 64°C for 30 s. d) 30 mL bottle, 96 - 97°C 10 s. e) petri dish, 48 - 55°C for 30 s. Data indicates number of cultures showing growth out of the total inoculated for each treatment.

Treatment	Growth in petri dish	Growth in UVM1
L. monocytogenes aliquot wet.		
a	10/10	5/5
b	3/3	2/2
c	nd#	nd
d	nd	0/5
e	5/5	nd
L. monocytogenes aliquot dry.		
a	3/5	1/5
b	4/4	3/5
c	2/2	nd
d	nd	0/5
e	5/5	nd

#nd = not done.

DISCUSSION INCLUDING BENEFITS

Clearly, *L. monocytogenes* does not grow in the model tanks, even over a 24 hour period (Table 2, Figure 3). As the harvesting of fish occurs over a much shorter time than used in the model system incubation, the likelihood of *L. monocytogenes* growing *in situ* is low. Various empirically-derived growth rates and predictive growth models for *L. monocytogenes* have been published. Table 4 lists measured and modelled growth rates, including values predicted by the model of Ross (1993) applied to the fish-harvesting environment described here. These data support the conclusion that growth of *L. monocytogenes* will be slow under the environmental conditions of fish harvesting. It is possible that different strains will demonstrate different growth rates. Barbosa *et al.* (1994) reported the mean generation time of 107 strains of *L. monocytogenes* at 4°C to be 43.1 ± 10.7 h and of 80 strains at 10°C to be 6.6 ± 1.3 h. At the cooler temperature the standard deviation is 25% of the mean, indicating a range of responses by different strains.

Using the predictive model of Ross (1993) the generation time of *L. monocytogenes* in sea water with blood at pH = 7.6 and temperature = 9°C is 8.9 h. Increasing temperature and organic content shorten both measured and modelled generation times (e.g. Buchanan and Phillips, 1990, Petran and Zottola, 1989, Walker *et al.*, 1990). The effect of temperature is clearly demonstrated with the predicted generation times derived from Ross's model for the fish tank environment. According to this model increasing the temperature from 9 to 20 °C decreases the generation time from 8.9 to 2.3 h. The impact of pH appears to be variable. For the fish tank system Ross's model predicts that at temperatures ≥ 15 °C pH will have little impact on generation time. However, at 1 °C changing pH from 7.6 to 5.6 increases the generation time from 8.9 to 109 h. The generation times derived from a cubic model by Buchanan and Phillips (1990) are shorter than the corresponding empirical values. They ascribed this to the conservatism of models which are not able to consider all possible factors that may affect growth. Therefore, models tend to overestimate the ability of microbes to grow. Thus the generation times predicted here (from Ross (1993)) for the fish tanks are likely to be minimum values.

The other aspect demonstrated in table 4 is the measured or modelled lag phase for *L. monocytogenes*. This can be very long at temperatures ≤ 20 °C irrespective of the medium and pH. The lag phase will be shortened by acclimation of the cells to temperatures similar to their incubation temperature (Walker *et al.*, 1990). In respect of both lag phase and generation time, any problems in fish harvesting due to *L. monocytogenes* are likely to be exacerbated in summer, because higher temperatures will lead to shorter generation and lag times. This will be particularly so if the daily harvesting period is lengthened. Thus, best practice would be to make use of the typically long lag phase of *L. monocytogenes* to ensure that the fish can be harvested, processed and frozen prior to any growth of contaminants occurring (Walker *et al.*, 1990).

Where the harvesting and processing times are kept short and the temperatures low, then little growth of *L. monocytogenes* is likely. However, if introduced into the tanks, *L. monocytogenes* will likely persist. Steam-jet cleaning was not able to kill *L. monocytogenes* because the temperatures that can be achieved are below the lethal level. Furthermore, steam cleaning would disperse cells throughout the tanks (Table 3). The possible sources of *L. monocytogenes* will be either the fish or water entering the harvest tanks. As it is unlikely that this contamination can be entirely eliminated and, given that *L. monocytogenes* does not grow *in situ*, then the problem that may arise will be one of low level contamination of fish during harvesting.

Hence, there is a risk that during the break between harvesting periods *L. monocytogenes* would grow, perhaps rapidly, in residual water containing blood or mucus, because the temperature can rise quickly, especially in summer. Helke and Wong (1994) and Palumbo and Willams (1990) demonstrated attachment and lengthy survival times for *L. monocytogenes* on stainless steel at 5 - 6°C and 25°C and at high or low relative humidity (32 - 35% and 75%). In addition Palumbo and Williams (1990) demonstrated that a high proportion of the inocula survived and that survival was better at 5 °C. In biofilms on stainless steel *L. moncytogenes* can grow at 10°C, even in mixed populations (Jeong and Frank, 1994). Given the possibility of persistance, then rinsing and cleaning of tanks to remove organic material, followed by effective drying of the tanks would be the best option. As low pH (e.g. 5.6) reduces the growth rate of *L. monocytogenes*, then a final rinse with an acidified water may be of use in further restricting growth between harvesting times. In this way the size of any residual population and its ability to initiate growth may be restricted. The nature of the acidulant requires further research directed at the fish-harvesting environment. Various authors have reported different rates of growth or survival with inorganic acids (Buchanan and Phillips, 1990, Johansen *et al.*, 1994, Parish and Higgins, 1989) and organic acids such as acetic, lactic, citric and malic (Buchanan and Golden, 1994, Glass *et al.*, 1995). Buchanan and Golden (1994) concluded that the effect of pH was dependent on the acidulant, its concentration and the pH.

Table 4: Measured and predicted generation times and lag phases of *L. monocytogenes*.

Medium	°C	pH	NaCl (%)	Generation time (h)	Lag phase (h)	ref.
whole milk	10	(6.7)	(0.5)	10	24	Marshall & Schmidt (1988)
whole milk	10	(6.7)	(0.5)	4.4	25	Buchanan & Phillips (1990)
chocolate milk	13	(6.7)	(2.5)	3.9-4.7	nd	Rosenow & Marth (1987)
chocolate milk	13	(6.7)	(2.5)	2.1	12	Buchanan & Phillips (1990)
tryptic soy broth	13	7.0	0.5	4.8	nd	Petran & Zottola (1989)
tryptic soy broth	13	7.0	0.5	2.8	17	Buchanan & Phillips (1990)
tryptic soy broth	4	7.0	0.5	33.5	nd	Petran & Zottola (1989)
chicken broth	9.3	6.4	nd	5-7	<24-48	Walker *et al.* (1990)
UHT milk	9.3	6.6	nd	5.5-9	≤24	Walker *et al.* (1990)
fish harvesting	9	7.6	3.5	8.9	nd	this study using Ross (1993)
fish harvesting	15	7.6	3.5	3.8	nd	this study using Ross (1993)
fish harvesting	20	7.6	3.5	2.3	nd	this study using Ross (1993)
tryptose broth	4	5.6	0.5	27.1	144	El-Shenawy & Marth (1988)
tryptose broth	21	5.0	0.5	2.1	9	El-Shenawy & Marth (1988)
tryptose broth	4	5.6	0.5	16.3	91	Buchanan & Phillips (1990)
tryptose broth	21	5.0	0.5	1.8	16	Buchanan & Phillips (1990)
fish harvesting	1	5.6	3.5	109.4	nd	this study using Ross (1993)
fish harvesting	15	5.6	3.5	3.9	nd	this study using Ross (1993)
fish harvesting	20	5.6	3.5	2.3	nd	this study using Ross (1993)

#nd = no data.

Clearly, processors of Atlantic salmon are the beneficiaries of this research as it has indicated that, in general, the techniques in present use should maintain a product of high microbiological quality. However, any lapse, especially if it leads to retention of "blood water" between harvests, could lead to undesirable proliferation of *L. monocytogenes*. Also, the inefficiency of steam-cleaning has been identified and, therefore, in some instances, cleaning methods may warrant re-assessment.

Further Development

(1) Because bacteria, particularly *L. monocytogenes*, could proliferate if temperatures were raised for a significant period during harvesting, operators should consider controlling and monitoring water temperatures during this process.

(2) Because steam-cleaning procedures are probably inadequate to decontaminate harvesting equipment, research into alternative procedures such as the use of acid-water washes followed by thorough drying should be undertaken.

Acknowledgments

Ms. M. Watts is thanked for experimental assistance. The helpful assistance of Dr R. Quintana and Mr K. Van Drunen of is very appreciated.

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POTENTIAL BACTERIAL HAZARDS AND HEALTH RISKS ASSOCIATED WITH THE COMMERCIAL PROCESSING OF FISH, PARTICULARLY ATLANTIC SALMON, FOR HUMAN CONSUMPTION

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SUMMARY

The incidence of disease outbreaks attributable to bacterologically contaminated seafoods (and many other types of foods) is likely to increase over the next 10 years due to changing patterns of human consumption. Strict hygiene practices such as HACCP (Hazard Analysis and Critical Control Points) are needed to counter the increasing risk of possible disease outbreaks due to chilled ready-to-eat seafoods. Various styles of commercial chilled ready-to-eat seafood products can be identified as high-risk, including: raw shellfish in the half shell, sashimi, cold- and hot-smoked, gravalaks, roe, pate, terrine, roulade, marinated, surimi and cold salad. Seven human bacterial pathogens (*E.coli, C.perfingens,* Coagulase Positive (C.P.) *S.aureus, L.monocytogenes, A.hydrophila, V.parahaemolyticus* and *V.vulnificus*) and the diseases they cause are described. Five separate stages in the food delivery chain can be identified whereby commercial finfish and shellfish products can become contaminated with potential human bacterial pathogens before reaching the consumer's plate.

Detailed methodology to recover the 7 pathogens above from seafood, water and swabs is presented. Using these methods, tissue surfaces (n=162) and flesh (n=27) of Atlantic salmon and blood water (n=9) were surveyed at 3 major factories using HACCP in SE Tasmania during harvesting and initial processing. The prevalence of these potential pathogens on fish was found to be low (CP *S.aureus, A.hydrophila*) to very low (*E.coli, C.perfingens, L.monocytogenes, V.parahaemolyticus, V.vulnificus*). Wastewater produced at 4 major fish processing plants in SE Tasmania was also investigated. At two plants processing Atlantic salmon, some bacterial hazards present in wastewater (faecal coliforms including *E.coli,* and CP *S.aureus;* also Standard Plate Counts) were substantially lower (by >2 orders of magnitude) than in two plants processing wildcatch fish and shellfish. Compared to fish flesh and tissue surfaces however, the levels of potential pathogens were generally much higher in wastwater, especially in influent. At one plant studied, ultra-violet treatment of effluent reduced some bacterial hazards (faecal coliforms including *E.coli,* CP *S.aureus* and *L.monocytogenes*) to a low level.

The risks of food poisoning of the human consumer, and also of wound infections of fishing industry staff, due to the 7 pathogens were provisionally assessed as minimal and low respectively. These predictions were made on the basis that HACCP systems were in operation during harvesting and initial processing. Critical control points were identified as: starvation of pre-slaughter Atlantic salmon, use of disinfected process water and equipment, temperature control (freezing or chilling at 0-2^{0}C), personal hygiene of staff, ambient water quality and staff training.

We recommend that the Australian fishing industry be encouraged strongly and widely to implement HACCP in order to minimise possible future bacterial disease outbreaks due to contaminated chilled ready-to-eat seafoods.

