The Retention of Picoplankton by the Pacific Oyster, *Crassostrea gigas*, and Implications for Oyster Culture

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Abstract

Pacific oyster (*Crassostrea gigas*) farming in New Zealand has reached a point where the pressures on resources appear likely to constrain current, and future, development. To maintain industry growth, security of juvenile oyster (spat) supply and productivity gains within the existing farm leases, are becoming industry imperatives. The use of hatchery technology could achieve both of these requirements, but it is expensive to establish and maintain. The additional expense of a hatchery could be offset by the establishment of, for example, a selective breeding program to enhance oyster productivity and/or marketability. Consequently a pilot-scale oyster hatchery facility was constructed to investigate the potential for establishing hatchery production of Pacific oysters in New Zealand.

This study showed that in the pilot-scale hatchery, oysters could be successfully spawned from in-season broodstock, the eggs fertilised and the resultant larvae reared through to settlement for on-growing to adult size. This process was successful for both oysters selected for morphological characteristics and those which were not. On-growing of the resultant stock indicated growth rate could be normal relative to wild caught oysters spat, although data was limited by the small scale of the experiment. However, an investigation of broodstock conditioning, to achieve out-of-season spawning, was less successful. Disease occurred and condition was lost in some broodstock, suggesting they were enduring stress within the conditioning system. The microalgal food supply was examined but the clearance rate of the microalgal species suggested they were an acceptable feed supply which agreed with previous reports of successful conditioning techniques. Comparing the pilot-scale facility in this study with descriptions of facilities which reported successful broodstock conditioning suggested that the use of a microfiltered recirculating water supply, as opposed to the more common flow-through, natural seawater systems containing a range of small size particles, limited necessary nutrient and/or maturation factors and may have had a significant impact on conditioning.

The nanoplanktonic (< 10 μm), food resource, which includes key microalgal species such as *Chaetoceros* spp. and *Isochrysis* spp., is generally considered the primary food resource for suspension feeding bivalves, including *C. gigas*. However, the picoplanktonic fraction (< 3 μm) can provide the largest proportion of this food resource in the water column in

terms of abundance and biomass. Consequently, an investigation of the *in situ* retention of picoplankton populations (picoeukaryotes, *Synechococcus*-type cyanobacteria and heterotrophic bacteria) by oysters was undertaken. Flow cytometry was used to quantify the picoplankton populations in water samples taken from the inhalant and exhalant feeding currents of individual oysters, allowing retention efficiency of the particles to be calculated. Five picoplankton populations were identifiable by flow cytometry (picoeukaryotes, heterotrophic bacteria and 3 populations tentatively identified as cyanobacteria) and accounted for a large proportion (up to 97 %) of the estimated available carbon (picoplankton + microalgae) in Kerikeri Inlet water. Generally the heterotrophic bacteria accounted for the largest proportion of the biomass with up to 564 ± 51 ng C ml^{-1}.

Retention of each picoplankton population was found to be variable and not directly related to particle concentration. Cyanobacteria (Cy2 population) were retained with the greatest efficiency (up to 42 ± 4.4 %), followed by heterotrophic bacteria (up to 38 ± 4.5 %) and picoeukaryotes (up to 12 ± 3.8 %). Overall more picoplankton biomass was retained during the summer months, of which the heterotrophic bacteria made the largest contribution in either cell number or estimated carbon retained. Tracking of the condition and constituent fractions (glycogen, lipid and protein) of the subject oysters showed that in the summer months, post-spawn, these levels were lowest, indicting a period of nutritional stress. This appeared to suggest that *C. gigas* was able to alter its retention efficiency to expand the range of particles captured, and consequently the available nutrient pool. The retention of greater quantities of heterotrophic bacteria may allow for the acquisition of essential nutrients required for growth and later gametogenesis (such as B vitamins). However, it is also possible that the heterotrophic bacteria mediate access to otherwise inaccessible, or inefficiently accessed, nutrient resources through their degradation of, for example, crystalline cellulose.

Consequently, the microbial flora associated with the oyster gut was investigated. An initial investigation, cultivating bacteria from gut contents, showed considerable variability in the numbers of colonies present within and between samples, but was inconclusive for identifying differences in species diversity. Using culture independent histological and 16S rDNA PCR/RFLP techniques to investigate the oyster gut microflora a spirochaete flora, commonly associated with bivalve crystalline styles, was clearly present. Molecular analyses provided evidence of other bacterial in the gut. A signature RFLP band pattern was found in oysters at low tide and this generally reoccurred in oysters that had been

immersed for varying lengths of time up to high tide. However, the signature RFLP pattern became more dilute as immersion time/potential feeding time extended.

The isolation of culturable bacteria from the oyster gut allowed characterisation and identification of a subset of the oyster gut microflora. 16S rDNA sequence analysis from selected isolates showed a predominance of *Vibrio* spp. These bacteria had previously been associated with marine molluscs, including as symbionts. Characterisation of these and other isolates from oyster gut showed a diversity of attributes including the ability to degrade cellulose. This suggests the bacterial production of enzymes, such as cellulases, which have been reported by other researchers as being present in ineffectual or low native levels in oysters. Consequently the bacterial presence in the oyster gut may be essential to efficient nutrient acquisition.

The results of these investigations have highlighted the potential importance of the heterotrophic bacteria to *C. gigas*. To date, bacteria have received relatively little attention in terms of their potential nutritive contribution to oysters, primarily due to observations that they are retained with low efficiency. However, even at low retention efficiencies the potential nutritive contribution is large due to the available abundances of heterotrophic bacteria. While the mechanisms and controls of bivalve suspension feeding have yet to be fully elucidated, the published literature indicates that selective mechanisms are available to bivalves including *C. gigas* and this current research suggests that even pico-sized particles, retained with apparently low efficiency, can be subject to selection. The importance of the heterotrophic bacteria to *C. gigas* requires further investigation as it will have implications for not only hatchery production, but also farm management, public health and environmental impact monitoring.

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I would like to dedicate this thesis to my parents, Bruce and Margaret

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Abbreviations

S.I. (Systéme International d'unités) abbreviations for units and standard notations for chemical elements, formulae, and chemical abbreviations are used in this work. Other abbreviations used are listed below.

A_{xxx}	absorbance at xxx nm
bp	base pairs
cc	cubic centimetres
C.I.	colour index
dH_2O	sterile, glass distilled water
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylenediamine-tetra-acetic-acid (disodium salt)
Exh	exhalant
g	grams
g	acceleration due to gravity
h	hour
Inh	inhalant
kb	kilobase pairs (1000 base pairs)
kDa	kiloDaltons
l	litre(s)
M	molarity (moles litre^{-1})
MW	molecular weight
min	minute(s)
ml	millilitre(s)
N	normal (concentration)
PCR	Polymerase Chain Reaction
PEG	polyethylene glycol
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
s.e.	standard error (sample)
TBE	Tris-borate-EDTA-buffer
TE	Tris-EDTA buffer
TEMED	Tetramethlethylenediamine
Tris	[2-amino-2(hydroxymethyl) propane-1,3-diol, (tris)]
Tris-HCl	Tris solution pH adjusted with HCl
U	units of enzyme (manufacturer defined)
UV	ultraviolet light
v/v	volume:volume
w/v	weight:volume

Chapter 1.0
General Introduction

1.1 Background

In New Zealand the Pacific oyster (*Crassostrea gigas*) is of economic, recreational and environmental significance. It has been identified as an effective marine invader, known to displace the native rock oyster, *Crassostrea glomerata* (Dinamani 1987) and to modify habitat (Crawford *et al.* 2003). The resulting dominance of the Pacific oyster over the native rock oyster has led to it becoming a significant resource for traditional (kai moana) and recreational harvesting. The fast growth, high fecundity and consistent recruitment also make the Pacific oyster the species of choice for the oyster farming industry (Curtin 1979) where they reach marketable size in a third of the time required for culture of native rock oysters (Dinamani 1974, 1991).

Since the introduction of the Pacific oyster to New Zealand oyster farming has grown from small scale, owner-operator ventures with local markets, to larger operations in which companies hold large lease areas. These larger companies have made significant investments in processing facilities and service both national and international markets. However, the growth of the industry is now limited by conflicts in resource use and a shift in paradigm regarding the acceptability of exotic species in New Zealand. Consequently, expansion of the industry within traditional growing areas is declining and greater efficiencies within existing lease areas are being sought.

Improving efficiency within existing lease areas requires farmers to maximise carrying capacity, improve oyster quality and sale price, and to reduce production costs. To achieve these improvements different approaches need evaluation. The spat (juvenile oyster) supply for stocking the majority of the oyster farms in New Zealand comes from wild caught spat, primarily from the Kaipara Harbour. Therefore, there is potential for production improvement by developing a stock enhancement program. Stock enhancement programs, such as Oregon State University's selective breeding program (Hedgecock *et al.* 1997), are still fairly novel but have shown promise for both oyster growth rate and phenotype enhancement (e.g. Brake *et al.* 2004). Stock enhancement

requires strictly controlled conditions and, in New Zealand, hatcheries must be developed to provide such conditions.

Hatcheries have been previously investigated in New Zealand in terms of their ability to provide spat as a safeguard against spatfall failure (Curtin 1979) with the emphasis on cheap and simple methods of spat production. However, for a successful stock enhancement program there are some specific considerations. In particular, specifically bred broodstock 'families' must be maintained and isolated from environmental risks (such as harmful algal blooms and disease), and other oysters (with which they could breed and degrade the integrity of the breeding programme) in a facility with recirculating, or limited exchange seawater. However, recirculating technology for bivalve facilities is still developing and there are aspects of oyster biology which are still not well understood. For example, gaps exist in our knowledge of what constitutes a complete, or balanced, diet, particularly in terms of gametogenic demands. Investigations of such knowledge gaps are integral to our ability to progress oyster hatchery technology.

1.2 Biology, Life History and Ecology of *Crassostrea gigas*

Taxonomy Phylum *Mollusca*,
Class *Bivalvia*,
Subclass *Pteriomorpha*,
Order *Pterioida*
Family *Ostreidae*
(Spencer *et al.* 2004)

1.2.1 General biology

Oysters are bivalve molluscs which are characterised by having two hinged, calcareous shells that fully enclose the soft body parts. The soft parts are, in turn, retained within the mantle tissue. The mantle provides some protection for the body parts from the calcareous shell but arguably it's most important functions occur along the mantle edge (Figure 1.1). Characterised by three folds, the mantle edge is involved in shell growth (outer fold and mantle surface (Galstoff 1964, Quayle 1969)), sensation of the external environment (middle fold), current control and tactile sensation (inner fold or tentacles; Yonge 1960).

The primary response that oysters make to environmental stimuli is to open or close the valves. The regulation of shell gape is facilitated by the adductor muscle, which is equipped with quick and catch components. The quick component enables rapid opening or closing of the shell, while the catch can provide intense resistance to external forces opening or closing the valves. The capacity of the catch muscle to seal the valves shut, coupled with changes in metabolic processes, enables intertidal oysters to survive periods of emersion without irrecoverable dehydration or hypoxia. Metabolic responses to anoxia in oysters include, the fermentation of glycogen and asparate, with an accumulation of succinate and alanine as end products (Collicutt & Hochachka 1977, Eberlee *et al.* 1983, Foreman & Ellington 1983), the repression of metabolic rate, frequently to less than 10% of resting aerobic rate (De Zwaan *et al.* 1991) and tissue specific changes in enzyme activities (Greenway & Storey 1999).

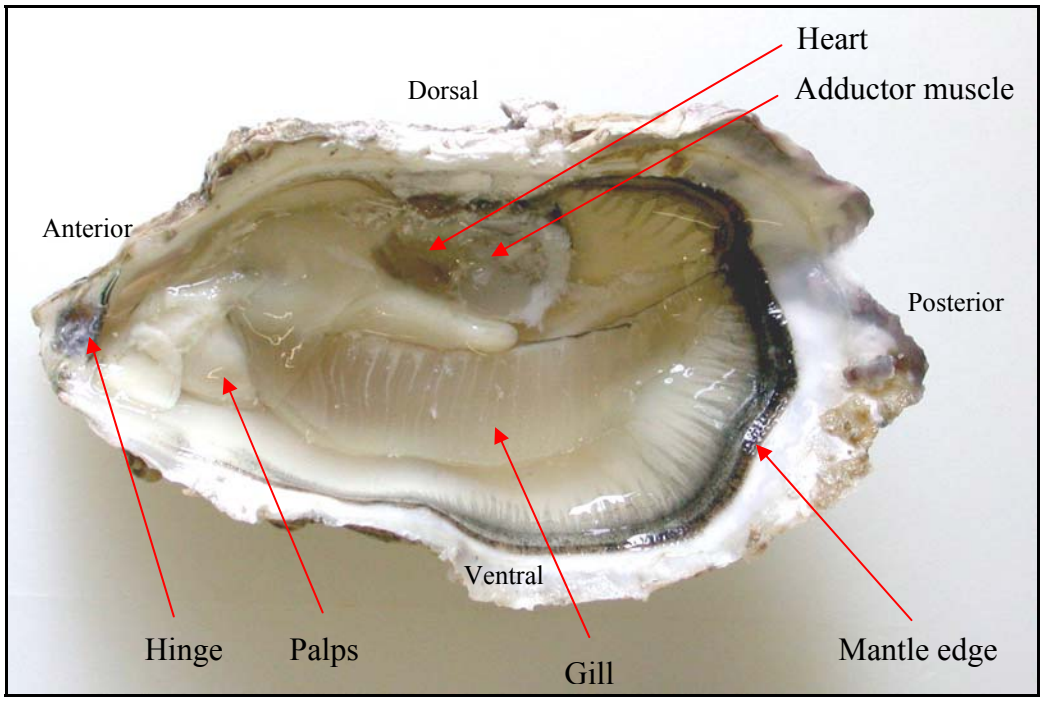


Figure 1.1 General orientation of a Pacific oyster with the top shell and upper mantle removed. (Photo credit R. Wong)

Oysters are filter, or suspension, feeders, using ciliary action to create a water current that passes between the valves. The water is drawn between the plicae of their heterorhabdic ctenidia allowing both respiration and the capture of food particles to occur. Food particles are captured by the cilia of the ctenidia and directed to the dorsal or ventral grooves where they are entrained in a flow of mucus to the labial palps (Ward *et al.* 1998). The palps

(two pairs) "are complex structures consisting of many ciliated ridges, grooves, and tracts (Newell & Langdon 1996), which carry particles ventrally toward the palp margin, dorsally toward oral grooves, and anteriorly toward the mouth" (p. 165, Milke & Ward 2003). The filter feeding process is discussed more fully in section 1.3 below.