BACKGROUND

Serious diseases of the human consumer may be caused by processed fish and shellfish products which have been contaminated by microorganisms, bacteria being the most common pathogenic agents (reviewed by Gibson 1992, Hughes and Tauxe 1990). The severity of symptoms varies considerably, ranging from general malaise, to vomiting and/or diarrhoea, to invasive disease causing temporary or permanent damage to tissues or organs, to death.

The incidence of disease outbreaks attributable to microbiologically contaminated seafoods (and many other types of foods) is likely to increase over the next 10 years due to changing patterns of human consumption especially as a much wider range of ready-to eat forms of food products is becoming commercially available. This trend reflects that consumers have less time to prepare food for themselves than previously. They typically purchase ready-to eat foods from various retail outlets including supermarkets, "take-away" shops and restaurants once or twice per week, or eat it at catered functions less frequently;

Also, during commercial processing, ready-to eat foods are increasingly receiving minimal treatment to inhibit or destroy potential human pathogens. This trend reflects greater consumer demand for fresh, uncooked or chemically unpreserved foods.

In 1995 and 1996 in Australia, examples of serious food poisoning outbreaks associated with ready-to-eat foods contaminated with pathogenic bacteria included: *E.coli* O157 H7 in salami (Adelaide), *Clostridium perfringens* in rewarmed meat (Melbourne), *Salmonella* in peanut butter (Australia-wide), *E.coli* O157 in delicatessen items (Gold Coast), *Listeria monocytogenes* in processed chicken meat (Adelaide) and *Salmonella* in chocolate dairy food (Australian commercial airflight to Japan).

Various styles of commercial chilled ready-to-eat seafood products can be identified as high-risk, including: raw shellfish in the half shell, sashimi, cold- and hot-smoked, gravalaks, roe, pate, terrine, roulade, marinated, surimi and cold salad. The populations at risk include western countries such as Australia and the USA, as well as Asian countries with growing economies such as Japan and South Korea. To counter the increasing risk of possible disease outbreaks due to ready-to-eat seafoods, the HACCP (Hazard Analysis and Critical Control Points) system (Huss 1992) is gradually being implemented in the fishing industry (eg Garland and Mellefont 1996).

During the manufacture of ready-to-eat and other types of food, seafood processing factories generate liquid and solid wastes which vary according to factors such as type of fish species processed, type of processing and plant wastewater treatment design. According to a survey of the Tasmanian fish processing industry by Nation (1990), the volume of wastewater generated is 5-10 kL/tonne of raw material processed. This corresponds to approximately 500 ML of wastewater being discharged into Tasmanian waterways annually, based on a raw products yield of ~58,000 tonnes in 1990 (Leonard 1993). This large volume of wastewater contains substantial inorganic and organic chemical loadings as well as microbiological loadings, and has the potential to cause adverse effects on the receiving environment such as a reduction in the dissolved oxygen content, stimulation of excessive algal growth and introduction of potential

human pathogens into the wateway (Otwell 1990; Howett 1991). Thus it is possible that edible fish or shellfish in waterways receiving the discharged effluent can become contaminated with these pathogens and cause foodborne diseases associated with seafood consumption.

While a wide range of viruses, bacteria and other microorganisms can conceivably contaminate seafood products this study targeted seven pathogenic organisms which the authors perceived to be among those most likely to contaminate Atlantic salmon and, to a lesser extent, other seafood products. On the basis of previous experience it was not expected that significant contamination existed, but this opinion needed to be tested and the results documented.

Information on the organisms of interest is provided hereunder.

Vibrio vulnificus* and *V.parahaemolyticus

Vibrio species are curved or straight Gram negative rods which are indigenous in water and include several human pathogens. *V.vulnificus* and *V.parahaemolyticus* cause conditions of vaying severity. *V.vulnificus* is recognised as a highly virulent pathogen which mainly attacks the immunocompromised eg. AIDS patients, subjects on chemotherapy or with underlying liver disease. With *V.cholerae* it is considered one of the most pathogenic of the *Vibrio* species (Hagen *et al* 1994). The more common clinical manifestation is gastrointestinal disease with diarrhoea and vomiting; this has been typically associated with the consumption of raw oysters. A second manifestation is septicaemia, which is the most dangerous form as it often causes death within 24 hours, even after admission to hospital. Different to the onset of the gastrointestinal form of disease, the septicaemia does not cause vomiting or diarrhoea (Janda *et al* 1988). Septicaemia can also develop from wounds and is a concern for "hands-on" staff working in areas where they are likely to incur wounds, such as shellfish splitters, divers and deckhands (Editor 1990). If *V. vulnificus* is present in water, the temperature is usually $>15^0$C.

V. parahaemolyticus is common in aquatic environments, from temperate to tropical. It occurs in seawater and brackish water, sediment, finfish and shellfish, and has also been isolated from plankton (Bartley and Slanetz 1971). The strains of greatest human health importance are Kanagawa positive and able to haemolyse human blood cells, a characteristic which is identified in the laboratory on agar plates (Desmarchelier 1989; Fung 1992). Generally the more common environmental strains of *V.parahaemolyticus* are Kanagawa negative (Fung 1992).

V.parahaemolyticus is a major cause of epidemic gastroenteritis in countries where raw seafood is often consumed. Foods implicated include crabs, oysters, shrimp and lobster (Chang *et al* 1994). The incidence of *V. parahaemolyticus* infection appears to be closely related to the number of bacteria present in the environment (Kelly and Stroh 1988; Tepedino 1982). In Japan *V. parahaemolyticus* has been responsible for approximately 70% of cases of all foodborne illnesses for several decades (Farmer 1992), which has been attributed to the popularity of eating raw (uncooked) fish and shellfish. Symptoms of infection by *V. parahaemolyticus* include abdominal pain, diarrhoea, cramps, chills and headaches (Desmarchelier 1989). Development of infection requires ingestion of viable bacteria, typically ~10^5/g (Fung 1992). Prevention

of infection is through thorough cooking and elimination of cross contamination between processed products and raw products (Desmarchelier 1989). It may be almost impossible to prevent *V. parahaemolyticus* infection in countries where the consumption of raw fish is common ethnic practice, such as Japan.

Aeromonas hydrophila

Aeromonas hydrophila is also a Gram negative rod which is indigenous to aquatic habitats. It has been recognised only relatively recently as a human pathogen (Majeed and Macrae 1989). The organism has been implicated in cases of gastoenteritis since 1970 (Ingham & Potter, 1988) and also a number of extraintestinal infections including wound infections, septicaemia and meningitis (Majeed and Macrae 1989, Freij 1987, Gross 1987, Wadstom 1987). *A.hydrophila* is also known as a fish pathogen and has been isolated from a number of different fish species (Patirak *et al* 1988). Thus, fish for processing may be carrying these bacteria, and they may be passed on to human consumers. *A.hydrophila* can survive refrigeration temperatures and is also able to survive in modified atmosphere packaging (MAP) (Ingham and Potter 1988).

Pathogenicity of *A.hydrophila* infection is thought to be mediated by a number of toxins, the most virulent possibly being an enterotoxin (Gravenitz 1987). Other toxins may include cytotoxins and endotoxins (Ljungh 1987). Other virulence factors implicated are proteases and surface adhesins. Adhesion factors are important as they protect the organism from host defence mechanisms (Neves *et al* 1994). ß-haemolysins may also be related to enteropathogenicity (Stelma *et al* 1986).

Symptoms of gastroenteritis involving suspected *A.hydrophila* infection include diarrhoea, vomiting and fever (Gross 1987). There does not seem to be a relationship however, between faecal carriage of *A.hydrophila* and infection resulting in gastroenteritis. Healthy individuals in Thailand were found to have the same faecal carriage rate as individuals with gastroenteritis (Wadstrom 1987). Why infections developed or whether they were associated with predispositions to disease was not indicated by Wadstrom (1987).

A separate and relatively rare disease involves wound infections following contact with waterborne *A.hydrophila* (Freij 1987). These can range from mild localised infections to sepsis which is generally associated with individuals with predispositions eg leukemia, aplastic anaemia and AIDS (Freij 1987, Majeed and Macrae 1989). Similar to *V. vulnificus* however, it is still important that healthy "hands-on" workers are informed of the risks and take precautions to avoid skin cuts or open wounds coming into contact with *A.hydrophila* in water or food.

Clostridium perfringens

C.perfringens is a Gram positive spore-forming heat-resistant rod which grows only in anaerobic conditions and can survive in water and sediment for months, even years. Similar to *E.coli* and *L.monocytogenes*, its presence on fish products indicates that the waterway from which it was harvested has been contaminated with faeces of warm blooded animals. Alternatively it may be sourced from human or animal faecal contamination in the factory eg via inadequately washed hands of processing staff.

C.perfringens has been recognised as a foodborne pathogen for many years and is now considered one of the principal agents of foodborne disease (Gilbert 1987).

C.perfringens belongs to that group of food poisoning bacteria that rely on high numbers of bacteria (in vegetative or spore from) to be ingested for infection to develop. After ingestion the spores germinate in the gut and/or bacteria multiply, and produce enterotoxins (Madden *et al* 1986). In some instances however, the spores may have germinated in food beforehand and toxin is produced which is subsequently ingested. Of five types of enterotoxin, only types A and C are associated with human foodborne illness (Jay 1986, Murrell 1989). Type A causes the more common type of foodborne illness, with symptoms including severe cramps and diarrhoea but generally no vomiting, nausea or fever. Onset of the symptoms occurs 6-22 hours after ingestion of the contaminated food but earlier if preformed toxin is present (Gilbert 1987). Death due to *C.perfringens* type A illness is rare and in most cases involves the immunocompromised, aged or infants. Type C toxin causes a severe form of foodborne illness, necrotic enteritis, which is quite rare (Murrell 1989).

C.perfringens occassionally causes food poisoning associated with fisheries products (Cann 1987). Most cases occur in institutional cooking where large pieces of meat are cooked and then cooled inefficiently, allowing germination of spores, bacterial growth and toxin formation (Jay 1986). An outbreak of food poisoning caused by boiled salmon in 1985 in the UK caused 500 cases with no deaths (Gilbert 1987). Other outbreaks have involved cooked fish or chowder. Handling of raw foods and inadequate hygiene of processing staff have been suggested as means of cross contamination of cooked seafood products (Bryan 1973).

Following wound infection, *C.perfringens* can also cause gas gangrene, septicaemia and other serious illnesses (Jay 1986).

Listeria monocytogenes

Listeria monocytogenes is a Gram positive rod with considerable environmental adaptability. It occurs commonly in soil, fresh water and sediments and less commonly in estuarine habitats (Soontharanont and Garland 1995), being sourced from the gastrointestinal tract of humans and terrestrial animals (Botzler *et al* 1974). Until recently listeriosis was quite rare (Jones 1990). There have been few outbreaks but these often occur as epidemics with mortalities as high as 30%, demonstrating that *L.monocytogenes* is one of the most dangerous potential foodborne pathogens. The at-risk population groups include immunocompromised individuals, the elderly, young children and pregnant women, their foetuses and neonates. In a large outbreak however, normally healthy individuals have also been affected (Fuchs 1991, Ryser and Marth 1991). The infectious dose of *L.monocytogenes* has not been clearly established but is likely to be $>10^3$/g (McLauchlin 1995).