A simple heart distributes haemolymph through a thin walled vascular system. The haemolymph carries haemocytes and a range of proteins, including at least 10 enzymes with high activity levels which have been implicated in bacterial defence (Hubert *et al.* 1996, Xue & Renault 2000). Auffret (1989) has identified four types of haemocyte, the granulocytes, acidophilic granulocytes, hyalinocytes and vesicular haemocytes. The haemocytes are very mobile throughout the body, congregating where needed for nutrition acquisition, respiration or defence. However, the relative proportions of the different haemocytes appear to be important in the immuno-response and this can be affected by physical (e.g. temperature) and/or chemical (e.g. trace metals) changes in the environment (Chu *et al*. 1995, Auffret & Oubella 1997).

C. gigas conforms to this basic pattern but differs morphologically from other oysters as set out in Table 1.1.

Table 1.1 Morphological characteristics of four oyster species (from Quayle 1969, Elliott 1966, Dinamani 1971). Morphological characteristics will vary with growing environment and may not be the most appropriate tool for taxonomic differentiation.

Scientific Name	***Crassostrea gigas***	***Crassostrea glomerata***	***Crassostrea virginica***	***Ostrea lurida***
Common Name	Pacific or Japanese oyster	New Zealand Rock oyster	Eastern or Atlantic oyster	Canadian or Olympia oyster
Anatomy	With promyal chamber	With promyal chamber	With promyal chamber	Without promyal chamber
Adductor muscle scar	Mauve or white, not clearly outlined	White with blueish/creamy markings, distinctly outlined	Dark purple or brown, sharply outlined	No pigmentation, clearly outlined
Concentric growth	Projecting with flutings	Flat	Flat but clear	Indistinct
Colour inside shell	Brilliant white	Pearly or solid white	White	Green iridescent
Colour outside shell	Creamy - Grey with purple	Grey/purple	Yellow brown	Grey with dark purple
Radial grooves	Generally deep	Indistinct	Barely apparent	Not apparent

1.2.2 Life history characteristics

A simplified life cycle of *Crassostrea gigas* is shown in Figure 1.2 below. *C. gigas* are protandric, first reaching sexual maturity as males, with progressively larger proportions of females developing in each age cohort over time (Guo *et al.* 1998). Work by Dinamani (1987) has shown that in New Zealand, *C. gigas* can reach sexual maturity as young as six months, compared to 2-3 years in British Columbia (Quayle 1969). Spawning events in New Zealand occur from early spring until late summer with the bulk of spawning occurring between December and March (Dinamani 1987).

C. gigas is a broadcast spawner, releasing eggs and sperm into the water column in a synchronous event that appears to be regulated by a combination of tide and temperature stimuli (18 – 20 °C in New Zealand; Dinamani 1987). However, individuals are unlikely to spawn all of their gametes in a single event and there is evidence that smaller spawning events occur both before and after the major spawning events (Dinamani 1991). Flexibility in the reproductive process in *C. gigas* has also been reported in terms of spawning temperature by Loosanoff and Davis (1950) who found spawning could take place in temperatures as low as 15 ^{o}C, rather than the usually accepted 18 °C (Mann 1979).

Fertilisation occurs in the water column with the fertilised eggs developing cilia and beginning to swim within about 5 hours. The shell rapidly develops and encloses the body within 24 hours of fertilisation and the larvae remain mobile via a ciliated velum. The velum also allows the larvae to feed upon microalgae in the plankton as they grow to approximately 250 μm (shell width). At this size the larvae begin protruding the foot. Upon contact with a hard surface the foot allows the larvae to crawl along the surface searching for a suitable position. When this position is found the larvae will cement itself in position with the lower, or left, valve attached to the substrate. In northern New Zealand the newly settled spat will take approximately 18 months to reach the premium market size of 120 mm shell length (Dinamani 1991, Brown pers comm.[1]).

[1] Simon Brown (Bay Oysters Ltd) reply to authors questions

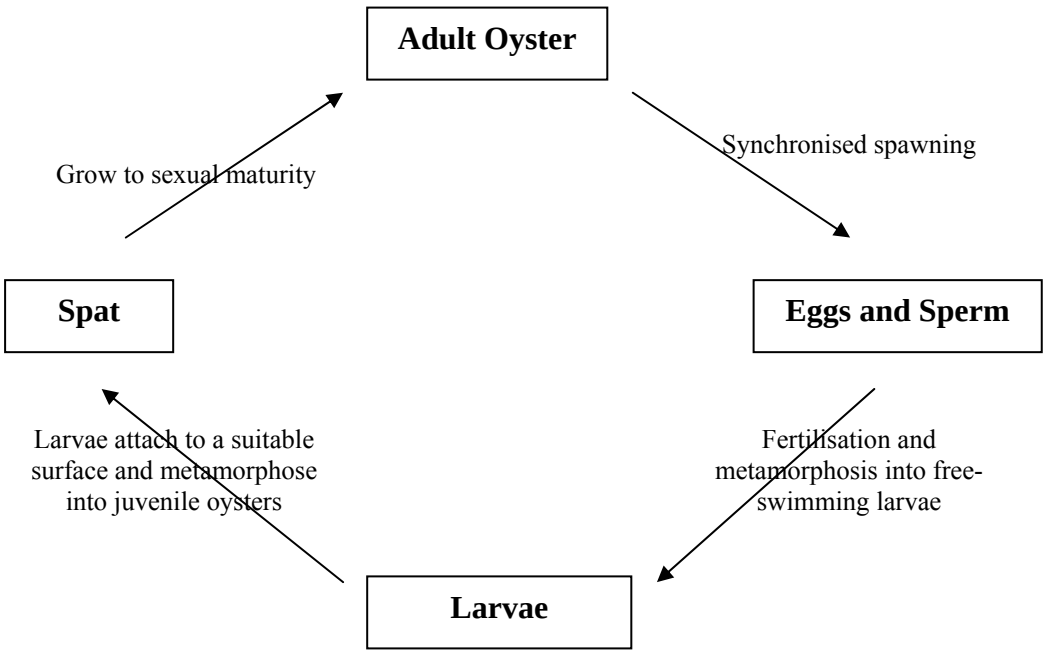


Figure 1.2 Simplified life-cycle of the Pacific oyster (*Crassostrea gigas*).

1.2.3 Ecology

The Pacific oyster originated in Japan and was accidentally introduced to New Zealand in the late 1960's (Dinamani 1971) rapidly becoming established, throughout northern New Zealand. An illegal transplantation into the Kaipara harbour in the mid-1970's (Tong 1989) led to the establishment of highly productive populations. The Pacific oyster out-competed the native New Zealand rock oyster (*Crassostrea glomerata*) in the mid intertidal zone and was soon adopted as the preferred species by the oyster farming industry (Wyborn 1980, Dinamani 1987). Oyster spat collections in the Mahurangi harbour, conducted by the New Zealand Ministry of Agriculture and Fisheries, showed the rapidity of establishment with the ratio of rock to Pacific oysters 1000:1 in 1972, 1:1 in 1977 and 1:4 in 1978 (Dinamani 1991). While the oyster can tolerate the sediment loads frequently encountered in the estuaries and inlets where oyster leases exist in New Zealand, it still requires a hard surface for initial settlement. The presence of other oysters appears to stimulate the settlement of oysters so rocky shores can become overcrowded with dense aggregations of oysters growing upon one another.

1.3 Feeding and Digestion

Knowledge of the mechanisms of feeding and digestion in Pacific oysters is incomplete yet is important for effective management of the species either on farms or in the hatchery. At the most basic level, suspension feeding can be divided into three processes: 1) production of currents to pass water across the feeding structures, 2) removal of particles from the water and 3) transport of particles to the mouth (Bernard 1974, LaBarbera 1981, Ward 1996). Much of the literature on bivalve suspension feeding has used the mussel, *Mytilus edulis,* as the model yet the mechanisms of suspension feeding have remained contentious. Riisgård (2001), for example, comments that "heaps of conflicting data may pose an insurmountable barrier for a meaningful understanding of bivalve filter feeding" (p. 275). When considering the less studied *Crassostrea* spp. the applicability of general concepts of the feeding mechanisms must be reconsidered as *M. edulis* has flat, homorhabdic ctenidia as opposed to the plicate, heterorhabdic ctenidia of *Crassostrea* spp (Ward *et al.* 1993). Consequently the following discussion refers to studies of *Crassostrea* spp. where they are available.

1.3.1 Feeding currents and regulation

To remove matter from suspension, bivalves must draw the ambient water between the valves. To achieve this, ciliary action on the ctenidia produces a complex pattern of water flow between the valves and through the ctenidia. In *C. gigas,* water is drawn in along the ventro-posterior shell edge and exhaled along the dorsal shell edge within the vicinity of the adductor muscle (Figure 1.3). On entering the mantle cavity, the water flow courses between the ctenidia allowing for respiratory exchanges and the capture of particles from the water. The water currents across the ctenidia have been demonstrated by Ward (1996) and Ward *et al.* (1998 and, in particular, the video footage of 2000) showing a flow perpendicular to the ctenidia surface "produced mainly by the lateral cilia, [an anterior flow] more difficult to explain, but might be the result of several factors, [and a dorsally directed component] probably generated in part by cilia on the frontal surfaces of the principal filaments" (p. 220, Ward 1996). Particles moving in the anterior-ward flow are drawn toward the ctenidial filaments and consequently make contact at a low approach angle (30^{o} or less) and become entrained in the flow of the frontal cilia (Ward 1996, Ward *et al.* 1998).

Simple observation of feeding oysters shows that the water flow between the valves can be controlled with variation in shell gape, and shielding by the tentacles of the inner mantle fold (pers. obs., Yonge 1960, Jørgensen 1990). Jørgensen (1990, 1996), argued that shell gape was the only control of water flow, with suspension feeding bivalves being filter pumps operating "in the absence of physiological mechanisms for regulating water pumping" (p. 89, Jørgensen 1990). However, researchers have shown that pumping rate changes in response to environmental conditions. Winter (1978), for example, showed a relationship between beat frequency of cilia and temperature, which in turn regulates pumping rate, in *C. gigas.* Similarly, His (1982) demonstrated with *C. gigas* that size effects pumping rate (4.9 l h^{-1} for 20g individual; 9.04 l h^{-1} for 40g individual and 14.08 l h^{-1} for 120g individual), suggesting that ctenidia surface area, and thus cilia number will regulate maximal pumping rates. However, Jørgensen argues that such responses fit with the concept of a "leaky, viscous, constant force pump" (p. 89, 1990) that is adapted to the concentrations of food "that prevail in the biotope during the productive seasons of the year" (p. 287, 1996).

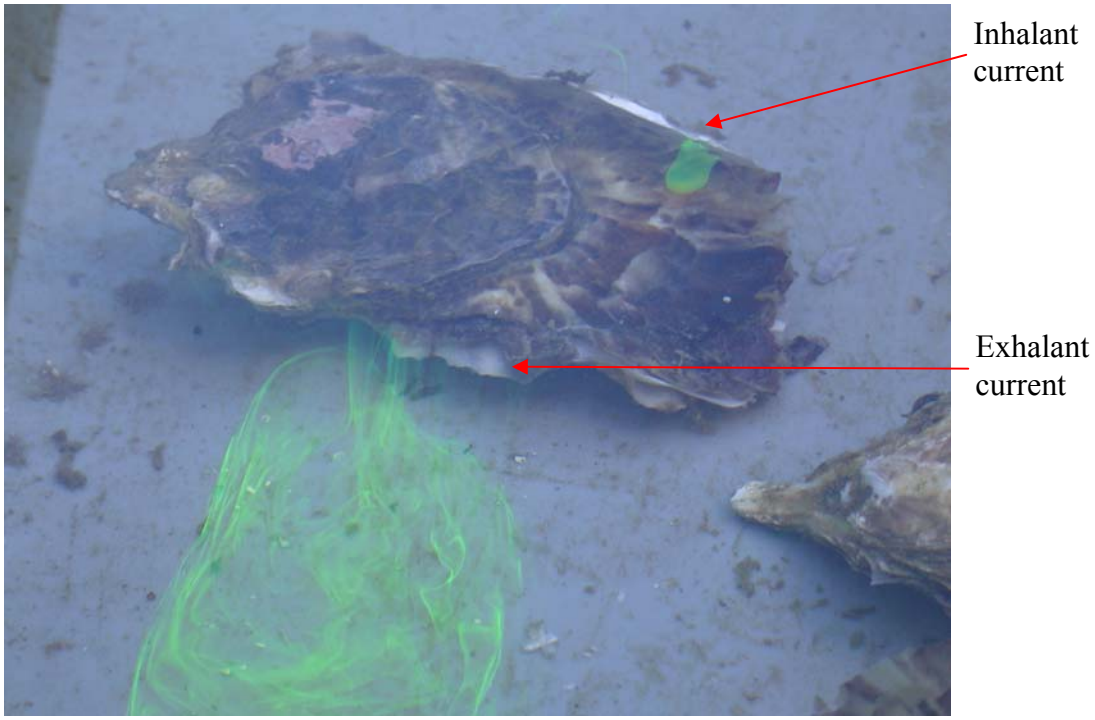


Figure 1.3 Location of the inhalant and exhalant feeding currents in a Pacific oyster. The current has been visualised with Fluorescein dye while the oyster was pumping in a tank of static seawater. (Photo credit T. Bootten)

Conversely, many authors propose that our knowledge of suspension feeding in bivalves has developed to an extent where 'filter pump' is "no longer an adequate short-hand descriptor of the bivalve feeding apparatus" (p. 5, Bayne 1998). This has been evidenced by a number of studies which have demonstrated oyster regulated responses to environmental stimuli. For example, Wilson (1983) showed increasing concentrations of the microalgae *Isochrysis galbana* caused *Ostrea edulis* to increase pumping up to a maximal concentration of 23.8 cells μl^{-1} after which further increases in algal concentration caused reduced pumping. Barillé *et al.* (1993) have also noted the *C. gigas* can regulate "gill leakage" in response to seston concentration so only larger particles are retained when concentrations are high. Ward *et al.* (1998), propose that the control of the width of interfilamentary spaces (as demonstrated by Galstoff 1964, Ward *et al.* 1994) could change the hydrodynamics within the ctenidium. Such control would change both the rate and efficiency of pumping for a given effort, allowing the oyster to respond to particulate load and quality.