The initial clinical manifestations of listeriosis may include diarrhoea ~20 hours after ingestion of contaminated food and flu-like symptoms which are often disregarded. The later symtoms of disease are far more severe and include meningitis, septicaemia, spontaneous abortion, granulomatosis infantiseptica (newborn listeriosis), conjunctivitis, oculoglandular listeriosis, cutaneous listeriosis, pneumonic listeriosis, cervicoglandular listeriosis and death (Jones 1990, Marth 1988, Ryser and Marth 1991). Since the incubation period for the disease ranges from one day to several weeks, investigation of the foodborne cause of the disease is usually very difficult, and sometimes impossible.

Previously it was thought that only dairy foods or other foods that came in contact with them were at risk from contamination with *L.monocytogenes*. Recently a wider variety of foods has come to be associated with *L.monocytogenes* disease outbreaks including marinated mussels, smoked food products, pate and coleslaw (Fuchs 1991, Ryser and Marth 1991). A small outbreak with one fatality in Adelaide very recently involved processed chicken meat. *L.monocytogenes* has become more common in the wholesaling. retailing and domestic environments with the greater use of refrigeration, since it is able to survive and grow at normal refrigeration temperatures and will often out-compete spoilage organisms at low temperatures. Various studies have shown that *L.monocytogenes* can be a component of surface flora of various foods including meat, poultry, shellfish, chilled ready-to-eat foods and dairy foods (Abdalla *et al* 1993, Arnold and Coble 1995).

Successful methods for the control of *L.monocytogenes* in the seafood industry require scrupulous attention to hygiene in the processing factory (AQIS 1995, Garland 1995, Maple 1995).

***Staphylococcus aureus* (coagulase positive)**

S.aureus is a Gram positive coccus and very well documented foodborne pathogen but is rarely waterborne. It is a normal inhabitant of human skin, hair and mucous membranes eg. nasal passages, and as such its presence in food may indicate inadequate staff hygiene in the processing factory. Coagulase positive strains of *S.aureus* can cause a variety of illnesses. It may also cause severe food poisoning due to heat stable toxins (A, B, C1, C2, C3, D and E) (Fung 1992). These toxins are of low molecular weight, approximately 30 000, and induce symptoms including severe nausea, abdominal cramps, vomiting, diarrhoea and prostration (Fung 1992). In more severe cases, headaches, muscle cramps and transient changes in blood pressure and pulse rate can occur. Similar to *C perfringens*, some outbreaks of disease are due to preformed toxin. Also similar to *C perfringens*, it can cause wound infections.

S.aureus grows best on high protein foods and within 4 hours can product enough enterotoxin to induce disease (Fung 1992). Fish is a very high protein food and can readily act as a substrate for *S. aureus* growth. Between 1960 and 1969 *S.aureus* was responsible for more than 23% of cases of food poisoning caused by fish and shellfish products, mostly due to poor processing hygiene (Bryan 1973), whereas between 1975 and 1981 only 3 of 226 cases of staphylococcal food poisoning were caused by contaminated finfish (Bennet 1986).

Escherichia coli

E. coli is a Gram negative rod and was first identified in 1885 (Padye and Doyle 1992). It occurs in the gut of almost all warm blooded animal species including man (Eyles and Davey 1989). For many years *E.coli* has been used as an indicator of recent faecal contamination especially with regard to shellfish and water since it tends not to persist in the environment for more than 1-2 weeks unless recontamination has occurred (Eyles and Davey 1989, Kornacki and Marth 1992).

E.coli strains isolated from the intestinal tract of humans are generally regarded as harmless commensal organisms not normally posing a threat to human health (Padye and Doyle 1992). In rare instances however, specific *E. coli* strains from the gut of humans and other animals can become pathogenic. Most of these infections occur

after ingestion of a large dose of these bacteria by the immunocompromised, infants or young children (Kornacki and Marth 1992). The highly virulent pathogenic strain O157 H7 is rare but receiving increasing attention eg due to an outbreak of HUS (haemolytic uraemic syndrome) in Adelaide in early 1995 caused by contaminated salami, and a continuing outbreak during 1996 in Japan which has affected >11 000 people with 11 deaths and has been associated with a variety of foods including smoked eel and potato salad (AQIS Canberra, pers comm).

Unless heavily contaminated, it must be emphasised that foods containing *E.coli* or the other bacteria above will not necessarily cause disease of the human consumer. It is prerequisite for bacteria to multiply to the infectious dose, which usually occurs in conditions of temperature abuse eg due to inadequate chilling, prolonged inadequate cooling after cooking or slow inadequate rewarming of chilled or frozen product.

Need

Five separate stages in the food delivery chain can be identified whereby commercial finfish and shellfish products can become contaminated with potential human microbial pathogens before reaching the consumer's plate:

1 during the growth and development of animals in water

2 at harvest when animals are removed from water and then stored and transported to the processing factory

3 during processing in the factory

4 post-processing during transport, storage, wholesaling and retailing

5 shortly before consumption when seafood products are in storage or at the final step of preparation in the domestic kitchen or food outlet serving area

This report is concerned with the first 3 stages of the food delivery chain and 7 of the more common types of potential human bacterial pathogens. Stages 2 and 3 (up to and including initial processing in the factory) were studied at 3 SE Tasmanian factories which harvest and process Atlantic salmon. Stage 1 (ambient water quality) was studied indirectly by examining the wastewater which 4 SE Tasmanian fish processing factories discharge into waterways where fish and shellfish grow or are harvested.

The literature on laboratory microbiological examination of seafood products, water and the manufacturing environment is moderately extensive for some organisms, scant for others and overall rather dispersed. Consequently a substantial section of the report details effective methodology, including the validated recovery of target bacteria from seafood products and water.

Objectives

(1) To survey the prevalence of potential human bacterial pathogens on Atlantic salmon and in water and swabs during harvesting and initial processing.

(2) To survey the prevalence of potential human bacterial pathogens in wastewater discharged by factories processing Atlantic salmon, other finfish and shellfish.

(3) Although not specifically an initial objective, it soon became obvious that another objective was to modify existing methods and/or develop new methods for efficaciously detecting the presence of the seven bacteria of interest.

(4) Provide a provisional risk assessment relating to:

(i) foodborne bacterial diseases of consumers of seafood products.

(ii) bacterial wound infections of fishing industry and fish processing staff.

(iii) bacterial cross-contamination of farmed or wild finfish and shellfish due to discharged wastewaters.

METHODS

METHODS TO RECOVER POTENTIAL HUMAN BACTERIAL PATHOGENS

General Comment

As mentioned earlier, the literature on methods to isolate and identify potential human bacterial pathogens in seafood and water is dispersed. Where possible we have used standard methods in APHA (1992) and AS1766 (1986+) for food and in AWWA/APHA/WPCF (1992) and HMSO (1982) for water. These methods are usually recognised internationally, including by countries which import Australian seafoods. Further information on methods to isolate and identify *Vibrio vulnificus* and *V.parahaemolyticus* was extracted from Farmer (1992) and Kaysner *et al* (1992), and from various research reports on the other bacteria of interest.

Where possible we have used commercially available bacteriological media and reagents.

Collection and Preparation of Samples for Bacteriological Examination

Food At the factory ~25g of flesh (skin-on) was aseptically excised from the belly flap of each of 5 harvest-size (3-5kg) Atlantic salmon and stored in sterile plastic jars at $<10^0$C during transport for 3-4hrs. In the laboratory a subsample of ~2g was removed from each of 5 samples and composited to a total of 10g for examination of *E.coli*, *C.perfringens*, *S.aureus* and *A.hydrophila*. The composite samples were homogenised (Colworth Stomacher 400 for 2 mins) in 90ml 0.1% peptone water pH 7.2 to yield a 10% homogenate; serial tenfold dilutions were also prepared in 0.1% peptone water pH 7.2. For the examination of *L.monocytogenes*, *V.vulnificus* and *V.parahaemolyticus*, ~5g subsamples of each of 5 samples were composited to a total of 25g which was homogenised (Colworth Stomacher 400 for 2 mins) in the media described below.

Water At the farm and factory, samples were collected aseptically in sterile 1L glass bottles and stored at $<10^0$C during transport for 3-4h. In the laboratory the membrane filtration technique (0.45um) was used to detect *E.coli* (and faecal coliforms),

C.perfringens, *S.aureus* and *L.monocytogenes* in 10-100ml sample volumes; serial dilutions were prepared in 0.1% peptone water pH 7.2. *A.hydrophila* and *Vibrio* spp were processed as described below, noting that for preparation of serial dilutions 0.1% peptone water pH 7.2 was used for *A.hydrophila*.

Swabs At the farm and factory, for each microbiological test 5 harvest-size fish were individually swabbed (non-absorbent cotton tip) over a surface area of at least $50cm^2$ (belly flap) or $10cm^2$ (rectum) per fish. The swab was applied to the same surface repetitively (5x) and rinsed in media between each sampling to achieve maximum recovery of organisms potentially present (APHA 1992, pp 57-59). The 5 swabs for each test were composited immediately in specific media, as described below, and stored at $<10^0C$ during transport for 3-4h. The composited swabs for each test represented total surface areas of $250cm^2$ belly flap) and $50cm^2$ (rectum) of the 5 fish sampled.

Bacteriological Examination

Food

Escherichia coli Method AS 1766.2.3 (1992) was used. The 10% homogenate of composite samples and serial dilutions were pour-plated (1ml) in duplicate with Violet Red Bile Agar. Plates were incubated at 30°C for 24h. Typical presumptive colonies were purple with surrounding purple haloes. Confirmatory tests on presumptive colonies were performed using lauryl tryptose broth (+ve gas production), eosin methylene blue agar (typical colony colours), EC broth (+ve gas production) and tryptone water (+ve indole production).

Clostridium perfringens Method AS 1766.2.8 (1991) was used. The 10% homogenate of composite samples and serial dilutions were spread-plated (0.1ml) in duplicate on tryptone sulfite cycloserine (TSC) agar with an overlay of TSC agar without egg yolk. Plates were incubated anaerobically at 37°C for 24h. Typical presumptive colonies were black with a halo (clearing) caused by lecithinase activity. Confirmatory tests included Gram stain and morphology and characteristic reactions in nitrate motility medium (non-motile, reduction of nitrate to nitrite) and lactose gelatin medium (+ve fermentation, +ve liquefaction).

Staphylococcus aureus (coagulase positive) Method AS 1766.2.4 (1986) was used. The 10% homogenate of composite samples and serial dilutions were spread-plated (0.1ml) in duplicate on Baird-Parker agar. Plates were incubated at 37°C for 48h. Typical presumptive colonies were black with a halo (clearing) caused by lecithinase activity. Confirmatory tests included Gram stain and morphology and characteristic oxidase (+ve) and catalase reactions (+ve). Commercial serological kits (eg Staphyltect, Oxoid) were used to confirm coagulase activity.