1.3.2 Particle capture

The capture and transport of particles on the ctenidia could result from a number of factors, including ciliary action (Moore 1971, Owen 1974), hydrodynamics (Jørgensen 1996) and mucous (Beninger *et al.* 1991). The hypothesis that particles are captured by the ciliary mesh of the ctenidia (in particular the eu-latero-frontal cilia), and hence capture efficiency is linked to the mesh size (0.6 x 2.4 or 4.8 μm in *M. edulis* (Owen 1974)), has lost credibility over recent years. Jørgensen (1981, 1982, 1989) argues that particle capture is governed by low Reynolds number principles. Thus, particles don't have to come into contact with the cilia of the ctenidia, but merely become entrained within the boundary flow created by ciliary action. However, this theory was based upon studies using excised portions of gill filament (e.g. Jørgensen 1982, Nielsen *et al.* 1993, Silverman *et al.* 1996) which could show altered functioning and/or hydrodynamic processes around the excised portions. Endoscopic studies of intact feeding bivalves (e.g. Beninger *et al.* 1992, Ward 1996, Ward *et al.* 1994, 1998) have been used to address these concerns.

Ward and co-workers (1998) proposed a new model for particle capture based upon their observations using endoscopy. They suggest that species specific combinations of forces act as the ctenidia actively creates the conditions for 1) direct interception, 2) hydrodynamic entrainment, and 3) mucociliary transport of particles. The conditions for direct interception are created as the combination of anterior, dorsal and perpendicular

water flows leads to particles approaching the ctenidia at a low angle (approximately 30°). The low approach angle leads to a reduced boundary layer, caused by streamline compression, and is at least twice as likely to result in particle interception compared to a 90° approach angle. Hydrodynamic entrainment is facilitated by the laterofrontal cirri which create vortices between the ctenidial filaments which redirect more particles onto the frontal cilia. Mucociliary transport operates on the frontal edge of the filaments where the combination of hydrodynamic entrainment by the frontal cilia and mucus adhesion. In the case of the plicate ctenidium of *Crassostrea* spp. there is a further localised flow from the plical crest toward the primary filaments, at the base of the plicae. Particles are either entrained in a ventrally directed flow along the plical crest or a dorsally directed flow along the primary filaments. Captured particles are transferred to the mucus slurry of the dorsal groove or mucus string of the ventral edge where they are transported anteriorly (Ward et al. 1998).

Mucus masses, transported by ciliary action, are considered essential for the capture and transport of particles and appear to be specialised for their role (Beninger *et al.* 1991). For example, Beninger and Dufour (1996) reported that in *C. virginica* the ventral groove and psuedofaeces had viscous, acid mucopolysaccharides while, in the dorsal groove and entering the mouth, lower viscosity neutral mucopolysaccahrides occurred. This is similar to the mucus characteristics observed by Ward *et al.* (1997) in the dorsal and ventral grooves of *C. gigas*. Examining five bivalve species, Beninger and St-Jean (1997) reported common principles of mucociliary transport across bivalve species. Chiefly, that variation of mucus type and viscosity occurs with position on feeding structures. "There thus appears to be a specialization of mucus type corresponding to functional specialization of the various pallial organs in suspension feeding bivalves." (p. 389, Beninger & St-Jean 1997).

1.3.3 Retention efficiency and particle selection

Particles can be retained with considerable efficiency by bivalves. Héral (1985), for example, showed 100 % retention of particles over the size range 6 - 10 µm by *C. gigas*. For the heterorhabdic ctenidia found in oysters it is generally thought that 'efficient' particle capture begins in the 4 to 5 µm size (Møhlenberg & Riisgård 1978, Riisgård 1988).

Retention efficiency may be modulated by particle size. For example, Jørgensen (1989) reported 90 % retention efficiency of 5 µm particles in *C. virginica* and 4.5 µm particles in

O. edulis, but only 50 % retention efficiency of particles 2.5 µm (*C. virginica*) and 2µm (*O. edulis*). Concentration may also affect particle retention efficiency (Loosanoff & Davis 1963, Wilson 1983, Heral 1985, Barillé *et al.* 1993). For example, Barillé *et al.* (1993) found that retention of 3 - 4 µm particles by *C. gigas* reduced with increasing seston load from a retention efficiency, for 3.92 µm particles, of 83.99 % at 11.06 mg l^{-1} seston load, to 65.33 % at 27.65 mg l^{-1}. Smaller particles, including bacteria, are retained by bivalves, although the efficiency and contribution of retained bacteria is unclear from the literature. Crosby *et al.* (1990) for example, reported that *C. virginica* fed cellulolytic bacteria had an assimilation efficiency of 52.5 % and McHenry and Birkbeck (1985) found radio-labelled *E. coli* were cleared by *O. edulis*, especially when in the presence of the microalgae *Tetraselmis suecica*. In contrast, Dupuy *et al.* (2000a, b) found that *C. gigas* could not significantly retain picoplankton or bacteria. Instead they hypothesised that *C. gigas* "access the strong bacterioplanktonic production through [consumption of the] hetero/mixotrophic protists" (p. 227, Dupuy *et al.* 2000a). Indeed Le Gall *et al.* (1997) have provided experimental evidence of *C. gigas* retaining and ingesting a ciliate that had been fed on cyanobacteria. This suggests that ciliates may provide a link between the abundant bacterioplankton and suspension feeders.

Bivalve stomach contents do not fully represent the particulate matter composition of the ambient seawater in which they live (e.g. Grave 1916, Morse 1944, Cognie *et al.* 2001). The mechanism(s) and site(s) of particle selection have been the subject of much debate. Bernard (1974) suggested that an element of selection occurred on the ctenidia where "Over stimulation of the filaments… will cause the release of the heavier rejectory mucus" (p. 8). Retained food particles are moved to the palps via the food grooves where the "palp lamellae are always closely appressed" (p. 7, Bernard 1974). Bernard (1974) argues that such positioning of the ciliated palp surfaces leads to a sorting of food particles and, consequently, the mucus entering the buccal cavity has a dry weight four-fold higher than that of the food grooves.

Shumway and co-workers (1985) observed three selective mechanisms in bivalves clearing mixed cell suspensions. These were (i) preferential clearance on the ctenidia, (ii) pre-ingestive selection on the labial palps, and (iii) differential absorption in the gut. While Shumway *et al.* (1985) concluded that these results suggested some organisms may be quantitatively more important in diet than relative abundance suggested, they made no conclusions on the nutritional merit of such selective processes.

Selection, based upon the qualitative aspects of food items, has been reported in numerous studies (Iglesias *et al.* 1992, Barillé *et al.* 1997, Bougrier *et al.* 1997, Bacon *et al.* 1998, Hawkins *et al.* 1998a, Ibarrola *et al.* 2000, Cognie *et al.* 2001 Velasco & Navarro 2002). Ward *et al.* (1997), observed the differential selection of living versus detrital material in *C. gigas* and noted that the higher quality, living cells were directed toward the dorsal tract for further processing at the palps, while lower quality, detrital material was directed to the ventral tract for rejection as pseudofaeces.

Ward *et al.* (1997) also observed feedback mechanisms whereby high quality particles were rejected after extended periods of feeding, suggesting that "gut fullness might influences (sic) rejection of even good particles" (p. 132). Seston composition and concentration has also been reported to effect food selection with Urban and Kirchman (1992) finding kaolinite clay disrupted selective processes in *C. virginica* and Barillé *et al.* (1997) reporting disrupted feeding processes in *C. gigas* when seston concentrations exceeded 90 mg l^{-1}. However, Cognie *et al.* (2003) noted that selective processes on the ctenidia were also dependant on the ability of *C. gigas* to handle the potential food items. Large particles, for example, have to be sorted primarily on the labial palps as they cannot fit the selective mechanisms of the ctenidial filaments. Similarly, Bougrier *et al.* (1997) speculated that observed differences in selection by *C. gigas* feeding on different microalgae was due to differences in algal shape and flexibility, which either inhibited or enhanced retention mechanisms, as opposed to any specific selective mechanism.

The labial palps are likely to be important in sorting food particles (Loosanoff 1949, Bernard 1974, Shumway *et al.* 1985, Ward 1996). While Jørgensen (1990) claims some food is passed directly from the ctenidial margin to the mouth, direct observations by Ward (1996), and conclusions by other authors (such as Bernard 1974) suggest all food passes via the labial palps. It has been suggested that species with less complex homorhabdic ctenidia (e.g. *M. edulis*) conduct more sorting on the palps than species with heterorhabdic ctenidia (e.g. *C. gigas*) that sort more on the ctenidia (Ward *et al.* 1997). More recently, Milke and Ward (2003) found that particle processing time on the palps of mussels was much lower than on the palps of oysters, primarily due to a more efficient sorting structure on the mussel palps. However, Milke and Ward (2003) concluded that while the additional sorting was a rate limiting step, the enrichment of food it caused was more energetically efficient than processing mixed assemblages in the gut. This supports the findings of

Barillé *et al.* (1997) who observed that net absorption efficiency increased as the organic content of ingested particles increased.

1.3.4 Ingestion and digestion

Mucus masses are directed into the mouth and progress along the digestive tract, where they are subjected to extracellular and intracellular digestive processes. As noted above, Shumway *et al.* (1985) found that these digestive processes could enable selective absorption (post-ingestive selection) of food items in *C. virginica* fed three different microalgal species. Specifically, the cryptomonad flagellate *Chroomonas salina* was not preferentially retained or rejected in the pseudofaeces yet did not occur in the faeces unlike the diatom *Phaeodactylum tricornutum* which was over-represented in the faeces. This evidence of selective digestion has been more recently confirmed by Cognie *et al.* (2001) in *C. gigas* fed natural microphytobenthic assemblages where the retention efficiency was not significantly different between the four dominant species.

Changes in food quality and quantity have been reported to induce corresponding changes in bivalve clearance rate, selection efficiency, absorption efficiency and digestive enzymes, by Ibarrola and colleagues (Ibarrola *et al.* 1998a, 1998b, 1999, 2000, Iglesias *et al.* 1992, 1996, Navarro & Iglesias 1993, Navarro *et al.* 2003) predominantly with the infaunal cockle *Cerastoderma edule*. Both acute and acclimated changes were identified for absorption rate, gut retention time, and digestive processes (such as, production rates of different enzymes). These studies found evidence that physiological controls operate to maximise energetic gain from the available food. In contrast, Gardner (2002), and previously Gardner and Thompson (2001), reported that they could not find a relationship which suggested that clearance rate could be modulated to enhance absorption efficiency in three species of mussel. This was despite finding that seston variation explained 15 – 20 % of variation in clearance rate, and 52 – 59 % of variation in absorption efficiency. The use of natural seston in Gardner's work, as opposed to artificial mixes of microalgae and sediment in much of the work by Ibarrola and colleagues, and coarse measures such as particulate organic matter as opposed to counts of individual feed species, may have made it difficult to detect significant enhancement of absorption efficiency. Alternatively these results may reflect the variation between infaunal and epifaunal habit.

Differences in the strategies of infaunal and epifaunal bivalves to maximise absorption efficiency have previously been demonstrated by direct comparison. For example, Hawkins *et al.* (1990) reported that, compared to *M. edulis*, *C. edule* ingested 3 - 4 times as much food per hour, had gut contents 5 - 6 times greater and a gut passage time 2.5 times longer. Such strategies may also differ between epifaunal bivalves with Hawkins *et al.* (1998a) reporting that, in the bay of Marennes-Oléron, *C. gigas* was not as efficient as *M. edulis* at digesting or assimilating organic material. Similarly, Gardner (2002) found variable responses to seston quantity and quality between co-occurring mussel species although ultimately these differences led to similar absorption efficiencies.

Seasonal variations in seston quality and bivalve nutritional requirements may lead to seasonal variations in nutrient acquisition. Hawkins and Bayne (1984, 1985) have examined the responses of *M. edulis* to seasonal variations in food utilisation and concluded that the "significant mechanisms effecting… regulation of nutrient acquisition" (p. 233) were the ingestion rate and gut organic content (Hawkins & Bayne 1984). While they suggested, in 1984, that absorption efficiency was rather insignificant in influencing nutrient acquisition, the conclusions of their 1985 work were that seasonal changes in nutrient requirements were the key determinants of absorption and net growth efficiency, if the nutrient supply is standardised.

In *C. gigas* the link between ingested food quality and absorption efficiency has been further elucidated by Barillé *et al.* (1997) who found that absorption efficiency increased with increasing organic content. This suggests that in *C. gigas*, absorption rate is constrained by digestive processes which respond to the quality and composition of ingested food. This is supported by the findings of Brock (1989) that *C. gigas* feeding on *Tetraselmis suecica* were cellulase competent with hepatopancreas extract lysing algal cell walls where extracts from starved individuals could not. However, similarity in the action of cellulases from the oyster extract with those from bacteria suggested a possible link to a bacterial flora. Barillé *et al.* (1997) have also suggested that bacterial mediation, as described by Crosby *et al.* (1990), may be important due to the large detrital content of food consumed when *C. gigas* attained absorption efficiencies greater than 50 %.

1.3.5 Bacteria and digestive processes

The role of bacteria in oyster nutrition is not well understood. Bacteria were thought not to require the symbiotic relationships they utilise in the terrestrial environment as, in the marine environment, conditions are generally ideal for bacteria to flourish (Harris 1993). Consequently, studies that have detected the presence of bacteria in bivalves have often assumed that they will be digested (Crosby *et al.* 1990). Indeed, some strong evidence, in the form of radio-label uptake, has been presented to demonstrate bacterial digestion in a range of bivalves (McHenry & Birkbeck 1985, Crosby *et al.* 1990). It has also been suggested that the primary role of lysozyme in bivalves is for the digestion of bacteria (McHenry *et al.* 1979). Other researchers have also demonstrated the nutritive value of bacteria (e.g. ZoBell & Feltham 1938, Seiderer *et al.* 1984, Douillet & Langdon 1993), but whether this nutrition was attained by direct digestion of bacteria, or assimilation of bacterial by-products, is less certain. Crosby and Peele (1987), for example, noted that the assimilation of cellulose by *C. virginica* was significantly reduced in oysters treated with antibiotics, suggesting that bacteria may mediate nutrient acquisition. Similarly, Brock (1989) reported that "cellulolysis by hepatopancreas extract from the fed oysters [*C. gigas*] resembled that of the bacterial cellulase" (p. 157).

Given the evidence that bacteria could mediate nutrient transfer, particularly from detrital sources, the existence of a resident, commensal flora does not seem unreasonable. Yet Simon and McQuaid (1999) reported that the association between mussels (*Perna perna*) and their enteric bacteria was transitory and incidental. In contrast, Kueh and Chan (1985) reported that the bacterial flora in shellfish (including *C. gigas*) was mainly derived from the external environment in the stomach but was "replaced by a more indigenous population which dominates the lower digestive tract" (p. 41). Similarly, Colwell and Liston (1960) speculated that oysters had an indigenous microflora, although they did not determine if this was specifically associated with the gut.

Olafsen *et al.* (1993) have reported that healthy *C. gigas* can contain bacteria in the haemolymph and tissues which can be considered commensal. However, only the crystalline style associated bacteria are, generally, considered to have a commensal or obligate relationship (Simon & McQuaid 1999) with spirochaete bacteria frequently identified in the style (Dimitroff 1926, Bernard 1970, Tall & Nauman 1981). The crystalline style is known to provide a viscous environment containing a wide array of enzymes and is an effective emulsifying agent for gut contents in the vicinity of the gastric

shield (Kristensen 1972, Bernard 1974). The spirochaetes, usually assigned as *Cristispira* sp., are thought to contribute to the enzymatic activity associated with the style. Judd (1977) for example, has shown *Cristispira* sp. in *Amphidesma australe* to effectively digest carbohydrates in the style, suggesting that these spirochaetes may contribute to digestion through their processes within the style itself. The contribution any commensal flora may make by digesting food items when the tide recedes, feeding stops and the style dissolves, is as yet uninvestigated.

Reviewing the literature on bivalve feeding shows that, while our understanding grows, so does the realisation of how complex the feeding systems appear to be. The more attempts that are made to produce effective, predictive models of bivalve feeding, especially in relation to the carrying capacity of water bodies, the greater our understanding of the complexities and differences at species, temporal and spatial scales. Hyun *et al.* (2001) in attempting to create a population dynamics model for *C. gigas* in Korea found that chlorophyll *a* was an inadequate measure of food available to oysters, yet measured labile lipid + protein + carbohydrate suggested excess food was available. Hyun *et al.* (2001) thus concluded that much of the potential food "is not utilized by oysters and that little of it is present as chlorophyll containing cells" (p41). In contrast Ren and Ross (2001) created a model for *C. gigas* where growth was regulated by phytoplankton concentration estimated by chlorophyll *a*. Such contrast may reflect the differences in the areas used to test the models (Korea versus France) and consequently the manner in which the populations have adapted their feeding habits to the local conditions. The implication appears to be that *C. gigas* has the capacity to alter its feeding behaviour to attain nutrient in the most energetically favourable manner possible. Microalgae may thus be the dominant source of food but, as microalgal populations wax and wane other sources must be available. The largest potential resource is the picoplankton (< 3 µm cell size) which includes picoeukaryotes, cyanobacteria and heterotrophic bacteria. The importance of this resource, either as a substantial or minor component of overall oyster nutrition, remains uninvestigated but could have significant implications for the management of *C. gigas* in both natural and artificial settings either as a commercial prospect, bioremediator or invasive pest.

1.4 Aims

The research presented in this thesis investigated three interrelated aims. These aims were;

1. Investigate potential for the production of Pacific oysters using a pilot-scale, recirculating seawater hatchery for selective breeding.

2. Determine the efficiency with which picoplankton (< 3 μm) are retained by Pacific oysters *in situ* and whether any temporal variation occurs in response to changing nutritional demands.

3. Locate and characterise the bacterial flora associated with the Pacific oyster gut.

Chapter 2.0
Investigation of Pacific Oyster Breeding and Conditioning

2.1 Introduction

2.1.1 Oyster farming in New Zealand

Oyster farming in New Zealand began in the 1960's with the establishment of government trial farms in the Mahurangi Harbour and Bay of Islands. The New Zealand rock oyster (*Crassostrea* (formerly *Saccostrea*) *glomerata*) was cultivated on wooden or Fibrolite battens attached to long rails in the intertidal zone (Curtin 1968). This is still the dominant farming technique in New Zealand although Fibrolite is no longer used and some operators have begun using Netlon bags, Tooltec trays and longline ongrowing techniques.

The oyster industry in New Zealand was established primarily by small scale, hobby farmers. Inconsistencies with the natural spat supply meant the industry struggled. In an attempt to support the industry the use of hatchery technology for spat production was investigated by the New Zealand Ministry of Agriculture and Fisheries (MAF) (Curtin 1979). This simple hatchery was based upon American and European examples, using published techniques, such as Loosanoff and Davis (1963), and overseas expertise. The hatchery was successful in producing both New Zealand rock oyster and Pacific oyster (*Crassostrea gigas*) spat. However, at this time, the small scale, owner/operator nature of most of the industry meant that even basic hatchery technology was "beyond their [the farmers] financial means" (p. 15, Curtin 1979). At this time the introduced Pacific oyster was becoming established in New Zealand and the high productivity of the illegally established Kaipara Harbour population (Tong 1989) saw a shift away from the difficult, slower growing, New Zealand rock oyster.

By the late 1970's the New Zealand oyster farming industry had moved exclusively to Pacific oysters. Currently there are 830 ha of gazetted oyster farm leases in 14 harbours throughout northern New Zealand. These farms account for most of the Pacific oyster production in New Zealand (some farms exist in the northern part of the South Island). The ownership of oyster farm leases has consolidated in the last decade (Rennie 2002) with

four companies accounting for 43 % of all leases by 2002. These four companies have all made significant investments in processing facilities and the development of markets, leading the industry to $32 million in sales (2002), of which $15 million came from exports (McCallum 2004). However, the threat of spatfall failure still exists and the financial implications are now much greater.

For the oyster industry, reliance on the natural spatfall has occurred due to 1) the consistent supply of natural spat, and 2) the historical owner/operator nature of most farms. While the system of spat collection has become more refined to take advantage of the highly productive Kaipara Harbour, it has also reached a level of demand such that a spat failure could cripple the industry. A recent example was the summer 2000/2001 closure of the Kaipara Harbour due to a toxic algal bloom (*Gymnodinium catenatum*, MacKenzie & Beauchamp 2001), which postponed spat collection and created great anxiety within the industry.

An obvious solution to the threat of spatfall failure is to produce spat in a hatchery as has been done elsewhere (e.g. Pacific Northwest America, Mann 1984). As noted above, this solution was examined in New Zealand as a response to spat supply difficulties with the New Zealand rock oyster. The hatchery established by MAF was designed to utilise the natural gametogenic cycle, operating over the period of natural spawning and settlement, so as to minimise operating costs (Curtin 1979). The concept of having a hatchery facility as a 'back-up' in case of shortages of spat received some attention but the expense of establishing and maintaining a facility and trained staff made such an option undesirable for the industry. A commercial hatchery providing an annual supply of spat would, necessarily, be at a higher cost to the farmer. Until recently, attempts to establish hatcheries in New Zealand have all met with a similar lack of commitment to invest from the industry. The situation is similar worldwide with hatcheries struggling to maintain industry enthusiasm for their product in the face of natural spatfalls and/or low profit margins (Robert & Gérard 1999).

The costs of hatchery production can be offset, however, if the financial benefit of operation can be improved by, for example, improving stock quality. This has been the focus of long term stock enhancement programs such as the Molluscan Broodstock Program (USA) which has concentrated on genetic improvement to improve productivity (Hedgecock *et al.* 1997), and the New South Wales Fisheries program (Australia), which

has directed effort toward triploid production (Nell & Maguire 1998, Benzie *et al.* 2002, Nell 2002). Such programs use careful management of broodstock to improve the desirable characteristics of each generation of progeny. Generally, these characteristics relate to improving productivity, such as faster growth and lower mortality, but characteristics that enhance market desirability, and hence demand, can also be selected for (Brake *et al.* 2004).

2.1.2 Hatchery technology

An oyster hatchery exploits what is known of oyster life history in order to produce large amounts of spat. The development of hatcheries has allowed the establishment and continuation of industries in areas where oysters are outside of their physiological range (e.g. Pacific Northwest America; Mann 1984, Robinson 1992). At its simplest, hatcheries succeed by reducing mortality through the larval rearing stages (removing predators and minimising disease exposure) thus setting large numbers of spat for farm growout. The process becomes more complex as it is further refined. Selection of "good" oysters for broodstock, conditioning of broodstock to maximise gamete production, selected pair matings, culling of slow growing larvae, and remote setting of larvae, are some examples of refinements used to obtain a commercially more desirable product.

Figure 2.1 provides a simple overview of hatchery production which can be broken into five basic sections: microalgal food production, broodstock selection and conditioning, spawning and fertilisation, larval rearing and setting of spat. Whilst carried out routinely in hatcheries worldwide and thoroughly described in publications (Loosanoff & Davis 1963, Utting & Spencer 1991, Helm *et al.* 2004), they remain empirical due to our limited knowledge of the reproductive physiology of *C. gigas* (Chávez-Villalba *et al.* 2002b).

1) Microalgal food production. This component is critical to the success of a hatchery and usually entails the greatest operational expense (Borowitzka 1997). Microalgal species (such as the diatoms *Chaetoceros calcitrans* and *Thalassiosira pseudonana* and the flagellates *Isochrysis galbana* and *Pavlova lutheri*) are cultured to provide a disease free, bulk food supply that will maximise the success of the various stages. Culture procedures are based on the maintenance of axenic microalgal isolates (provided from commercial collections such as CSIRO Microalgal Research Centre, Tasmania, Australia) under conditions that allow for logarithmic growth. A system of batch culture, where isolates are divided into progressively larger volume culture containers, is the simplest to establish and

maintain. Batch culture is usually used by smaller hatcheries with more efficient continuous and semi-continuous culture systems more desirable as hatchery size grows (Borowitzka 1997). All microalgae produced must not only have the desired quantity of cells (nutritional potential) but they must be free of potential oyster pathogens and of a water quality (e.g. pH and salinity) comparable to the oyster culture tanks. The procedures for the mass culture of microalgae have been well described (Guillard 1975, Richmond 1986, Laing 1991).

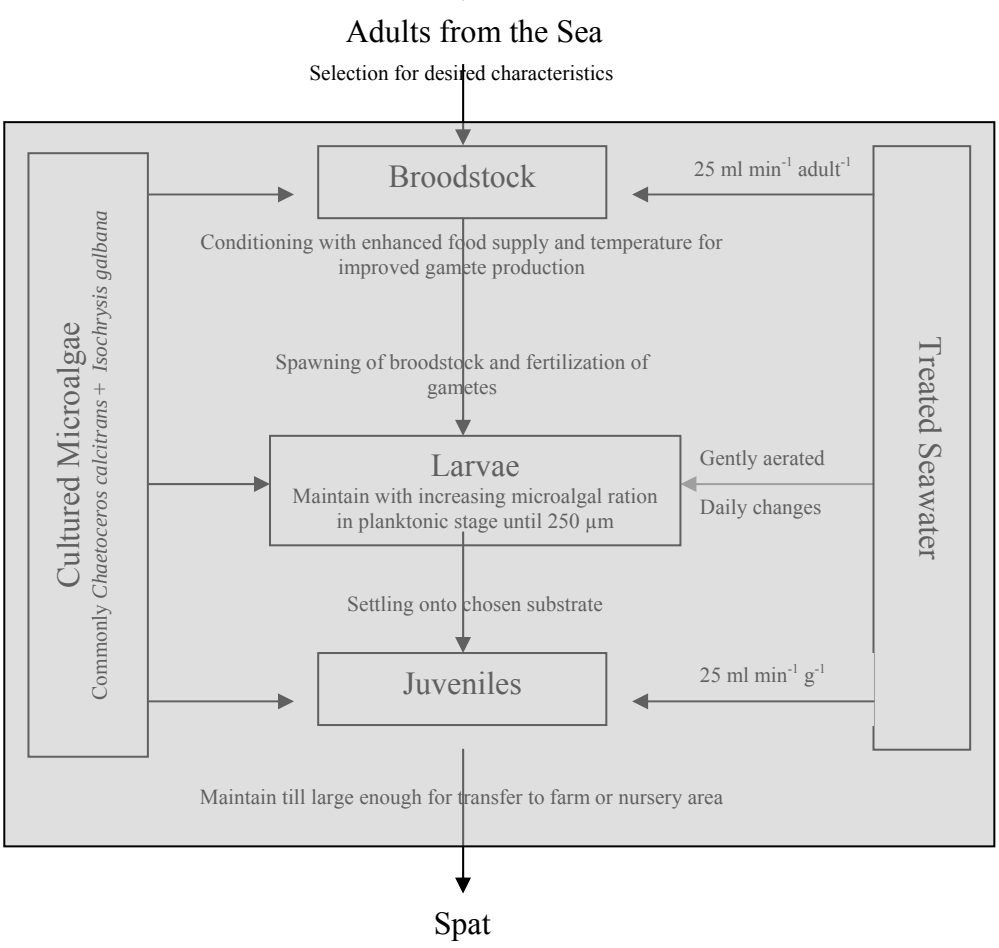


Figure 2.1 An overview of the processes for the hatchery production of Pacific oysters (*Crassostrea gigas*). This model assumes a flow through seawater system (modified from Utting & Spencer 1991).