Aeromonas hydrophila The method was modified from Palumbo *et al* (1992). The 10% homogenate of composite samples and serial dilutions were spread-plated (0.1ml) in duplicate on starch ampicillin agar containing 10mg/L ampicillin. Plates were incubated at 28°C overnight. Part of the plates was flooded with 5 ml of Lugol's iodine solution, typical presumptive *A. hydrophila* colonies being surrounded by clear zones of hydrolysed starch. (Note that iodine kills viable cells very quickly, so some typical colonies must be left unexposed to it for further testing). Confirmatory tests included Gram stain and morphology and characteristic reactions for catalase (?check), oxidase (+ve) and sensitivity to vibriostatic agent 0/129 (resistant to 150ug

disc). Further characteristic reactions included esculin hydrolysis (+ve), production of gas (+ve) or acetoin (+ve) from glucose, Voges-Proskauer (+ve) and suicide pheomenon (-ve) (Namdari and Cabelli 1989). In addition, colonies were inoculated onto Kaper's medium (Kaper *et al* 1979); *A. hydrophila* produces the following reactions-alkaline band at the top, acid butt, +ve motility, -ve H_2S and +ve indole. When identification of the isolates was not clear from the tests described, API 20NE kits were used.

Note that trials with Ryan's medium were not successful due to poor selectivity for *A. hydrophila* and overgrowth by other aeromonads.

Listeria spp including *L.monocytogenes* The USDA/FSIS (1989) method was used. Composite 25g samples were homogenised in 225ml *Listeria* enrichment broth (UVM1) and incubated at 30°C for 20-24h. Secondary enrichments were performed by transferring 0.1 ml UVM1 suspension to 10ml Fraser broth which was incubated at 35°C for 48h. Suspensions were streaked onto MOX agar (modified Oxford agar containing 10mg/L colistin and 15mg/L moxalactam) and incubated at 35°C for 48h. Typical presumptive colonies with surrounding black zones, due to esculin hydrolysis, were confirmed as *Listeria* by Gram stain and morphology and characteristic catalase (+ve) and oxidase (-ve) reactions. Further confirmatory tests included ß-haemolysis on 4% horse blood overlay agar at 35°C overnight, tumbling motility in BHI broth at 20-25°C overnight and umbrella growth in bacto motility test medium at 20-25°C for 2 days. To distinguish *L.monocytogenes* from other *Listeria* spp, characteristic reactions were determined for the CAMP test (enhancement of ß-haemolysis to *S.aureus* on 5% sheep blood overlay agar) as well as for various biochemical tests including bile esculin agar (blackening), MR-VP medium (+ve, +ve), O/F medium (+ve, +ve), nitrate broth (-ve reduction) and rhamnose (+ve), xylose (-ve) and mannitol (-ve) fermentation broths, which were usually undertaken by means of the API *Listeria* kit (BioMerieux).

Vibrio parahaemolyticus Method AS 1766.2.9 (1991) was modified in terms of the initial diluent; composite 25g samples were homogenised in 225ml 0.1% peptone water pH 8.6 with 3%NaCl and 0.5% Tween 80. The Most Probable Number (MPN) 3x3-tube technique as described in AS 1766.1.6 (1991) was used for enumeration. The 10% homogenate of composite samples (10ml) and serial dilutions (1 and 0.1ml) were inoculated into alkaline peptone water (APW) pH 8.6 (double or single strength). Tubes were incubated at 37°C for 6-8h. A loop of primary enrichment was then streaked on thiosulfate citrate bile-salts sucrose (TCBS) agar. One ml of the primary enrichment was also transferred to a series of fresh tubes of APW for secondary enrichment. TCBS plates and APW tubes were incubated at 37°C for 18h. Loopfuls of secondary enrichments were then streaked on TCBS plates which were incubated at 37°C for 18h. Typical presumptive colonies were large (eg 3-5mm) and blue-green on TCBS agar. Characteristic confirmatory test reactions are shown in Table 1 and included: triple sugar iron media (TSI), decarboxylisation of lysine, ornithine and arginine, ONPG, Voges-Proskauer, growth at 42°C and salt tolerance (0, 8 and 11%). In addition, the following confirmatory tests (Table 1) were performed to obtain greater confidence of identification: sensitivity to vibriostatic agent 0/129 (10 and 150ug discs) and sugar fermentation tests using L-arabinose, D-sorbitol, D-mannitol, myo-inosytol and D-galactose (Kaysner *et al* 1992).

Vibrio vulnificus The method was the same as that for *V.parahaemolyticus* except that colistin-polymyxin B-cellobiose (mCPC) agar was used in place of TCBS and the plates were incubated at 40°C for 24h (Tamplin *et al* 1991, Parker *et al* 1994). Typical presumptive colonies were yellow on mCPC agar. Confirmatory tests were performed as described above for *V. parahaemolyticus* and characteristic test reactions are shown in Table 1.

Water

Faecal coliforms including *E.coli* The method in HMSO (1982) was used. After filtration of samples and appropriate serial dilutions, 0.45um filters were placed on membrane lauryl sulphate agar (MLSA). Plates were incubated at 30°C for 2-4h, followed by 14-18h at 44°C. Typical presumptive colonies were yellow. They were confirmed as faecal coliforms in lauryl tryptose broth (+ve gas production), and as *E.coli* in tryptone water (+ve indole production).

Clostridium perfringens The method of Ball and Shipway (1993) was used. Samples with an expected low count were processed directly whereas wastewaters with an expected high count were firstly heat shocked at 75°C for 10 minutes to kill vegetative cells. After filtration of samples and appropriate serial dilutions, 0.45um filters were placed on Perfringens agar (OPSP, Oxoid) supplemented with 85 mg/L MUP (4-methylumbelliferyl phosphate) which had been membrane-filter sterilised and added separately to cooled OPSP media after autoclaving. The plates were incubated for 24 hours at 35°C anaerobically. Typical presumptive colonies produced black fluorescence. Confirmatory tests included Gram stain and morphology and characteristic reactions in nitrate motility medium and lactose-gelatine medium, as described above for food.

Staphylococcus aureus (coagulase positive) The method of Borrego *et al.* (1988) was used. After filtration of samples and appropriate serial dilutions, 0.45um filters were placed on Borrego-Florido-Romero-O (BFR-O) medium consisting of tryptone 10g, yeast extract 3g, beef extract 1.5g, D - mannitol 20g, glycine 12g, Na pyruvate 10g, K thiocyanate 25g, NaCl 100g, Na azide 49mg, phenol red 25mg, agar 15g, distilled water 1L, pH 7.2$\pm$0.1; and which had been autoclaved at 121^{0}C for 15min and allowed to cool after plate pouring. The plates were incubated at 35 °C for 48 - 72 hours. Typical presumptive colonies were yellow and discrete. Confirmatory tests included Gram stain and morphology and characteristic oxidase, catalase and coagulase reactions, as described above for food.

Listeria spp. including *L. monocytogenes* The USDA FSIS (1989) method was modified. After filtration of 100ml-1L samples, the 0.45 um filters were placed into 100ml UVM1 for 20 - 24 hours at 30°C. The samples were processed further and media examined for presumptive colonies and confirmed, as described above for food.

*Vibrio vulnificus*and *V.parahaemolyticus* Method AS 1766.2.9 (1991) was used in conjunction with the Most Probable Number (MPN) 3x3-tube technique described in AS 1766.1.6 (1991). The samples (10ml) and serial dilutions (1 and 0.1ml) were inoculated into alkaline peptone water (APW) pH8.6 (double or single strength). Tubes were incubated at 37°C for 6-8h. The samples were processed further and media examined for presumptive colonies and confirmed, as described above for food.

Standard Plate Count (SPC) The APHA/AWWA/WPCF (1992) method was used. Samples (wastewater only) and appropriate serial dilutions prepared in 0.1% peptone water pH7.2 were pour-plated (1ml) in duplicate on Plate Count Agar. Plates were incubated at 22°C for 5 days and colonies counted.

Swabs

E.coli, C.perfringens, S.aureus (coagulase positive) and *A.hydrophila* For each test, 5 swabs were composited in 10ml 0.1% peptone water pH 7.2 immediately after sample collection and vortex mixed for 2min on arrival at the laboratory. One ml volumes and serial dilutions were then processed as described above for food.

Listeria spp. including *L.monocytogenes* Swabs (5) were composited in 20ml UVM 1 broth immediately after sample collection and vortex mixed for 2min on arrival at the laboratory. They were then processed as described above for food.

V.vulnificus and *V.parahaemolyticus* Swabs (5) were composited in 5ml 0.1% peptone water with 3%NaCl and 0.5% Tween 80 immediately after sample collection and vortex mixed for 2min on arrival at the laboratory. One ml volumes and serial dilutions (0.1 and 0.01ml) were then inoculated into APW pH 8.6 (single strength) and processed as described above for food.

Validation of Methods

The bacteriological methods described above were tested for validated recovery of target organisms by inoculating sterile fish flesh (Atlantic salmon) and sterile water (0.1% peptone water pH 7.2) with medium and low numbers of the pathogens of interest. Additional tests were performed for *L. monocytogenes* in smoked Atlantic salmon and Pacific oysters. The seafood flesh was first sterilised by swabbing with ethanol and allowing the surface to air dry in a laminar flow cabinet for 10min. Pathogens were grown to high population density (~10^9/ml) in appropriate broth media. Cell densities were enumerated and size of inocula calculated. Pathogens were recovered from food and water using the methods exactly as described above.

As laboratory trials indicated poor recovery of *V.vulnificus* and *V.parahaemolyticus* from food using the AS 1766.2.9 (1991) method, 0.5% Tween 80 was added to the diluent used for sample homogenisation, to determine if recovery could be improved.

SURVEY OF POTENTIAL HUMAN BACTERIAL PATHOGENS FROM FISH (ATLANTIC SALMON) AND WATER

Validated Recovery

Food Using the test methods detailed in this report, *C.perfringens, E.coli,* coagulase positive *S.aureus, L.monocytogenes* and *A.hydrophila* were recovered from seafood flesh in numbers very similar to the medium and low levels at which they were inoculated (Table 2).

In the case of *V.vulnificus* and *V.parahaemolyticus,* their recovery was achieved in much lower numbers compared to their inoculation levels if Tween 80 was absent from the initial diluent, and was only achieved in similar numbers if Tween 80 was present in the initial diluent. In particular *V.vulnicus* failed to be recovered from low

inoculum fish flesh when Tween 80 was absent from the initial diluent. Tween 80 is known to adhere to the outer surface of the bacterial cell wall and protect cell viability during storage after sample collection and homogenisation.

Water Using the test methods detailed in this report, *C.perfringens*, *E.coli* and coagulase positive *S.aureus* were recovered from seafood flesh in numbers very similar to the medium and low levels at which they were inoculated (Table 3). *V.vulnificus*, *V.parahaemolyticus* and *L.monocytogenes* were also recovered after addition of low and medium inoculum levels to water (Table 3).
Swabs Validated recovery testing was not undertaken on swabs as the swabbing technique is regarded as semi-quantitative, not quantitative.

Fish : Sampling Strategy
Sampling was undertaken at the premises of the 3 major companies which farm and process Atlantic salmon in SE Tasmania.

Number of Substages Five substages of stages 2 and 3 (described in the Introduction) were investigated:

2A the surface of fish after they had been removed from water and anaesthetised on the farm harvest jetty

2B the water in bins in which fish were killed on the farm harvest jetty (termed blood water)

3A the surface of fish when they arrived at the factory after transport from the farm; note that frozen fish were examined at company 3 on 15/5/1996 (Table 4), as fresh fish were not being harvested at that time.

3B the rectum of fish when they arrived at the factory after transport from the farm.

3C the flesh of fish after they had been gutted, gilled and graded ie after initial processing in the factory; note that frozen fish were examined at company 3 on 15/5/1996 (Table 4), as fresh fish were not being harvested.