Microalgal species selection for hatcheries is based upon 1) availability of stock cultures, 2) ease of culture and 3) nutritional composition. For bivalves the presence of 20:5n-3 and 22:6n-3 polyunsaturated fatty acids (PUFA) have been identified as being of particular importance (Volkman *et al.* 1989, Utting & Millican 1997). Generally, these fatty acids

are deficient in green algae compared to brown (Volkman *et al.* 1989) although greens may have proportionately greater quantities of total polyunsaturated fatty acids. Table 2.1 provides an overview of the composition of five microalgal species commonly used in bivalve hatcheries. Brown (1991) concluded that current knowledge of the ability of bivalves to digest carbohydrates was insufficient for microalgal nutritional quality to be judged either on carbohydrate content or sugar composition. However, Brown (1991) noted that there were significant differences in composition between species which may, in conjunction with other attributes, determine the nutritional quality of a species.

Table 2.1 Nutritional analysis of the different microalgal species used in the pilot-scale hatchery (from Brown 1991). [1] PUFA = Polyunsaturated fatty acids from Volkman *et al.* 1989. *C. calcitrans* and *T. pseudonana* are Baciilariophyceae *Isochrysis galbana* (T-iso strain) and *P. lutheri* are Prymnesiophyceae; *Tetraselmis suecica* is Prasinophyceae.

Component	**Species**				
	Chaetoceros calcitrans	***Isochrysis galbana***	***Pavlova lutheri***	***Thalassiosira pseudonana***	***Tetraselmis suecica***
Protein (% dry wt)	34	23	29	34	31
Carbohydrate (% dry wt)	6	6	9	8.8	12
Lipid (% dry wt)	16	20	12	19	10
All PUFA's (% dry weight)	33.7	41.3	42.0	52.6	59.5
PUFA 20:5n-3 (% dry wt) [1]	11.1	19.3	0.2	19.7	4.3
PUFA 22:6n-3 (% dry wt) [1]	0.8	3.9	8.3	9.4	Trace
Essential amino acids (% total amino acids/protein)					
Arginine	6.4	7.4	8.4	6.3	13.2
Histidine	1.9	2	2	1.6	1.8
Isoleucine	5.5	4.6	4.9	5.5	3.5
Leucine	8.2	8.7	8.1	8.4	8
Lysine	6.3	6	5.6	5.9	6
Methionine	2.6	2.6	3.2	2.2	2.3
Phenylalanine	6.7	6.2	6.6	6.8	5.9
Proline	5.6	5.9	5.7	4.8	4.7
Thneonine	4.5	4.5	4.3	5.2	4.1
Tryptophan	1.4	1.6	1.5	0.87	1.2
Valine	5.9	6.1	6.7	6.1	5.7
Sugars (% polysaccharides)					
Arabinose	0.2	7.1	11.7	0.19	0.9
Fucose	14.3	1	3.6	2.8	0.0
Galactose	20.5	8.8	12.9	5.4	15.7
Glucose	54.7	70.3	42.6	82.4	74.8
Mannose	2.0	5.9	13.2	5.0	3.0
Rhamnose	3.3	0.0	2.1	1.3	0.97
Ribose	3.3	3.8	3.5	1.4	4.5
Xylose	1.7	3.2	10.3	1.6	0.0

Whilst there are other examinations of the composition of microalgae (Whyte 1987; Viso & Marty 1993; Derrien *et al.* 1998) it is difficult to compare results due to the differences in methodology, predominantly that used for obtaining microalgae samples. Fernández-Reiriz *et al.* (1989) demonstrated this variability when following the levels of constituents of cultures sampled at different ages. Some large changes in proportional composition were noted such as the carbohydrate level in *Isochrysis galbana,* which ranged from 15.21 to 48.35 % organic weight for harvests at days 7 and 20 respectively.

2) Broodstock selection and conditioning. The selection of broodstock is usually based upon desired adult characteristics, such as fast growth or shell morphology (Hedgecock *et al.* 1997, Brake *et al.* 2004). However, to successfully breed from selected broodstock they must be in appropriate condition. The condition or stage of gonad development, particularly oocytes, will determine spawning, fertilisation and larval rearing success (Muranaka & Lannan 1984, Chávez-Villalba *et al.* 2002a & b). Egg quality can be assessed by various methods including biochemical analyses, histological examination and examination of a gonad smear (Steele & Mulcahy 1990, Lango-Reynoso *et al.* 2000, Ren *et al.* 2003). Appropriate gonad condition can be achieved by waiting until the gonads have reached maturity in the wild or, for more controlled production, the broodstock can be conditioned so their gonad reaches peak condition when the hatchery requires (Helm *et al.* 2004).

The process of broodstock conditioning exploits the direct relationship that exists between water temperature and gametogenesis in oysters (Muranaka & Lannan 1984). The basic technique is to combine elevated temperature (19 - 20 ^{o}C) with a suitable food ration to advance gametogenesis (Loosanoff & Davis 1963, Walne 1979, Lannan *et al.* 1980, Utting & Spencer 1991, Utting & Millican 1997, Caers *et al.* 2002, Chávez-Villalba *et al.* 2002b). The length of time, temperature regime, and food quantities required for conditioning, all depend upon gonad condition on arrival at the hatchery. Generally the requirements for conditioning are defined as a daily ration of 6 % of the initial dry meat weight in dry weight of microalgae and a constant water temperature between 20 and 22 ^{o}C (Utting & Millican 1997). For oysters collected in late winter or early spring gametes should be available after 4 - 6 weeks (Utting & Spencer 1991). However, if oysters are selected in late summer, a period of cooling, to stimulate reabsorption of unspent gametes and allow for the acquisition of suitable levels of storage metabolites, will be required before gametogenesis can be initiated (Loosanoff & Davis 1963, Shpigel *et al.* 1992). Under this

regime conditioning may take up to six months. Due to the time considerations and bulk of microalgal feed required, some assessments of hatchery production (such as Curtin 1979) consider broodstock conditioning an excessive burden and recommend obtaining in season broodstock.

3) The spawning of competent oysters can be induced by cycling oysters through temperature shocks (Loosanoff & Davis 1963, Chanley 1981), stimulation with stripped sperm (Galstoff & Smith 1932, Dupuy & Rivkin 1972, Helm & Millican 1977) and/or the injection of serotonin into the adductor muscle (Gibbons & Castagna 1984). Mechanical stripping of eggs and sperm is also commonly used but is fatal for the broodstock (Lannan 1980, Helm *et al.* 2004). Individual oysters are spawned in separate containers so eggs and sperm are kept separate until fertilisation is initiated by the operator, allowing specific pairings to be made if desired. This process helps ensure that future pairings avoid inbreeding depression and is essential for effective enhancement programs (Hedgecock & Sly 1990, Evans *et al.* 2004).

4) Larval rearing covers the period from fertilisation until the larvae start to become competent for settling. During the first 24 hours there is a rapid development of the eggs into "D-shell" larvae, with no feeding taking place. The next 14 - 20 days the larvae are free-swimming and feeding, growing from approximately 60 to 250 µm when they start to metamorphose. Feeding is generally started with a diet of *Isochrysis galbana* and *Chaetoceros calcitrans* (Helm & Millican 1977, Ponis *et al.* 2003) as *I. galbana* alone is deficient as a larval diet (Helm & Laing 1987). Larvae have been shown to select food items based upon size (Fritz *et al.* 1984) and can utilise dissolved nutrients and bacteria for nutritional benefit (Langdon 1983, Douillet & Langdon 1993). Gerdes (1983) has reported that *C. gigas* larvae can regulate their filtration rate in response to food concentration and composition, and filtration rate increases as size increases. As assimilation efficiency is constant relative to size, larvae require greater quantities of feed as they grow and a shift from a 2 species to 3 species microalgal diet has been recommended as larvae exceed 140 µm (Helm & Millican 1977). If larvae are not receiving enough nourishment the rate of development will be retarded but this will not necessarily result in a significant increase in larval mortality (Moran & Manahan 2004), although Powell *et al.* (2002) has reported a relationship between larval mortality and dietary lipid.

Larvae are generally reared in static cultures with water changes no more frequent than 48 hours apart to minimise handling stress (Helm & Millican 1977). Water changes require collection of the larvae from the outlet water, usually with sieves. This process allows size-selective culling of larvae; an effective tool in creating faster growing oysters (Collet *et al.* 1999), and also allowing densities to be lowered as larval size increases (Utting & Spencer 1991). At approximately 250 µm shell length the larvae become competent for settling and are transferred to a settling tank.

5) Settling oysters naturally metamorphose when they find an appropriate surface to settle upon. In hatcheries this surface ranges from oyster or scallop shell to PVC slats or plastic sheeting (Utting & Spencer 1991, Holliday 1996, Devakie & Ali 2002). As the production of 'single' oysters has become desirable, chemicals, such as epinephrine, have been used to induce metamorphosis without attachment to a substrate in *C. gigas* larvae (Coon *et al.* 1986). Oysters metamorphosed in this manner, or on shell chip, can be transferred to upwelling units for rearing until they reach a size suitable for the receiving farm (Rodhouse & O'Kelly 1981, Spencer 1988, Utting & Spencer 1991, Laing & Millican 1992). Settlement to sticks is preferred by some oyster farmers and the use of fibrolite or PVC slats allows easy removal of oysters, either at harvest time or prior to harvest, so final growout as singles can be achieved (Curtin 1979). In New Zealand the current farm culture systems are more amenable to oyster culture on sticks of some form, with bag or tray culture for final growout or the relaying of harvested oysters deemed too small for processing (Brown pers comm.[2]). Given the associations reported between parent oysters, their shell biofilm and larval settlement (Tamburri *et al.* 1992, Maldonado & Young 1996) stick substrates are usually conditioned in seawater prior to use in the settlement tanks (Curtin 1979). Anderson (1996) has identified the presence of leaching calcium hydroxide as a settlement cue for *Saccostrea commercialis* although Holliday (1996) found *S. commercialis* settlement wasn't as high on PVC slats with a lime and cement coating compared to without.

In the week after metamorphosis *C. gigas* spat undergo metabolic stress and embark upon a period of exponential growth, particularly shell growth (García-Esquivel *et al.* 2001). Growth and mortality during this period is reported to be governed by access to food, especially lipid and protein (Utting 1986, Laing 1995, Caers *et al.* 2000, García-Esquivel *et al.* 2001). To minimise mortality, maximise growth and keep hatchery costs down juvenile

[2] Simon Brown (Bay Oysters Ltd); reply to authors questions.

oysters are moved from cultured microalgae to natural seawater assemblages as soon as possible. For a hatchery that is setting spat on sticks this means a transfer of juveniles to nursery or holding leases approximately 2 weeks after the completion of settlement (Curtin 1979).

2.1.3 Aims

The work presented in this chapter relates to an investigation, conducted in association with Sanford Ltd and Bay Oysters Ltd, of the possibility of establishing a selective oyster breeding program. A pilot-scale hatchery was established with the following aims.

1. To investigate the potential for selective breeding using in-season oysters;

2. To investigate the conditioning of Pacific oysters for the production of out-of-season spat;

3. To investigate the suitability of the microalgal culture species and system for food production using clearance rate of microalgae by oysters as a de facto measure of acceptability.

2.2 Materials and Methods

2.2.1 Hatchery

2.2.1.1 Physical description

A pilot-scale hatchery was constructed in the grounds of the Mt Albert Research Centre in Auckland. The design was based upon the recommendations of Curtin (1979) with modifications to account for the limitations of the location. A building of approximately 8 x 6 m was divided into two multi-purpose rooms, the larger containing spawning, larval rearing and settling operations, while the smaller contained the algal rearing and conditioning operations. The two rooms were connected by a small area that contained the main seawater and air supply controls (Figure 2.2). A header tank, to warm and distribute filtered seawater through the hatchery, was housed on a platform above the larger room. Both rooms were insulated and the air temperature regulated by air conditioning units retro-fitted with Carel ir32 temperature controllers.

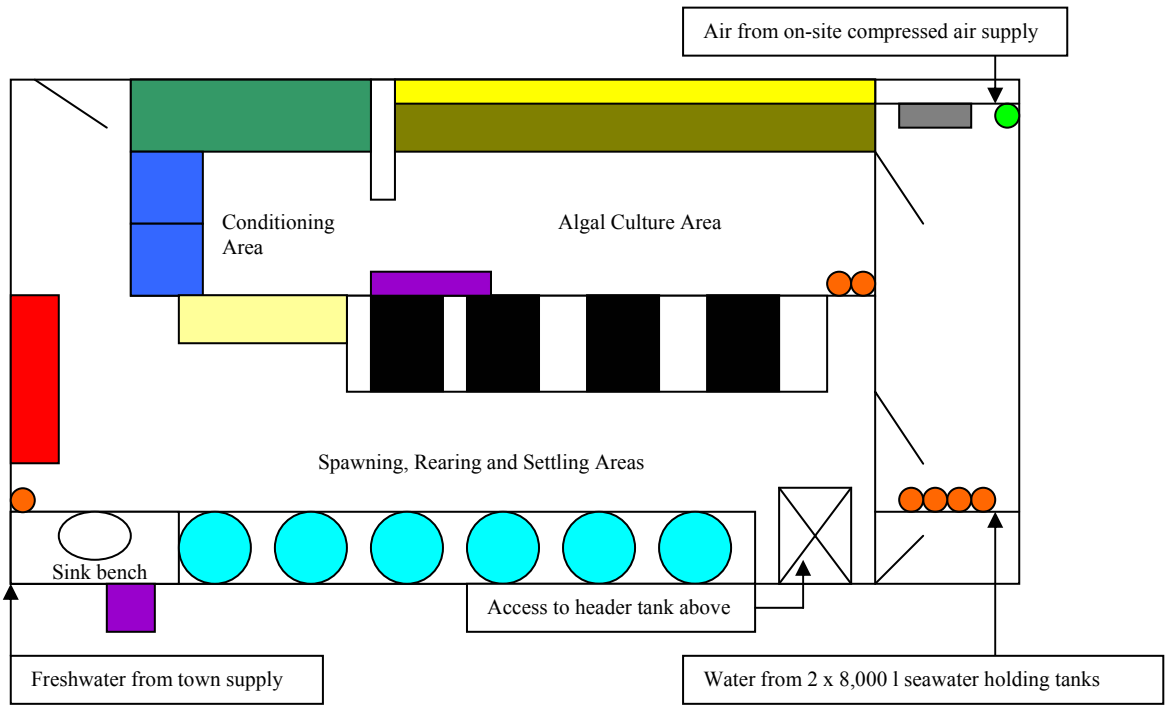


Figure 2.2 Floor plan of pilot-scale hatchery at the Mt Albert Research Centre. = conditioning system; = workbench and storage; = Algal culture racks; = Light bank; = Air conditioning units; = Air supply filtration and mixing; = CO_2 supply; = Water filters; = Larval rearing tanks; = Settling tanks; = dry bench; = Spawning bench. The overall floor area is approximately 8 m long by 6 m wide.

Seawater for the hatchery was delivered by tanker truck from a collection site at Wynyard Wharf, Auckland and held in storage tanks. As required, seawater was pumped from the storage tanks to the header tank via a series of filters (to a final 1 μm filter). Once in the header tank the seawater was continuously circulated through a UV sterilizer unit (35 W, Steriflow 369) and warmed to an appropriate temperature at which it could be distributed through the hatchery as required. The seawater used for microalgal culture was further filtered through 0.45 μm and 0.2 μm cartridge filters (as suggested by Lewis *et al.* 1988) to remove potentially contaminating particles. A compressed air supply (0.2 μm filtered) was distributed throughout the hatchery with CO_2 mixed (1 % v/v) into the supply for the microalgal cultures. Microalgal cultures were grown in batches to a maximum of 60 l d^{-1}. More detailed descriptions are provided in Appendix 2.1.

2.2.1.2 Microalgal species and culture

The selection of microalgal species for feeding to oysters in the hatchery was initially made by consulting a) published literature (Utting & Spencer 1991, Utting & Millican 1997), b) discussion with scientists experienced in oyster hatchery technology (particularly Peter Redfearn at the National Institute for Water and Atmospheric Research, Wellington, New Zealand and Mark Gluis at the South Australian Research and Development Institute, Adelaide, Australia) and c) the availability of axenic stock cultures. The five microalgal species selected for use in the pilot-scale hatchery were the bacillariophytes *Chaetoceros calcitrans* and *Thalassiosira pseudonana* (diatoms, high in eicosapentaenoic acid); the prymnesiophytes *Isochrysis galbana* and *Pavlova lutheri* (dinoflagellates, high in docosahexaenoic acid); and the prasinophyte *Tetraselmis suecica*.

The techniques for microalgal culture followed those of Guillard (1975), Lewis *et al.* (1986) and Laing (1991). All microalgal cultures were maintained using the F/2 culture medium (Guillard 1975) in which the stock cultures had been grown at the CSIRO Microalgal Research Centre. The components of this medium (Appendix 2.2) were held in bulk supply at 1 ^{o}C and added, in appropriate proportions, to the seawater prior to inoculation with microalgae.

Four culture lines were used for the batch production of microalgae;
1) The "inviolate line" which was derived from axenic stock cultures purchased from the CSIRO microalgal research collection. These cultures (100 ml) were maintained in static,

conical flasks (250 ml) and were used to establish working lines, by sub-culturing, as necessary;
2) The "working line" of cultures (200 ml) were maintained in static, conical flasks (500 ml). Sub-culturing, on a six day cycle, provided inoculum for the starter line as well as maintaining the working line;
3) The "starter line" cultures (1.5 l) were maintained in aerated, conical flasks (3 l) and harvested on a six day cycle to inoculate the bulk line;
4) The "bulk line" cultures (15 l) were maintained in suspended, aerated, plastic bags (70 μm, polyethylene (Davidson Plastics)) and harvested on a 6 – 7 day cycle for feeding to oysters. This resulted in a daily supply of 60 litres of microalgae at densities in excess of 10^6 cells ml^{-1}.
Procedures for the culturing of microalgal species are described in further detail in Appendix 2.3.

Microalgal densities were calculated from samples (50 μl) from which a subsample was loaded onto an improved Neubauer haemocytometer. The cells were counted in every second 0.004 mm^3 grid in each counting chamber using a light microscope (Nikon Labophot; 400x magnification). The average number per 0.004 mm^3 grid was calculated then converted to cells ml^{-1}. Motile cells such as *I. galbana* were fixed with one or two drops of 10 % (v/v) formalin prior to counting (Helm *et al.* 2004).

2.2.2 Oyster breeding and husbandry

2.2.2.1 Broodstock collection

Oysters (80 - 100 mm shell length) were collected from leases in Kerikeri Inlet, Kaipara Harbour and Whangaroa Harbour. Collections were made by Bay Oysters Ltd and delivered next day to the hatchery. The Kaipara and Whangaroa batches were collected with selection only for shell length. The Kerikeri batches were also selected for shell length but one batch was further selected by industry for morphological traits. The oysters were considered in-season at the time of collection but were held in the hatchery for four weeks prior to spawning due to technical problems.

2.2.2.2 Spawning and fertilisation

Spawning of broodstock was achieved using thermal shock stimulation (30 min alternations between 19 ^oC and 28 ^oC; Utting & Spencer 1991). Eggs and sperm were collected separately by spawning oysters in separate containers. Eggs were filtered

through a 50 µm mesh sieve into 20 l volumes to give a concentration of less than 1000 eggs ml^{-1}. Polyspermy was avoided by allowing eggs to be naturally spawned and incubated for approximately 60 minutes before sperm were added (Stephano & Gould 1988). Sperm were added (approximately 1 % v/v) to the eggs by passing them through a 35 µm sieve, and mixed with gentle stirring. A sample (0.5 ml) was observed for the presence of sperm on the eggs using a dissecting microscope (80 x magnifications) to ensure adequate sperm had been added.

Fertilisation and initial development was allowed to take place in the undisturbed 20 l buckets for at least 60 minutes. Buckets were then resampled (3 x 1 ml) and assessed for number and proportion of successfully fertilised eggs. The fertilised eggs were transferred into larval rearing tanks at a density less than 80 eggs ml^{-1}.

2.2.2.3 Larval rearing and settlement

Larval rearing was carried out at 25 ^{o}C. Since larvae do not begin feeding until 24 hours after fertilisation (Lucas & Rangel 1983), no food was added during this time and the tanks were kept static. Gentle aeration was initiated with the first supply of food approximately 24 hours after fertilisation. Feeding ration for larval cultures was that described by Utting and Spencer (1991) where optimal algal density in the larval rearing tanks is 125 cells μl^{-1} *Chaetoceros calcitrans* and 50 cells μl^{-1} *Isochrysis galbana*. Small (slow growing) larvae were culled with every water change (at 48 hour intervals). This also enabled the density of larvae to be controlled so a density of 1 larvae ml^{-1} was achieved by the time larvae were large enough to be retained on a 200 µm sieve. Larvae were transferred to settling tanks on reaching 250 µm, or when they were found to be settling on the sides of the rearing tank.

Figure 2.3 Location of Kerikeri Inlet and Whangaroa Harbour where on-growing and broodstock collections were made. Map courtesy of Frontier Mapping NZ Ltd.

Settlement of larvae took place in well aerated settling tanks that contained PVC plastic slats (1000 x 50 x 3 mm) that had been roughened and conditioned in seawater for at least 3 months prior to use. Water in the settling tanks was changed every day by draining through a 250 µm sieve until a negligible number of larvae were retained on the sieve. Food ration for settling and settled larvae was made up of four microalgal species with feeding events scheduled to maintain a density of 100,000 cells ml^{-1} (Curtin 1979). *Chaetoceros calcitrans* and *Isochrysis galbana* (50:50 v/v) were fed to the larvae early in the day followed by *Thalassiosira pseudonana* and *Pavlova lutheri*, as required, later in the day to maintain feed concentration. The *C. calcitrans*, *I. galbana*, *T. pseudonana* combination has previously been described as a very good reference diet for juvenile *C. gigas* (Knuckey *et al.* 2002).

2.2.2.4 On-growing of spat

On-growing of hatchery produced spat was carried out at either Kerikeri Inlet or Whangaroa Harbour (Figure 2.3) once the spat were 2 mm or greater in shell length. Samples of oysters (20 $stick^{-1}$) were measured at intervals, starting before they left the hatchery, to allow an estimation of growth rate. Oysters were transferred to Netlon bags or trays at the discretion of the farm management (between 10 and 11 months post-settlement for the batches monitored).

2.2.3 Conditioning of broodstock

Oysters were collected as broodstock for conditioning from Kerikeri Inlet by farm staff and sent to the hatchery by overnight delivery. Exceptions to this were the batch received in January which had been conditioned by the Cawthron Institute from oysters collected in Croiselles Harbour, Marlborough Sounds, and the October batches 1-4 (see Section 2.2.2.1). All broodstock were considered by the oyster farmers to be post-spawn when collected, excepting the fifth October batch.

Broodstock conditioning took place in a recirculating seawater system where six 45 l plastic bins, containing 12 oysters each, were fed with water from a sump to which overflow from the plastic bins returned (see Appendix 2.1 for further detail). Approximately 30 % of the seawater in the system was renewed, with filtered seawater from the header tank, every second day when the plastic bins were vacuumed clear of any debris. Salinity was maintained at 32 ± 1 ‰ and the dissolved oxygen level remained

relatively stable at 6.6 mg l^{-1}. Temperature was maintained at an average of 20.5 °C (range, 19.5 °C to 21 °C) during conditioning.

The conditioning regime followed that described by Utting and Spencer (1991) with food supplied as a microalgal diet with a daily ration calculated as 6 % of the initial dry meat weight of oysters in dry weight of microalgae. The conversions from dry weight to wet weight reported by Utting and Spencer (1991) were used for the microalgae, whereas the oyster dry weight was calculated by sacrificing three oysters on arrival and weighing the meat after drying at 80 °C for 24 hrs.

Assessments of oyster condition were made by visual inspection. To do this, oysters were placed in a bath of magnesium chloride (0.37 M in tap water) which caused persistent relaxation of the adductor muscle (Namba *et al.* 1995) thus allowing visual inspection of the meat. If numbers were sufficient oysters were sacrificed and the gonad dissected out to assess gamete development by light microscopy (80x magnification). The size (35 - 40 μm) and shape (rounded, slightly pear-like shape) of oocytes was considered indicative of readiness to spawn (Lango-Reynoso *et al.* 2000).

One batch of oysters (June) was assessed using a biometric condition index (CI) to determine if the conditioning regime was effective. To determine the CI each oyster was scrubbed clean and the whole weight (g) recorded. The oyster was then opened, drained (5 min) and the meat removed into a pre-weighed aluminium foil tray. Both shell and meat were then dried for 24 hours at 80 °C and their respective weights recorded. The CI was then calculated for each oyster using the formula described by Lawrence and Scott (1982) where;

CI = (dry meat weight x 100)/cavity volume

Cavity volume was calculated by subtracting the weight of the valves from the weight of the intact oyster as described by Lawrence and Scott (1982).

Spawning was attempted at intervals using a thermal shock stimulation where the oysters were cycled between a lower temperature (19 °C) and a higher temperature (26 °C) at 30 minute intervals as described in Section 2.2.2.2 above. Frozen sperm was used as a stimulant if the oysters appeared responsive but were not releasing gametes after the first

one or two thermal cycles. A successful spawning was defined as the release of gametes from at least 1 male and 1 female oyster. However, the success of conditioning was determined as the production of settled spat from any given spawning event.

Strip spawning was used on a single occasion. In this case the oysters had the top shell removed and a small sample of gonad was observed (80x magnification) to determine sex. Each oyster was removed from the shell and the gametes stripped by making many parallel incisions along the gonad with a sterile scalpel. The gametes were released from the gonad by this action and drained into a 5 l bucket of 0.2 µm filtered seawater, in a 24 °C water bath. The oyster was finally rinsed with 0.2 µm filtered water to flush remaining gametes into the bucket. Gametes were combined for fertilisation as described in Section 2.2.2.2.

2.2.4 Clearance of microalgae from suspension

The consumption of microalgae by oysters was investigated by both direct observation of oysters feeding in the conditioning system and controlled experimentation. The rates of clearance were determined, using the following methods, for each microalgal species (Table 2.2).

Table 2.2 Microalgal species used in the clearance rate experiments. References; [a] = Brown *et al.* (1997), [b] = Brown (1991), [c] = Helm *et al.* (2004), [d] = Utting & Spencer (1991).

Microalgal species	Average cell size (µm)	Cell volume (μm^3)	Organic weight ($\mu g\ cell^{-1} \times 10^6$)
Chaetoceros calcitrans	3-6[a]	5[b]; 35[c]	7[d]
Isochrysis galbana	3x5[a]	51[b]; 40-50[c]	19-24[d]
Pavlova lutheri	4x6[a]	91[b]; 40-50[c]	19-24[d]
Thalassiosira pseudonana	4-5[a]	32[b]; 45[c]	22[d]

Oysters from the conditioning system (80 – 100 mm shell length) were cleaned and placed in buckets (5 l) filled with filtered (0.45 µm) seawater (20 ± 1 ^{o}C). Each bucket was gently aerated and, after 30 minutes, microalgae were added. For each treatment, four buckets were used – three experimental and one control. To each experimental bucket one oyster was added, while the control bucket contained no oyster, allowing sedimentation to be assessed (Equation 3). Clearance rates could thus be calculated with correction for sedimentation (Equation 2). Samples (1 ml) were removed hourly from each bucket and cell concentration estimated by measuring sample absorbance (450 nm, Shimadzu UV-

visible spectrophotometer) in a 1 cm path length, plastic, disposable cuvette. Sampling ended after six hours.