Each substage above occurred over a period of 2-2.5h and sampling was undertaken at early, mid and late phase during the period; approximately 1000 to 2000 fish (total throughput volume) were handled by farm or factory staff in this time.

Seasonality Samples were collected in late summer and autumn.

Number of Samples and Tests Each composite sample for microbiological testing consisted of 5 subsamples and some composite samples were tested for more than one potential pathogen. The total number of composite samples and subsamples examined were respectively:

external surface of fish at harvest; 54 composite samples of swabs representing 270 fish

water in killing bins at harvest; 9 large volume samples representing ~3000-~6000 fish

external surface of fish on arrival at processing factory; 72 composite samples of swabs representing 360 fish

internal gut (rectal) surface of fish on arrival at processing factory; 36 composite samples of swabs representing 180 fish

flesh of fish after early processing; 27 composite samples representing 135 fish

Each sample was examined for 7 potential human bacterial pathogens (*L.monocytogenes, C.perfringens, S.aureus* (coagulase positive), *E.coli, A. hydrophila, V.parahaemolyticus* and *V.vulnificus.*

In total 315 microbiological tests were undertaken on 198 samples.

Representativeness At each of the 4 substages where swabs or fish flesh were examined, the 15 fish used for each microbiological test represented 0.75-1.5% of the total throughput volume (1000-2000 fish in 2-2.5h). If the data are combined for these 4 substages at companies 1 and 2 in March and April 1995, the 60 fish used for each microbiological test represented 3-6% of the total throughput volume.

Fish of 3-5kg total body weight have approximate dimensions (length x curved breadth) between 55x13cm and 70x20cm and approximate external body surface areas between 750 and 1500cm^2 respectively. Thus for each fish the external area used for swabbing (50 cm^2) represented 3.3-6.7% of the total external surface. The 2-5g sample of flesh used for analysis represented ~0.4-~1.5% of total body weight or ~0.35-~1.3% of body weight after gutting and gilling.

Reliability The information above suggests that the bacteriological survey results for Atlantic salmon can be regarded as reasonably reliable, as the number of samples represents 0.35-6.7% of the sample set. In comparison, approved sampling schemes for the bacteriological certification of Australian fish products for export typically involve n=5 samples (50-100g total sample weight). These represent a % value of the total export weight (usually tonnes) which is 2-3 orders of magnitude lower than the % value sampled in this study.

The reliability of the sampling technique can also be considered in terms of the minimum prevalence of a human pathogen on one (1) fish necessary for it to be detected. Since 60 fish of a total 1000-2000 were examined during substages 2A-3C, the minimum prevalence is 3-6% at company 1 and a little higher at the other two companies.

DETAILED RESULTS

Fish : Results of Bacteriological Survey

Overall the survey results in Table 4 demonstrate a very low prevalence of the 7 potential human bacterial pathogens on Atlantic salmon examined during harvesting at the farm and initial processing at the factory. Comments on individual pathogens include:

L.monocytogenes was absent from 100% (45 of 45) of samples tested. This result indicates that *L.monocytogenes* is very unlikely to be present in waterways where the fish are currently grown or harvested in bacterial numbers which can significantly contaminate fish. In a separate study, Soontharanont & Garland (1995) did not detect *L.monocytogenes* in water at a farm in SE Tasmania where Atlantic salmon were harvested and which was tested at 2-weekly intervals over a 12-month period. The results also suggest that if ready-to-wholesale final product is subsequently found to contain *L.monocytogenes*. it is highly likely to have been acquired in the factory.

C.perfringens was absent from 84.4% (38 of 45) of samples tested. It was detected at low to moderate levels (range 1-60/10ml) in 66.7% (6 of 9) of samples of blood water (substage 2B). *C.perfringens* was also found at a moderate level (100/250cm^2) in 3.7% (1of 27) swabs of fish on arrival at the factory (substage 3A). *C.perfringens* is well adapted to survival in aquatic systems and its presence in blood water probably derives from the water film present on fish skin when the fish are harvested, the organism being sourced from ambient water. Its occassional detection is not surprising.

S.aureus (coagulase positive) was absent from 84.4% (38 of 45) of samples tested. It was detected at moderate levels (2100 and 2200/250cm^2) in 22.2% (2 of 9) of swabs of fish at harvest (substage 2A) and at a low level (100/250cm^2) in 22.2% (2 of 9) of swabs of fish on arrival at the factory (substage 3A). It was also found at low levels (5-20/10ml) in 33.3% (3 of 9) of samples of blood water (substage 2B). *S.aureus* occurs in natural waterways and its occassional detection is not surprising, although it is not as well adapted to survival in aquatic systems as *C.perfringens*. A small number of organisms may have been transmitted to fish by slaughtering staff during handling.

E.coli was absent from 91.1% (41 of 45) of samples tested. It was found at a very low level (1/10ml) in 44.4% (4 of 9) of samples of blood water (substage 2B) but was absent from all other samples. *E.coli* is known to survive for only a few days in natural waterways. Its detection in water usually indicates recent rainfall and runoff, or an outfall in the vicinity which is discharging contaminated effluent or stormwater.

A.hydrophila was absent from 95.6% (43 of 45) of samples tested. It was found at low levels (100 and 400/10ml) in 22.2% (2 of 9) of samples of blood water (substage 2B) but was absent from all other samples. Aeromonads are regarded as indigenous aquatic organisms and it is not clear why *A.hydrophila* was not detected more frequently. Possibly a more sensitive testing technique (eg with a minimum level of detection of say 1/100ml, compared to 100/10ml as used in the current study) would demonstrate its presence in samples, particularly blood water.

V.parahaemolyticus was absent from 95.6% (42 of 45) of samples tested. It was detected at a very low level (2/250cm^2) in 8.3% (1 of 12) of swabs of fish on arrival at the factory (substage 3A). An organism intermediate between *V.parahaemolyticus* and *V.vulnificus* was found at a very low level (2/250cm^2) in 11.1% (1 of 9) of swabs of fish at harvest (substage 2A) and at a low level (1.5/10ml) in 11.1% (1 of 9) of samples of blood water (substage 2B). The organism is likely to be *V.parahaemolyticus* since *V.vulnificus* prefers tropical and subtropical waters.

V.vulnificus was absent from 93.3% (43 of 45) of samples tested. Its possible presence in two samples is mentioned in the section on *V.parahaemolyticus* above.

The results in Table 4 indicate that blood water (substage 2B) is by far the most likely sample in which a potential pathogen might be found, and thus the most sensitive indicator of the possible presence of potential pathogens during processing stages 2 and 3 as described in the Introduction. Potential pathogens were detected much less frequently on the external surface of fish at harvest (substage 2A) or on arrival at the factory (substage 3A). No potential human pathogens were found in the rectum of fish on arrival at the factory (substage 3B) or in the flesh of fish after initial processing (gutting, gilling and grading) (substage 3C).

Wastewater: Sampling Strategy

Four major seafood processing plants in SE Tasmania were studied. Plants A and B were Atlantic salmon processing factories and corresponded to Companies 2 and 1 in the first part of the study. Plants C and D processed wildcatch finfish and abalone respectively. Details of processing operations and wastewater treatment systems used at the 4 plants are shown in Figure 1 and Table 5; further details are given in Intrasunghka & Garland (1996).

Number of Substages Influent (raw untreated wastewater) samples were collected from the stream wastewater which had undergone preliminary treatment (usually screening and oil & grease skimming) on exiting from the factory and entering the main treatment system. Effluent (treated wastewater) samples were collected at a point close to the end of the pipe which discharged it into the receiving waterway.

Seasonality Samples were collected in late summer and autumn.

Number of Samples and Tests Samples were collected on three different days over a four week period during the operational season at each factory. Twelve samples of both influent and effluent were analysed.

Each sample was examined for SPC and 7 potential human bacterial pathogens (faecal coliforms, *E.coli*, *S.aureus* (coagulase positive), *C.perfringens*, *L.monocytogenes*, *V.parahaemolyticus* and *V.vulnificus*).

In total 192 microbiological tests were undertaken on 24 samples.

Representativeness Sampling was undertaken during peak processing, typically late morning. Samples (1L) were collected from tanks (10 000sL) containing mechanically well-mixed wastewater.

Reliability In view of the limited number of samples from each plant and the low % value which each sample represented of total volume, the results should be regarded as reasonably reliable only where a strong trend is evident.

Wastewater: Results of Bacteriological Survey

The mean and range values of 5 bacterial indicator tests for both influent and effluent samples are shown in Table 6 and the results of presence/absence detection of *Listeria*

and *Vibrio* spp are shown in Table 7. Note that two results are shown for effluent samples at Plant A : effluent before being UV treated (results discussed first) and after being UV treated (results discussed later).

Influent The quantitative results for SPC counts, faecal coliforms, *E. coli* and CP *S. aureus* in influent samples followed specific patterns depending on the type of fish/shellfish being processed. The SPC counts were ~100fold higher in Plants C and D which processed wildcatch fish and shellfish, than in Plants A and B which processed aquacultured Atlantic salmon. Two likely explanations are:

Atlantic salmon are starved for 1 - 2 days before harvest, so the gut has a very low bacterial content. Thus contamination of wastewater after evisceration was minimised compared to processing wildcatch species;

since many of the aquaculture products are in the ready-to-eat form, stricter hygiene procedures and temperature control are adopted at Plants A and B (eg AQIS 1995, Garland 1995). In contrast, wildcatch species are usually wholesaled as ready-to-cook, cooked or canned products, so attention to hygiene and temperature, though satisfactory, is not as strict at Plants C and D.

Faecal coliform and *E.coli* counts were variable but generally higher (10-100fold) at Plants C and D than at Plants A and B. This result is not attributable to the faecal coliform/*E.coli* content of the wildcatch fish and shellfish being processed, which is usually very low, but rather it reflects the intermittent input of human faecal waste to the wastewater stream at Plants C and D. Domestic sewage was not added to the wastewater stream at Plant B; it was added at Plant A but after the influent sampling point we used.

CP *S.aureus* counts were also much higher (100fold or more) at Plants C and D than at Plants A and B. A likely explanation for this result is cross-contamination from staff handling wildcatch fish and shellfish during harvesting, slaughtering and processing. Staphylococci including CP *S.aureus* are commonly found on the hands of staff, being sourced from the upper respiratory tract (especially the nose), and unless strict hygiene practices are in place they are readily transmitted onto food during handling.

The results for the other organisms followed different patterns. *C. perfringens* was detected at low to moderate levels, also being sourced from human faecal waste, and its presence in influent is not surprising. Interestingly, the *C. perfringens* counts were consistently 10-100fold higher in Plant D, which mainly processed abalone, than in the other 3 plants. The increased counts may be due to the gut content of abalone which live on the bottom and adjacent rocky outcrops, and graze areas where sediment particles accumulate. *C. perfringens* becomes incorporated in the sediment (and water column) after transport from land runoff and various effluent and stormwater outfalls discharging to the local waterway (Kator and Rhodes 1991).

Listeria spp were commonly found in influent (Table 7) but *L.monocytogenes* was detected only occassionally in influent at Plants A and B and not at Plants C and D. Plants A and B have operated *Listeria* control programs since mid-1993 and we know from regular testing results that the presence of *Listeria*, especially *L.monocytogenes*, in the manufacturing area is now rare (Garland and Mellefont 1996). Its likely source

in influent is the subfloor drains exiting to the wastewater treatment plant, which are notorious for harbouring the organism (Ryser and Marth 1991).