A standard curve of absorbance (A_{450}) was produced for each species by measuring mean absorbance at known cell concentrations. Cell concentration from samples could be calculated by the equation;

$$\mathbf{C\ (cells\ ml^{-1}) = A_{450}/m - b} \qquad \text{(Equation 1)}$$

Where C = cell concentration, A_{450} = absorbance at 450 nm, m = slope of the standard curve, and b = intercept of the standard curve.

The clearance rate (CR) was calculated using the following equation based on Coughlan (1969);

$$\mathbf{CR\ (cells\ ml^{-1}\ h^{-1}\ oyster^{-1}) = [((C_0 - C_x) - s) / t]} \qquad \text{(Equation 2)}$$

Where C_0 = initial algal concentration (cells ml^{-1}); C_x = algal concentration at time x (cells ml^{-1}); t = time elapsed (hours); s = sedimentation rate (cells ml^{-1} h^{-1}) which was calculated as;

$$\mathbf{s = (C'_0 - C'_x)/t} \qquad \text{(Equation 3)}$$

Where C'_0 = initial concentration in the control bucket (cells ml^{-1}) and C'_x = concentration at time x in the control bucket (cells ml^{-1}).

2.3 Results

2.3.1 Spawning and husbandry of oysters

In-season oysters were collected as being either selected, or not, for morphological characteristics. Spawning, larval rearing and on-growing from the different batches of broodstock then allowed comparison of select, or not, batch performance. All four batches of in-season broodstock successfully produced spat. The numbers of spat produced from each batch of oysters were variable and this is reflected in the number of slats sent to the farms and the densities of spat on the slats, which range from an average of 18 (Kaipara) to 216 (Kerikeri (3)) spat per slat (Table 2.3). All of the spat were sent to Kerikeri Inlet farms for ongrowing except for those from the selected broodstock which were divided into two lots (Kerikeri 2 & 3) for on-growing. The Kerikeri (3) lot was sent to Whangaroa Harbour farms while the Kerikeri (2) lot were sent to Kerikeri Inlet farms for ongrowing.

Shell lengths of the Kerikeri (2) and (3) batches indicated that these oysters were growing at a similar rate to those not from selected broodstock (Table 2.3). The other three batches are difficult to assess as the number of sticks, and consequently number of spat, were low and had declined to even lower numbers by the time measurements were made at 7 months. At this point the remaining Kerikeri (1) slats had the oysters removed into a netlon bag for further ongrowing while the slats from the remaining Kaipara and Whangaroa batches were cleared of oysters and added to the farmed oysters being bagged that day. The Kerikeri (1) batch was sampled for size measurements at month 11 when it was found to have been heavily fouled by mud. It was consequently moved within the farm but became lost.

The Kerikeri (2) and (3) batches were removed from slats and placed in bags (Kerikeri (2)) or trays (Kerikeri (3)) after 10 months. Kerikeri (2) had been separated into 4 bags of 6 dozen oysters per bag, of which only 2 bags were found for the 14 month measurement and they were harvested soon thereafter. The rails upon which the Kerikeri (3) batch trays had been placed collapsed between month 11 and 15. The measurement from month 15 came from salvaged trays which were found to be heavily fouled with marine growth. These trays were cleaned and relayed by the farmers and harvested before any further measurements could be taken.

Table 2.3 Average size (mm ± 1 s.e.) and density (number slat^{-1} ± 1 s.e.) of hatchery-produced spat on leaving the hatchery (Month 0) and in subsequent months. All batches were identified by the location from which the broodstock were sourced. The Kerikeri (2) and (3) batches are from the same spawning where the resultant settled slats were divided for ongrowing between Kerikeri Inlet and Whangaroa harbour (Kerikeri 3). Number of slats refers to the number of slats dispatched from the hatchery for ongrowing. Sizes in months 11 – 15 indicate average size of oysters after transfer to Netlon bags (Kerikeri farms) or Tooltech trays (Whangaroa). Where no data is available the squares have been left blank.

Batch identification	Number of slats		Months after settlement								
			0	1	3	6	7	10	11	14	15
Kaipara	11	Average size (mm)	5.2 ± 0.1	8.2 ± 0.6			56 ± 5				
		No./slat	18 ± 4	6 ± 2			3 ± 2				
Whangaroa	13	Average size (mm)	3.7 ± 0.09	5.9 ± 0.3			45 ± 2				
		No./slat	35 ± 10	11 ± 3			5 ± 3				
Kerikeri (1)	34	Average size (mm)	3.6 ± 0.06	6.2 ± 0.2			47 ± 1		59 ± 1		
		No./slat	69 ± 13	26 ± 5			5 ± 1				
Kerikeri (2)	56	Average size (mm)	2.8 ± 0.03		23.3 ±0.6	41.6 ± 0.6		55 ± 0.1		71.5 ± 1	
		No./slat	189 ± 24		23 ± 10	8 ± 1		8 ± 1			
Kerikeri (3) (On-grown in Whangaroa Harbour)	90	Average size (mm)	2 ± 0.01			54.2 ± 1.4			81.2 ± 0.8		85.4 ± 1
		No./slat	216 ± 18			16 ± 2					

2.3.2 Broodstock conditioning

In order to investigate the possibility of out-of-season oyster production conditioning of broodstock was attempted using eight batches of oysters collected in different seasons. Four of these batches produced successful spawns and four batches failed (Table 2.4). Of the successful spawns, all the larval cultures had high mortalities with only the January batch reaching settlement. However, the very low settlement densities of the January batch meant the settled slats were not transferred to the farms for on-growing. The time spent in the conditioning system varied from 1 – 13 weeks for each batch. Generally, the longer the time spent in the conditioning system the less likely it was that a successful spawning event would occur. The July batch did successfully spawn after 13 weeks conditioning but the larval cultures failed soon after fertilisation. The December batch was the only batch to be strip spawned. This was carried out after 5 weeks as the batch had been assessed as ripe on arrival at the hatchery and the failure to stimulate spawning unexpected. However, the subsequent larval cultures failed in 5 days.

The conditioning process of the June batch was tracked by condition indexing three oysters every 17 days. As the condition index relates the meat mass to the shell cavity volume, successful conditioning should cause the CI to increase as gonad can account for greater

than 50 % of meat mass in mature Pacific oysters (Quayle 1988). Initially, the condition index increased, as would be expected under an effective conditioning regime, and a spawning was attempted. This spawn failed and conditioning was continued. However, as shown in Figure 2.4, condition index declined and a final spawn attempt also failed.

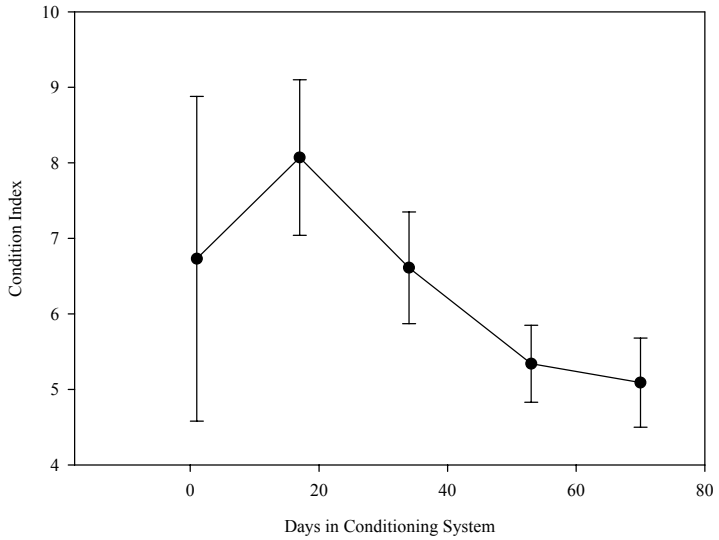


Figure 2.4 Average (n = 3) oyster condition index (± 1 s.e.) as a function of days held in the conditioning system. The conditioning system was maintained at 20 ^{o}C and oysters were fed on a daily ration equivalent to 6 % of the initial dry meat weight in dry weight of algae. Spawning attempts were made at days 14 and 20.

Table 2.4 Summary of the success of conditioning broodstock oysters in a recirculating seawater system. Broodstock were held at 20 °C with a daily ration equivalent to 6 % initial dry meat weight in dry weight of microalgae. The October batches (shaded) were sexually mature on arrival and their time in the conditioning system related to practical constraints as these were the first oysters introduced to the hatchery. n = number of oysters in the batch, F = failed spawning attempt.

Month Commenced	Batch Number	n	Spawning Attempts (weeks after commencement of conditioning)										Comments
			1	2	3	4	5	6	7	9	10	13	
January	**1**	36	6 Male 18 Female										From oysters conditioned by the Cawthron Institute (Nelson) and not used in their last spawn of the season. Mortality was high and settlement density was low (< 20 spat slat^{-1}).
February	**1**	8			F								5 oysters were sacrificed after the first spawn attempt and found to be in very poor condition. The 3 remaining oysters were discarded.
	2	36			1 Male 5 Female								The larvae from this spawn failed soon after fertilisation with many failing to properly begin cell division after fertilisation. All the broodstock died 8 days after the spawn.
April	**1**	36			F	F	F	F	F				Consecutive spawns used a different set of 6 oysters for each
June	**1**	36		F							F		These oysters were discarded as condition index continued to fall throughout the conditioning period (see text). A final spawn attempt confirmed they had not produced gametes.
July	**1**	36								F	F	8 Male 3 Female	Larvae failed after 3 days
October	**1**	8			3 Male 5 Female								These became the Kaipara batch in 2.3.3 above.
	2	13				1 Male 2 Female							These became the Whangaroa batch in 2.3.3 above.
	3	13				1 Male 1 Female							These became the Kerikeri 1 batch in 2.3.3 above.
	4	30				5 Male 10 Female							These became the Kerikeri 2 and 3 batches in 2.3.3 above.
	5	20		F	F	F					F		Died soon after the spawning attempt at 10 weeks
December	**1**	36	F	F		F	4 Male 6 Female						These gametes were physically stripped and the larval cultures failed within 5 days.

2.3.3 Clearance of microalgal feed species from suspension

Clearance rate was used as a de facto measure of the acceptability of the microalgae supplied to the broodstock. An early visual assessment of oysters in the conditioning system appeared to indicate that oyster pseudofaecal production increased in the presence of *Tetraselmis suecica*. This suggested that *T. suecica* was not a preferred food and it was thus removed from the ration and use in the hatchery.

Investigation of the clearance of microalgae showed that clearance rates (cells ml^{-1} h^{-1}) were higher when the initial microalgal concentrations were higher (Figure 2.5). Clearance rate (CR) decreased once concentrations passed a certain point for *C. calcitrans* (138 x 10^3 cells ml^{-1}), *P. lutheri* (238 x 10^3 cells ml^{-1}) and *T. pseudonana* (172 x 10^3 cells ml^{-1}). However, *I. galbana* concentrations did not reach a point where a decline in clearance rate was observed. *P. lutheri* had the highest CR (34.3 ± 1.1 x 10^3 cells ml^{-1} h^{-1}) although the initial concentration was significantly higher than any used for *C. calcitrans* or *I. galbana* ($P<0.05$) and in between the upper concentrations used for *T. pseudonana*.

Proportionally, *C. calcitrans* was the most cleared species (91.9 ± 4.1 %) although *I. galbana* (86.4 ± 13.6 %), *T. pseudonana* (85.7 ± 0.63 %) and *P. lutheri* (88.6 ± 0.7 %) all had similarly large proportions cleared from suspension (Figure 2.6). The proportion of the initial concentration cleared varied with the density of the initial concentration. The diatoms *C. calcitrans* and *T. pseudonana*, were proportionally more cleared with increasing initial concentration until 138 x 10^3 cells ml^{-1} (*C. calcitrans*) or 172 x 10^3 cells ml^{-1} (*T. pseudonana*) was exceeded which reflected the pattern for clearance rate.

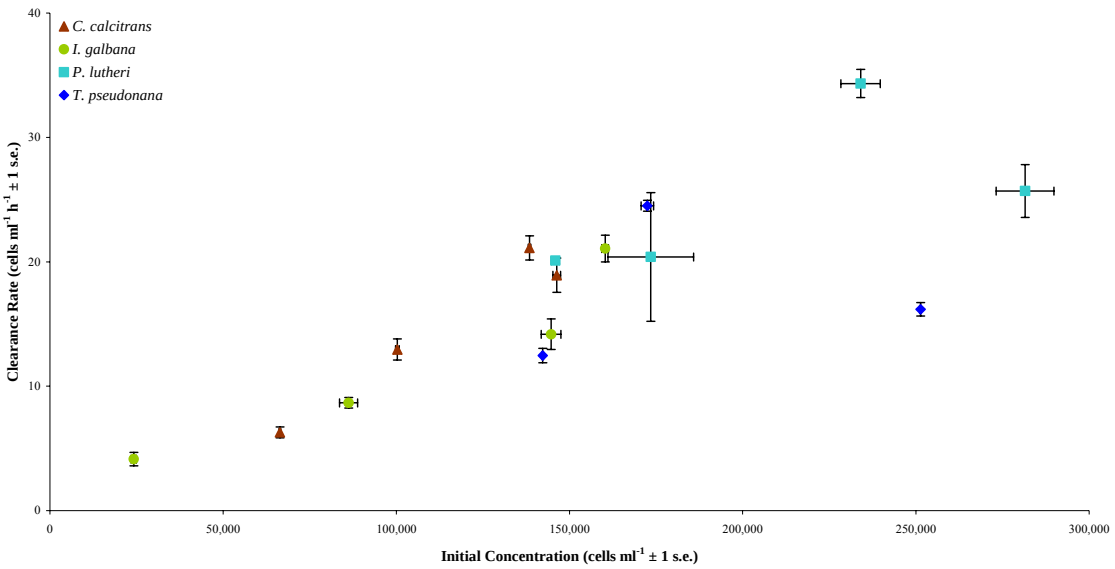


Figure 2.5 Oyster clearance rate (cells ml^{-1} h^{-1} ± 1 s.e.) for each microalgal species after six hours. Each point represents the average of three oysters both for the initial algal concentration (cells ml^{-1} ± 1 s.e.) and the rate of clearance (cells ml^{-1} h^{-1} ± 1 s.e.).