V.parahaemolyticus was found occassionally in influent at Plants A and C but not at Plants B and D. As *V.parahaemolyticus* is an indigenous marine organism, its detection from time to time is not surprising.

V.vulnificus was not found in influent at any factory, even in February when ambient water temperatures were >15^{0}C. Similar to the results for fish and blood water in processing substages 2A-3C, this result suggests that *V.vulnificus* is present rarely, if at all, in temperate SE Tasmanian waters or on fish and shellfish harvested from it.

Effluent The SPC counts (Table 6) were moderate to high (~1x10^5-~4x10^6/ml) in effluents and occurred at similar levels at the 4 plants. Wastewater treatment systems are designed to make specific use of bacteria to degrade organic loadings, so the result is not surprising in view of the similar organic levels entering the raw wastewater stream (Intrasunghka and Garland 1996).

Faecal coliform and *E.coli* counts in effluent were 10-100fold lower at Plants A and B than at Plants C and D. The faecal coliform and *E.coli* counts were similar in effluent compared to influent at Plants A and B but were lower (10-1000fold) at Plants C and D, indicating the effectiveness of the wastewater treatment systems in use (aerated ponds at Plant C, activated sludge at Plant D). Nonetheless, it is clear that significant loadings of faecal coliform and *E.coli*, ranging ~1x10^2-~1x10^4/ml, were being discharged into the receiving waterway by Plants C and D.

CP *S.aureus* counts were generally low (<1x10^2/ml) in effluents at the 4 plants, indicating the effectiveness of the wastewater treatment systems in use, particularly at Plants C and D where a 100-1000fold reduction of influent loading was achieved.

C. perfringens counts were generally low (~1x10^2/ml) and similar to CP *S.aureus*. *C.perfringens* was not detected in effluent at Plant B, similar to the results for influent, due to domestic sewage not being added to it. At the other 3 plants, *C.perfringens* counts in effluent varied little from those in influent, highlighting the marked ability of the organism to survive out of its normal habitat, the gut of warm-blooded animals.

Listeria spp (Table 7) were commonly found in effluent (100ml sample volume) at the 4 plants but *L.monocytogenes* was detected in efffluent (pre-UV treatment) only at Plant A. In a separate study, Soontharanont and Garland (1995) examined effluent (1L sample volume) at 1-month intervals over a 12-month period and found *L.monocytogenes* very commonly at Plant A and also occassionally at Plant D. The latter results probably reflect a more reliable sampling strategy, including higher sample numbers, greater sample volume and full seasonality. Similar to the earlier suggestion concerning detection of *A.hydrophila* in blood water (substage 2B), a more thorough sampling scheme may result in *L.monocytogenes* being found in effluent more frequently.

V.parahaemolyticus was not detected in effluent at Plants A-C but was found occassionally in effluent at Plant D. As *V.parahaemolyticus* is an indigenous marine organism, its detection from time to time is not surprising.

V.vulnificus was not found in effluent at the 4 plants, similar to its absence from influent samples.

Effect of UV-Treatment Ultraviolet (UV) radiation was used to treat effluent at Plant A shortly before discharge to the local waterway. Its effects on the bacteriological loading varied according to bacterial type (Tables 6 and 7). For example, UV treatment reduced low levels of faecal coliforms including *E.coli*, CP *S.aureus* and *Listeria* spp (including *L.monocytogenes*) to negligible levels. SPC and *C.perfringens* counts were less affected.

DISCUSSION INCLUDING

BENEFITS

The survey results in Table 4 showed that the 7 potential human bacterial pathogens studied were present on Atlantic salmon at very low levels, if at all, during harvesting and initial processing (noting that the minimum prevalence rate for their detection on fish was 3-6% or a little higher). This study was particularly valuable as it has enabled specific hazards and critical control points to be identified. As examples two scenarios will be considered:

Scenario 1
If these fish were to be eaten immediately after substage 3C without further processing (e.g. as sashimi 100g portion), the risk to human health would be minimal since the bacterial numbers on fish were several orders of magnitude below the infectious dose for disease to develop (typically 10^5-10^6/g). If the fish were to be cooked and consumed soon after, the risk would be even lower.

Referring to HACCP, various critical control points to achieve the safe microbiological status of this product can be identified:

Starvation of Fish Pre-slaughter Farmed Atlantic salmon are starved for 1-2 days prior to harvest so that the bacterial content of their gut is very low. This practice minimises the opportunity for bacterial contamination of the exposed surfaces of fish to occur during evisceration.

Water Rinses The use of a series of tanks containing iced chlorinated water to exsanguinate fish also serves to remove (desorb) bacteria from their external surfaces, as evidenced by the much higher occurrence rate of potential pathogens in blood water (substage 2B) than in fish flesh or swabs. The low temperature (0-2^{0}C) of the blood water is also critical, as it prevents desorbed bacteria from multiplying and possibly recontaminating slaughtered animals.

Water rinses and temperature control are also critical control points during initial processing in the factory.

Personal Hygiene of Staff For ease of handling fish, gloves are worn by staff during harvesting and initial processing. This practice also serves to minimise cross-contamination of fish with handborne bacteria including CP *S.aureus*. Clearly strict handwashing by staff after using the toilet is also critical in order to minimise cross-contamination of fish with handborne bacteria of human faecal origin such as *E.coli*, *C.perfringens* and *L.monocytogenes*.

Equipment The results in Table 4 indirectly show that equipment used during harvesting and initial processing had been adequately cleaned and disinfected beforehand, which is essential to minimise cross-contamination of fish with bacteria, particularly *L.monocytogenes* (Garland 1995, Jemmi and Keusch 1994).

Ambient Water Quality Potential human bacterial pathogens (particularly *E.coli* and *C.perfringens*, and possibly *L.monocytogenes*; Tables 6 and 7) in effluent discharged to waterways are of concern if they reach an area where fish or shellfish are located and contaminate them. Various physical and hydrological factors determine whether these bacteria will reach the fish or shellfish at risk, including: the bacterial loading in effluent, dilution rate of the effluent at the point of discharge into the receiving waterway, outfall design (point source or diffuser), distance between the outfall and where the fish or shellfish are located, and current speed and direction. Factors which influence bacterial survival during transport in waterways include: sensitivity to increased salinity, decreased temperature and UV radiation (and other wavelengths) in sunlight; predation eg by protozoa; and adhesion to particulates which settle out in the sediment. If potential pathogens reach an aquaculture or fishing area, their adsorption to external surfaces or uptake into the gut of animals must then occur for contamination to occur.

In this report, Atlantic salmon and wastewater were both studied at companies 1 and 2 (= Plants B and A). Since the salmon were relatively free of the 7 potential pathogens studied, it appears that the selection of the effluent outfall sites and related factors was satisfactory. Nevertheless the use of UV radiation as a final treatment of effluent at Plant A to reduce levels of *E.coli*, CP *S.aureus* and *L.monocytogenes* (Tables 6 and 7) is an effective general control point, possibly becoming a critical control point if occassional very high influent loadings overload the wastewater treatment system.

Staff Training Although it was not the subject of the study, a comment on the value of staff training will be made. From our wide experience in the food production and service industry (as well as the water and pharmaceutical industries), it is highly desirable that all "hands-on" factory staff undertake training in bacterial hazards and control points associated with manufacturing products for human use. For example, in the past 5 years we have conducted staff training sessions based on HACCP for ~15 companies, usually after a bacterial pathogen or spoilage incident had occurred. In all instances, 3-6 months later the microbiological quality of the product improved by 2 or more orders of magnitude and the product became microbiologically safe consistently (but not invariably) and more stable (longer shelf life).

Scenario 2

In the case where Atlantic salmon are processed further after substage 3C (e.g. gravalaks, cold- or hot smoked), the risk to the human consumer will depend on how strictly various hygiene practices are implemented during the remaining stages in the

food delivery chain. In the factory critical control points include: quality of water for rinsing, brining, washdown and staff handwashing; cleaning and disinfection of food contact and secondary surfaces; staff hygiene; temperature control (freezing or chilling at 0-2^{0}C); separation of functions; air quality; staff training; and restriction of entry by visitors including tradesmen. Further details are given in AQIS (1995), and Sikorski (1990). If hygiene control is satisfactory, then wholesale products will leave the factory with a continued safe microbiological status.

Also cases of wound infection caused by *V.vulnificus, A.hydrophila, C.perfringens* and CP *S.aureus* were described earlier in the report in the section Bacterial Hazards. There are very few data concerning the infectious dose necessary to induce disease but nonetheless the very low numbers of potential pathogens on Atlantic salmon suggest that staff handling fish are at low risk. Several critical control points can be identified:

Hand Protection If not already, staff should be encouraged by management to wear gloves and handle fish or equipment in a manner which minimises the opportunities for skin abrasions to occur. If staff have sustained a minor skin abrasion prior to reporting for work, they should protect the wound (eg with a non-absorbent bandage). If the wound is deep, consideration should be given to redirecting them from processing until the wound has healed.

Bacteriological Status of Fish It is preferable to handle fish which are consistently free of potential human bacterial pathogens.

Staff Training If not already, staff should be informed by management of the occupational health and safety issues relating to bacterial wound infections which can occassionally occur during fish processing. This is perhaps more important for at-risk groups such as immunocompromised or pregnant workers.

Referring to staff working at the wastewater treatment plant, the risks of bacterial disease are increased, due to the higher loadings of potential pathogens in influent and effluent, particularly if human faeces are added. Staff should be advised to take appropriate protective measures.

FUTURE DEVELOPMENT

Provisional Risk Assessments and HACCP

The prediction of whether a bacterial hazard becomes a health risk requires an extensive information base and a series of complex considerations. Quantifiable risk assessment (Gerba *et al* 1996) can be used to predict the probability of a specific disease occurring in an individual and involves 4 steps: hazard identification, dose response assessment, exposure assessment, and risk characterisation. For the estimated probability to be judged as significant or not, an incidence rate of disease which is acceptable to the community needs to have been established.

Strict hygiene control through HACCP is apparently effective in minimising contamination of Atlantic salmon with 7 human bacterial pathogens (*E.coli, C.perfingens*, CP *S.aureus, E.coli, A.hydrophila, V.parahaemolyticus* and *V.vulnificus*) during harvesting and initial processing in the factory. To minimise

possible future bacterial disease outbreaks due to contaminated seafoods, the Australian fishing industry should be encouraged strongly and widely to implement HACCP. Where HACCP is adopted, its effectiveness should be monitored.

Survey of ready-to-eat seafoods

Ready-to-eat products will usually be packaged and frozen or chilled but they remain susceptible to opportunities for bacterial contamination and growth. In Dr Garland's experience, the greatest opportunities occur at retailing and domestic premises e.g. in the restaurant food preparation area, self serve buffet, supermarket cabinet or domestic refigerator or kitchen bench. In these conditions, ready-to-eat foods are particularly susceptible to cross-contamination and temperature abuse. Consideration should be given to undertaking a survey of ready-to-eat seafoods at the retail and domestic levels, to determine the extent of their contamination with potential human bacterial pathogens. The study should also assess risks to human health, based on any identified bacterial hazards.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the support of the Tasmanian Fish Marketers, Processors and Exporters Association and member companies which provided access to their factories and wastewater treatment plants for sampling. The statistical assistance of Dr. David Ratkowsky was appreciated.

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TABLE 1. Characteristic Test Reactions for *V. parahaemolyticus* and *V. vulnificus* (modified from Farmer 1992 and Kaysner *et al* 1992).
Specific tests to distinguish the *Vibrio* spp are in bold.

Tests	*V. parahaemolyticus*	*V. vulnificus*
morphology	Gram -ve rods	Gram -ve rods
motility	+	+
TSI	**K/A***, H_2S (-), gas (-)	**A/A****, H_2S (-), gas (-)
oxidase	-	-
lysine decarboxylase	+	+
ornithine decarboxylase	+	+
arginine decarboxylase	-	-
growth with 0%NaCl	-	-
growth with 8%NaCl	**+**	**-**
growth with 11%NaCl	-	-
growth at 42°C	+	+
Voges-Proskauer	-	-
ONPG	**-**	**+**
arabinose fermentation	**+**	**-**
mannitol fermentation	+	+
galactose fermentation	+	+
sorbitol fermentation	-	-
myo-inositol fermentation	-	-
Vibriostatic agent 0/129 (10mg)	**resistant**	**sensitive**
Vibriostatic agent 0/129 (150mg)	sensitive	sensitive

TSI= Triple Sugar Iron agar, ONPG=o-Nitrophenyl-ß-D-galactopyranoside

* **K/A**=Alkaline slant/acid butt; glucose has been fermented but not sucrose or lactose

** **A/A**=Acid slant/acid butt; lactose and/or sucrose has been fermented

TABLE 2. Recovery of 7 Potential Human Bacterial Pathogens from Fish Flesh (Atlantic salmon) and Recovery of *L. monocytogenes* from smoked Atlantic salmon and Pacific oysters.

	Medium Inoculum			Low Inoculum		
	Added (/g)	Recovered (-Tween 80) (/g)	Recovered (+Tween 80) (/g)	Added (/g)	Recovered (-Tween 80) (/g)	Recovered (+Tween 80) (/g)
V. parahaemolyticus	$7.3x10^2$	$4.3x10^1$	$2.4x10^2$	$1.5x10^1$	3	4
V. vulnificus	$2.0x10^3$	$4.6x10^2$	$>1.1x10^3$	$3.9x10^1$	<3	$4.3x10^1$
C. perfringens	$2.1x10^3$	$1.3x10^3$		$8.2x10^1$	$5.0x10^1$	
E. coli	$1.6x10^3$	$1.2x10^3$		$4.4x10^1$	$4.0x10^1$	
S. aureus	$3.1x10^2$	$2.8x10^2$		3	4	
*L. monocytogenes**						
fish flesh	$1.6x10^2$	present		2	present	
smoked salmon	$8.8x10^6$	present		1	present	
oysters	$8.8x10^6$	present		1	present	
A. hydrophila	$1.3x10^2$	$1.6x10^2$		1	1	

**L.monocytogenes* was examined as a presence/absence test in 25g samples

TABLE 3. Recovery of 6 Potential Human Bacterial Pathogens from Water (0.1% Peptone Water pH 7.2).

	Medium Inoculum		Low Inoculum	
	Added (/ml)	Recovered (/ml)	Added (/ml)	Recovered (/ml)
C. perfringens	$7.6x10^3$	$2.5x10^3$	$7.6x10^1$	$4.0x10^1$
E. coli	$2.1x10^3$	$1.9x10^3$	$2.1x10^1$	$2.2x10^1$
S. aureus	$3.0x10^2$	$1.4x10^2$	3	3
V. parahaemolyticus	$2.7x10^4$	present*	$2.7x10^1$	present*
V. vulnificus	$7.3x10^5$	present*	$7.3x10^3$	present*
L. monocytogenes	$5.6x10^1$	present*	3	present*
A.hydrophila	not tested		not tested	

**V.parahaemolyticu s,V.vulnificus* and*L.monocytogenes* were examined (recovered) as a presence/absence test in 10, 10 and 25ml samples respectively.

TABLE 4 Survey of Potential Human Bacterial Pathogens on Fish (Atlantic Salmon), Water and Swabs

Company (date of sampling)	Prod'n Phase	Swabs of Surface of Ready-to-Kill Fish (Farm) (substage 2A)							Water in Killing Bin at Harvest (Farm) (substage 2B)						
		L.m /250cm^2	*C.p* /250cm^2	*S.a* /250cm^2	*E.c* /250cm^2	*A.h* /250cm^2	*V.p* /250cm^2	*V.v* /250cm^2	*L.m* /25ml	*C.p* /10ml	*S.a* /10ml	*E.c* /10ml	*A.h* /10ml	*V.p* /10ml	*Vv* /10ml
1	Early	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5	absent	<1	<1	<1	<1x10^2	<1.5	<1.5
(5/4/95)	Mid	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5	absent	<1	<1	<1	<1x10^2	<1.5	<1.5
	Late	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5	absent	<1	<1	1	<1x10^2	<1.5	<1.5
1	Early	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5	absent	1	1.2x10^1	<1	<1x10^2	<1.5	<1.5
(15/5/96)	Mid	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5	absent	1	2.0x10^1	<1	<1x10^2	<1.5	<1.5
	Late	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5	absent	1	5	1	<1x10^2	<1.5	<1.5
2	Early	absent	<1x10^2	2.2x10^3	<1x10^1	<1x10^2		2*	absent	3.8x10^1	<1	1	1x10^2	<1.5	<1.5
(30/3/95)	Mid	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5	absent	6.0x10^1	<1	<1	<1x10^2		1.5*
	Late	absent	<1x10^2	2.1x10^3	<1x10^1	<1x10^2	<1.5	<1.5	absent	6.0x10^1	<1	1	4x10^2	<1.5	<1.5

* Differentiation of species not achieved but probably *V.parahaemolyticus*

Key *L.m=L.monocytogenes, C.p=C.perfringens, S.a=S.aureus* (coagulase positive), *E.c=E.coli, A.h=A.hydrophila, V.p=V.parahaemolyticus, V.v=V.vulnificus*

TABLE 4 Cont'd

Company (date of sampling)	Prod'n Phase	Swabs of Surface of Fish on Arrival (Factory) (substage 3A)							Rectal Swabs of Fish on Arrival (Factory) (substage 3B)						
		L.m /250cm^2	*C.p* /250cm^2	*S.a* /250cm^2	*E.c* /250cm^2	*A.h* /250cm^2	*V.p* /250cm^2	*V.v* /250cm^2	*L.m* /50cm^2	*C.p* /50cm^2	*S.a* /50cm^2	*E.c* /50cm^2	*A.h* /50cm^2	*V.p* /50cm^2	*V.v* /50cm^2
1	Early	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5
(5/4/95)	Mid	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5
	Late	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5
2	Early	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5
(30/3/95)	Mid	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	2	<1.5	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5
	Late	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5
3	Early	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5							
		absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5							
(15/5/96**	Mid	absent	<1x10^2	<1x10^2	<1x10^1	<1x10^2	<1.5	<1.5							
22/5/96)		absent	<1x10^2	1x10^2	<1x10^1	<1x10^2	<1.5	<1.5							

	Late	absent	<1x10	<1x10	<1x10	<1x10	<1.5	<1.5
		absent	^{2}1x10^{2}	^{2}1x10^{2}	^{1}<1x10^{1}	^{2}<1x10^{2}	<1.5	<1.5

** These fish had been maintained in frozen storage and were not freshly harvested

Key *L.m=L.monocytogenes, C.p=C.perfringens, S.a=S.aureus* (coagulase positive), *E.c=E.coli, A.h=A.hydrophila, V.p=V.parahaemolyticus, V.v=V.vulnificus*

TABLE 4 Cont'd

Company (date of sampling)	Prod'n Phase	Flesh of Gutted, Gilled and Graded Fish (Factory) (substage 3C)						
		L.m /25g	*C.p* /g	*S.a* /g	*E.c* /g	*A.h* /g	*V.p* /g	*V.v* /g
1 (5/4/96)	Early	absent	<1x10^{2}	<1x10^{2}	<1x10^{1}	<1x10^{2}	<0.3	<0.3
	Mid	absent	<1x10^{2}	<1x10^{2}	<1x10^{1}	<1x10^{2}	<0.3	<0.3
	Late	absent	<1x10^{2}	<1x10^{2}	<1x10^{1}	<1x10^{2}	<0.3	<0.3
2 (30/3/95)	Early	absent	<1x10^{2}	<1x10^{2}	<1x10^{1}	<1x10^{2}	<0.3	<0.3
	Mid	absent	<1x10^{2}	<1x10^{2}	<1x10^{1}	<1x10^{2}	<0.3	<0.3
	Late	absent	<1x10^{2}	<1x10^{2}	<1x10^{1}	<1x10^{2}	<0.3	<0.3
3 (15/5/96*)	Early	absent	<1x10^{2}	<1x10^{2}	<1x10^{1}	<1x10^{2}	<0.3	<0.3
	Mid	absent	<1x10^{2}	<1x10^{2}	<1x10^{1}	<1x10^{2}	<0.3	<0.3

Late	absent	$<1x10^2$	$<1x10^2$	$<1x10^1$	$<1x10^2$	<0.3	<0.3

* These fish had been maintained in frozen storage and were not freshly harvested

Key *L.m=L.monocytogenes, C.p=C.perfringens, S.a=S.aureus* (coagulase positive), *E.c=E.coli, A.h=A.hydrophila, V.p=V.parahaemolyticus, V.v=V.vulnificus*

TABLE 5 Production Characteristics of Seafood Plants Processing Fish or Shellfish and Design Features of their Wastewater Treatment Systems This table is excerpted from Intrasunghka and Garland, 1996

Description	Plant A (salmon)	Plant B (wild fishery)	Plant C (salmon)	Plant D (abalone)
Fish species processed Common name (Scientific name)	Atlantic Salmon (*Salmo salar*)	Pacific Cod (*Gadus macro-cephalus*), Orange Roughy (*Haplostethus atlanticus*) and Abalone (*Haliotis* spp)	Atlantic Salmon (*Salmo salar*)	Abalone(*Haliotis* spp)
Processing stages	heading, gilling, gutting, filleting, brining, smoking	filleting, canning	heading, gilling, gutting, filleting, brining, smoking	canning
Processing days (/year)	280 - 300	200 - 250	150 - 200	200 - 250
Volume used water (kL/day)	20 - 50	20 - 50	50 - 100	30 - 50
Total volume of raw products processed prior to sampling date*	1st 4.6 tonnessample: 2nd sample: 3.2 tonnes 3rd sample: 3.1 tonnes	1st sample: 16.18 tonnes 2nd sample: 33.82 tonnes 3rd sample: 22 tonnes	1st sample: 15 tonnes 2nd sample: 7.8 tonnes 3rd sample: 10.1 tonnes	1st sample: 9.5 tonnes 2nd sample: 7.9 tonnes 3rd sample: 2.8 tonnes
Production yield (tonnes/year)	900 - 1000	1000 - 2500	2000	500 - 1000
No. of staff (person)	80 - 110	50 - 60	20 - 80	50 - 60
Human sewage added to wastewater	received	received	not received	received
Storm water from sealed areas around plant added to wastewater	not received	received	not received	received
Preliminary wastewater treatment	Bar screen and Oil separator tank	Bar screen and Oil separator tank	Tangential screen	Oil separator tank
Type of wastewater treatment system	Activated sludge with UV Treatment	Aerated pond	Aerated pond	Activated sludge
Retention time in wastewater treatment system	3 - 4 days	25 - 30 days	8 - 11 days	3 - 4 days

* The volume of processed products prior to sampling was estimated after taking into account the retention time of each wastewater treatment system.

FIGURE 1 Layout of Plants A-D (Table 1) and Diagrammatical Representation of their Wastewater

TABLE 6 Survey of Potential Human Bacterial Pathogens and SPC Counts in Wastewater

Parameter	Plant A (salmon)		Plant B (salmon)		Plant C (wild catch)		Plant D (abalone)	
	mean* /ml	range*/ml	mean/ml	range/ml	mean/ml	range/ml	mean/ml	range/ml
Influent								
SPC	$2.1x10^4$	$1.0x10^3$-$2.3x10^5$	$8.7x10^3$	$1.0x10^3$-$4.0x10^4$	$2.7x10^6$	$1.8x10^6$-$3.8x10^6$	$3.2x10^6$	$6.5x10^5$-$8.1x10^6$
FC	$<4.7x10^1$	1-$<1.0x10^4$	$<4.7x10^1$	$<1.0x10^1$-$<1.0x10^2$	$<5.8x10^5$	$6.9x10^4$-$2.7x10^7$	$<1.3x10^6$	$4.5x10^5$-$5.0x10^6$
E.c	$<4.7x10^1$	1-$<1.0x10^4$	$<4.7x10^1$	$<1.0x10^1$-$<1.0x10^2$	$<1.9x10^5$	$<1.0x10^4$-$6.8x10^6$	$<6.0x10^5$	$<1x10^5$-$5.0x10^6$
S.a	$<1.0x10^1$	$<1.0x10^1$	$<1.4x10^1$	$<1.0x10^1$-$3.0x10^1$	$3.7x10^3$	$2.7x10^3$-$6.6x10^3$	$<4.7x10^3$	$1.1x10^3$-$<1.0x10^4$
C.p	<5	1-$<1.0x10^1$	$<1.3x10^1$	<1-$2.2x10^2$	$1.4x10^1$	$1.0x10^1$-$3.0x10^1$	$6.9x10^2$	$1.0x10^2$-$2.0x10^3$
Effluent**								
SPC - pre-UV	$1.4x10^5$	$2.3x10^4$-$1.0x10^6$	$5.1x10^4$	$3.5x10^4$-$7.6x10^4$	$3.0x10^6$	$2.2x10^6$-$3.9x10^6$	$2.9x10^5$	$1.3x10^5$-$4.5x10^5$
- post-UV	$2.5x10^4$	$5.6x10^3$-$2.0x10^5$						
FC - pre-UV	$5.9x10^1$	$1.0x10^1$-$6.0x10^2$	$<1.0x10^1$	$<1.0x10^1$	$<2.5x10^4$	$5.4x10^3$-$<1.0x10^5$	$2.3x10^2$	$2.0x10^1$-$1.2x10^3$
- post-UV	<5	1-$1.3x10^1$						
E.c - pre-UV	$4.9x10^1$	$<1.0x10^1$-$5.5x10^2$	$<1.0x10^1$	$<1.0x10^1$	$<2.1x10^4$	$1.0x10^4$-$<1.0x10^5$	$1.1x10^2$	$2.0x10^1$-$3.0x10^2$
- post-UV	<5	1-$<1.0x10^1$						
S.a - pre-UV	$5.4x10^1$	$<1.0x10^1$-$5.5x10^2$	$<3.3x10^1$	$1.0x10^1$-$4.4x10^2$	$<1.0x10^1$	$<1.0x10^1$	<5	1-$1.0x10^2$
- post-UV	<5	1-$<1.0x10^1$						
C.p - pre-UV	$1.8x10^2$	$1.0x10^2$-$6.0x10^2$	<3	1-$<1.0x10^1$	$1.2x10^2$	$2.4x10^1$-$3.0x10^2$	$5.2x10^1$	$1.1x10^1$-$1.3x10^2$
- post-UV	$7.4x10^1$	$1.4x10^1$-$4.3x10^2$						

* geometric mean and range for n=3 samples ** A UV treatment system for effluent was operational at Plant A

Key SPC=Standard Plate Count, FC=Faecal Coliforms, *E.c=E.coli*, *S.a=S.aureus* (coagulase positive), *C.p=C.perfringens*

TABLE 7 Survey of *Listeria* and *Vibrio* spp in Wastewater

	Listeria spp. (/100 mL)	*L.m* (/100 mL)	*V.p* (/10 mL)	*V.v* (/10 mL)
Influent				
Plant A	1(3)*	1(3)	1(3)	0(3)
Plant B	2(3)	1(3)	0(3)	0(3)
Plant C	3(3)	0(3)	1(3)	0(3)
Plant D	3(3)	0(3)	0(3)	0(3)
Effluent**				
Plant A pre-UV	3(3)	3(3)	0(3)	0(3)
Plant A post-UV	0(3)	0(3)	0(3)	0(3)
Plant B	3(3)	0(3)	0(3)	0(3)
Plant C	3(3)	0(3)	0(3)	0(3)
Plant D	2(3)	0(3)	1(3)	0(3)

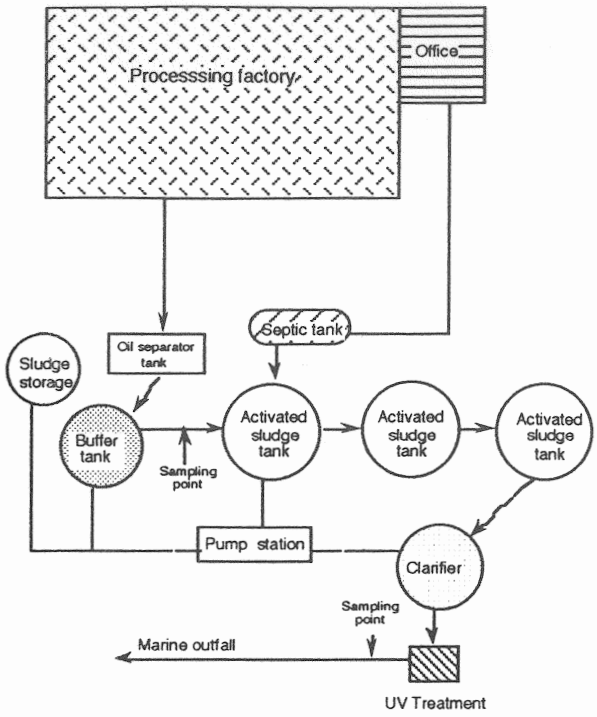
* no. of positive results (no. of samples tested) by presence/absence test

** A UV treatment system for effluent was operational at Plant A

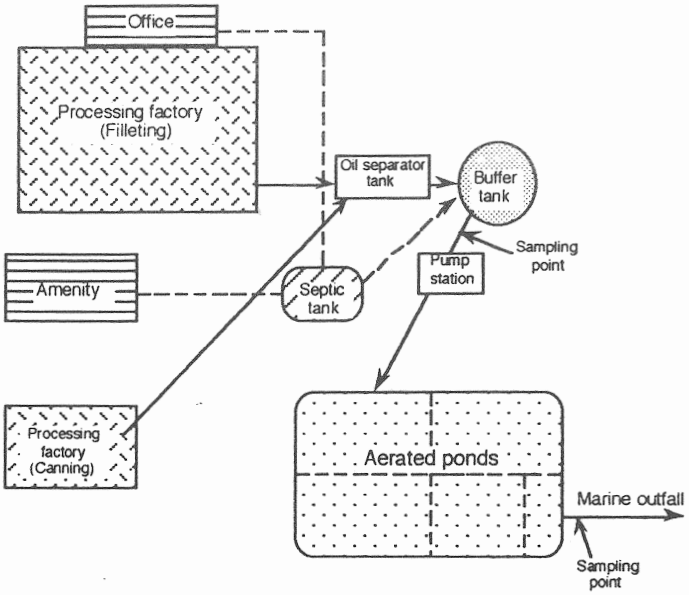
Key *L.m=L.monocytogenes, V.p=V.parahaemolyticus, V.v=V.vulnificus*

Treatment Systems. Sampling points for influent and effluent are indicated.

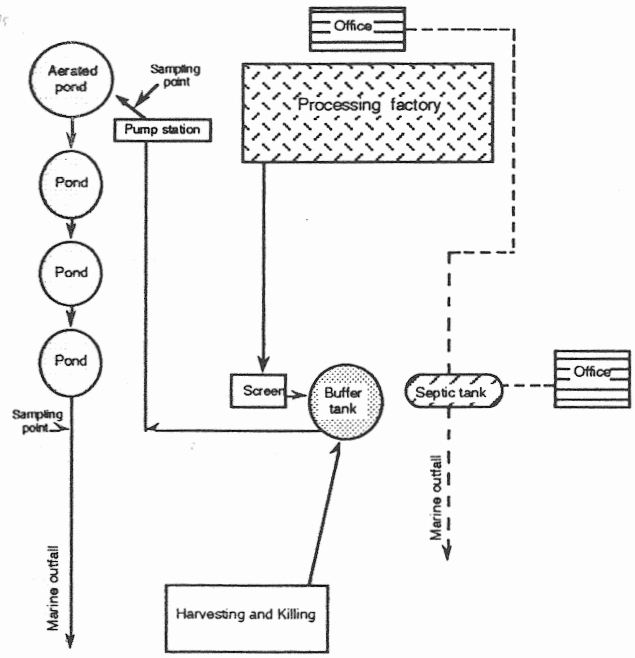
Plant A Plant B Plant C Plant D



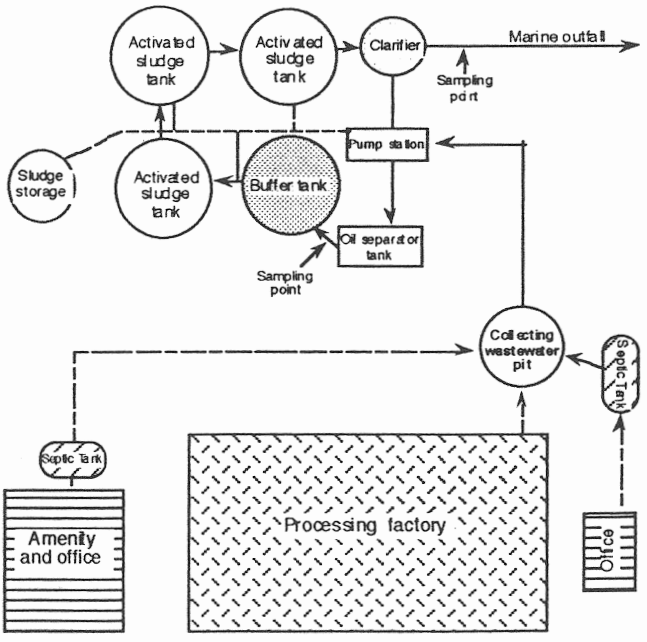
Plant A



Plant C



Plant B



Plant D

FIGURE 1 Layout of Plants A-D (Table 1) and Diagrammatical Representation of their Wastewater Treatment Systems. Sampling points for influent and effluent are indicated.