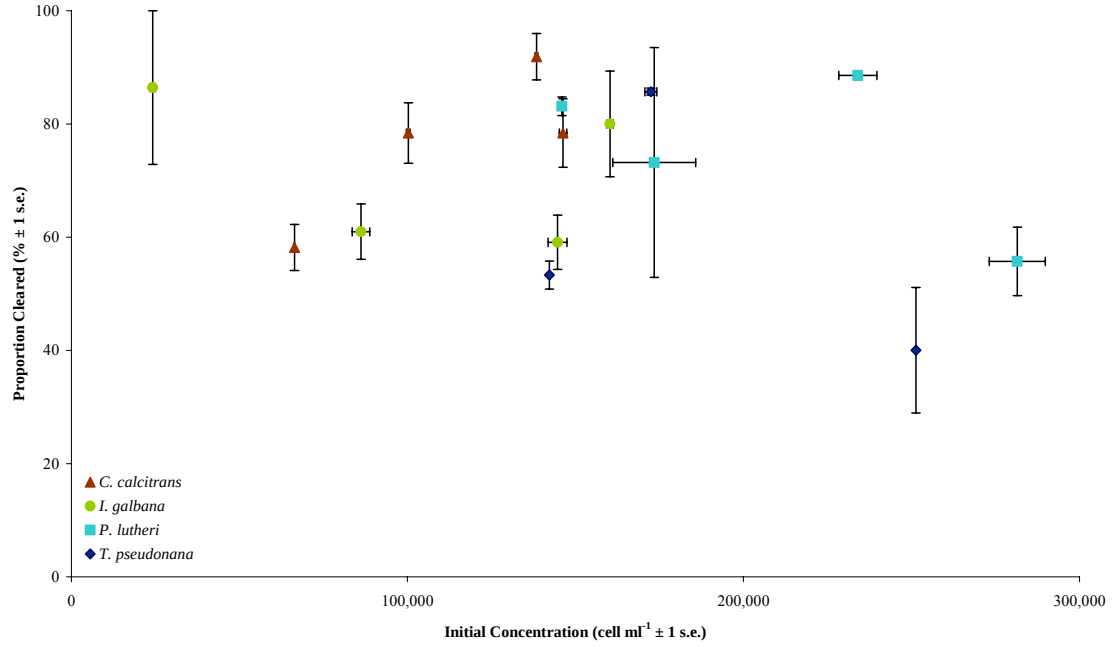


Figure 2.6 The proportion (% ± 1 s.e.) of each microalgal species cleared from suspension by oysters after six hours. Each point represents the average of three oysters (± 1 s.e.) for both the initial concentration of microalgal suspension (cells ml^{-1} ± 1 s.e.) and the percentage cleared.

2.4 Discussion

2.4.1 Oyster breeding and husbandry

To investigate the potential for selective breeding, in-season oysters were selected and bred. These broodstock were selected either on size, which defined mature oysters likely to contain large quantities of gametes, or both size and desired morphological characteristics. Consequently, variation in the growth and survival of spat from broodstock with standard or selected traits could be monitored almost simultaneously. The spawning of these oysters followed a protocol of thermal cycling (Utting & Spencer 1991) which proved to be effective. The selected broodstock provided more spawners which probably reflects the later collection (approximately 2 weeks), but may also reflect improving hatchery operations.

The thermal cycling method was used to induce spawning as the future direction of hatchery operations was thought to include the use of 'stud' broodstock which would require their reuse. At a later date this could include the use of specific male/female crosses, although in this work the larvae were pooled so individual crosses were not explored. The spawned gametes were successfully fertilised and reared through to settlement on PVC slats (Table 2.3). The procedures from fertilisation to settlement were found to be effective although some adjustment is required to optimise larval survival and improve spat settlement onto the desired substrate, in future attempts. Through the larval rearing phase improvements in the environmental conditions, in particular temperature maintenance (which was later found to fluctuate over a range of as much as 10 °C every 24 hours), were considered most likely to cause improvement.

The use of a two species food mix for larval feeding appeared to be effective, which is in agreement with reports such as Ponis *et al.* (2003) where a combined *Chaetoceros calcitrans*, *Isochrysis galbana* diet produces as good survival and growth results as a combined *Pavlova lutheri, C. calcitrans, I. galbana* diet. The feeding of microalgae at a ration of 125,000 cells ml^{-1} *C. calcitrans* and 50,000 cells ml^{-1} *I. galbana* was higher than what Strathman *et al.* (1993) described as a high or near satiating concentration of 30,000 cells ml^{-1}, or the 100,000 cells ml^{-1} Ponis *et al.* (2003) used, but this did not appear to have a detrimental effect on larval rearing.

The settlement of these batches was carried out predominantly on white PVC slats that had previously been trialled by Bay Oysters Ltd in Kerikeri Inlet. Consequently, these slats were well 'weathered' in seawater and the removal of fouling material by water blasting had further roughened the surface. Evidence of previous oyster settlement existed on some slats when they reached the hatchery but these shell fragments were removed, where possible, before settlement. These slats could be bundled in the settling tanks at relatively high densities, providing approximately 16 m^2 of surface area in a 210 litre tank. Spat settled preferentially on the underside of slats with the highest densities occurring on the deepest slats. This is in agreement with previously published observations of Sydney rock oyster spat settlement on PVC slats (Holliday 1996), but is in contrast to settlement on the black tank surfaces which were densest on the sides near the waterline. This observation suggests settlement in *Crassostrea gigas* larvae may have a phototrophic component, related to surface reflectance, which requires further investigation if settlement to plastic slats or sticks is to be pursued.

The size, and consequent flexibility, of the PVC slats proved difficult when they were transferred to the farms. As the frames in which the slats were bundled were not robust enough for use in open water the slats were immediately attached to the farm structures. This meant oysters ranging from 5.2 mm to 2.0 mm (Table 2.3) were being exposed on a light background compared to the usual technique of keeping spatted sticks in bundles until the spat have reached approximately 20 mm length. The subsequent loss of spat from the PVC slats may have been as a result of this premature laying out. Indeed some of the slats in Kerikeri Inlet had clear scrape marks that the oyster farmers attributed to fish predation (Brown and Thurkle pers comm.[3]). However, the nature of the slat surface and flexibility may have disadvantaged the spat. PVC slats were originally investigated as an oyster growing substrate as the oysters could be removed simply by twisting the slat. With such easy removal the process of removing oysters from sticks and grading them into bags for final ongrowing could potentially be made economical through automation. However, the PVC slats are prone to flexing on the racks due to environmental influences. While the slats were positioned on-edge on sheltered racks, flexing undoubtedly took place. By twisting some of the newly caught slats deliberately it became clear that large clumps of spat were more susceptible than individual oysters to being dislodged, especially as sticks dried out (pers obs).

[3] Simon Brown (Bay Oysters Ltd) and Vic Thurkle (Sanford Ltd) reporting observations to the author.

The standard stick used in oyster farming in New Zealand is made of pine timber treated to H3 tanalising grade or greater. Under normal circumstances timber sticks are coated in cement slurry, bundled and placed on dedicated spat catching racks for up to 6 months. Trials of these wooden sticks, which ranged from 1 – 3 seasons of use, found them unsuitable for the hatchery setting environment. Placement of larvae, or even adult oysters in tanks containing the wooden sticks resulted in rapid mortality. Presumably this was due to the leaching of toxic treating agents (such as chromated copper arsenate, Weis *et al.* 1995) at, or above, fatal levels in the 24 hours between water changes. Continued industry desire for stick settled oysters, suggests further investigations of suitable substrates is warranted, but falls outside the scope of this work.

Data on the growth of the hatchery produced spat is unfortunately sparse. Oyster farmers in New Zealand generally report that Pacific oysters will reach the premium size of 100 g (80 - 100 mm shell length) in 15 – 18 months after spatfall (Dinamani 1991, Handley 2002, Brown pers comm.[4]). With such low numbers of oysters it is difficult to determine if growth rates of hatchery produced oysters would be equitable with wild set. However, the Kerikeri (2 and 3) sizes would probably have fitted the 80 – 100 mm range within 18 months (Table 2.3). The 'selected' broodstock batches (Kerikeri (2) & (3) Table 2.3) survived in slightly better numbers than the other batches but this is probably due to the larger number of spat initially settled. Given that samples of the batches had shown good replication of the traits upon which the broodstock had been selected (80 %, Pokae pers comm.[5]), these preliminary results provided encouragement for the continuation of the hatchery.

2.4.2 Conditioning of broodstock

The provision of spat to farms out-of-season, so farms can be restocked as space becomes available, has been suggested to be a potential offset of the increased cost of hatchery production[6]. To produce spat out-of-season the hatchery had to be able to bring broodstock into a condition at which they could spawn viable gametes. Within the constraints of the hatchery, conditioning followed previously described successful techniques such as Utting and Spencer (1991), Utting and Millican (1997) and Robert and Gérard (1999). The conditioning procedures did result in spawnings on three occasions but

[4] Simon Brown (Bay Oysters Ltd); reply to authors questions.
[5] Rusty Pokae (Sanford Ltd); observations of Kerikeri 3 batch during harvest.
[6] Simon Brown (Bay Oysters Ltd); comments to author.

were unsuccessful in producing spat, suggesting further consideration of the processes is required. Oysters conditioned by the Cawthron Institute (January, Table 2.4) were the exception, but high mortality led to low settlement densities and they were consequently abandoned.

The time at which broodstock are introduced to the conditioning system can have a significant bearing on both the time and technique required (Loosanoff & Davis 1963, Shpigel *et al.* 1992). Without the ability to maintain lower temperatures required for reabsorption of old gametes and acquisition of storage metabolites (Robert & Gérard 1999, Caers *et al.* 2002, Cannuel & Beninger 2005), attempts to condition oysters collected in February and April were probably ill advised. The batches collected in June and July however, were likely to be near, if not initiating, gametogenesis (Dinamani 1987, Ren *et al.* 2003). Hence, the requisite diet and temperature conditions should advance the rate of gametogenesis as predicted by Muranaka and Lannan (1984). Utting and Spencer (1991) indicated that oysters collected in late winter will require approximately 6 weeks of conditioning. Thus, a period of 8 - 10 weeks would appear to be an adequate period of conditioning for oysters collected in June or July. The July batch was finally spawned after 13 weeks to produce larvae that only lasted 3 days while the June batch failed to spawn at all, suggesting inadequacy in the conditioning technique. This was confirmed by condition indexing of the June batch where, after the first 20 days, the condition index declined. This indicated that the meat mass was declining, something normally associated with spawning events in the wild. As 40 % of the broodstock was used for determining the condition index, conditioning of the June batch was discontinued after a final spawn attempt at 10 weeks.

Helm *et al.* (2004) note that broodstock conditioning at a daily ration of 6 % or greater of the initial dry meat weight in dry weight of algae can cause bivalves to pursue somatic, rather than gametogenic growth. Given the ration fed in this investigation it is possible that the condition index values of the June batch reflect shell growth as much as decline in meat mass. Indeed, new shell was observed in some oysters although this was discounted at the time as being due to some endogenous rhythm begun prior to collection. Cannuel and Beninger (2005) have questioned the need to feed broodstock in hatcheries suggesting that egg quality is maintained regardless of conditions with additional feeding merely increasing fecundity, which is similar to the results reported by Muranaka and Lannan (1984). In contrast, Uriarte *et al.* (2004) reported that a conditioning diet rich in protein

improved growth and survival of *C. gigas* larvae. This effect doesn't extend beyond metamorphosis however, which is thought to be influenced by the amassing of lipid reserves during larval feeding (Powell *et al.* 2002). Utting and Millican (1997) and, more recently, Caers *et al.* (2002), noted that rearing conditions define the importance of lipid and polyunsaturated fatty acid reserves in larvae. Thus, variability in the literature as to the value of conditioning may relate to the quality of the hatchery systems in minimising stress for broodstock and larvae. In the present work, stress on the October (5) and December batches during conditioning, and perhaps larval rearing, may have contributed to larval failures.

The capacity of the hatchery conditioning system constrained investigations of conditioning by limiting batch size. The number of broodstock conditioned, for example, may have been too small to account for the natural variation in reproductive success (Boudry *et al.* 2002) or timing of maturation (Dinamani 1987). The number of batches was also restricted with the June and July batches, for example, occupying all the space until October, precluding trials starting in August or September. The ability to assess gonad condition was restricted by not having perfected a non-lethal method of gonad biopsy and limited numbers of broodstock.

Visual assessment of the overall gonad and gonad smears under magnification was probably not as adequate a method as systematic histological or biochemical methods for the assessment of gonad condition. Histological examinations, in particular, provide information on overall gonad condition, including developmental stage and gonad follicle index (Dinamani 1974, 1987). Gonad smears, however, rely on a relationship between oocyte diameter and reproductive stage (Lango-Reynoso *et al.* 2000, Cannuel & Beninger 2005) based on individual oocyte size distributions, and assumes that males will attain gonad maturity synchronously with females. Hence, using the distributions of too few individuals will not accurately indicate population trends, failing to account for population heterogeneity. While this heterogeneity will decline as ripe condition is reached (Lango-Reynoso *et al.* 2000), use of oocyte size was, at best, a qualitative method, as used in this research. Histological analysis would have provided better information on gonad quality through conditioning and probably would have detected oysters not advancing gametogenesis. However, the larger batch sizes required could not be accommodated within the hatchery.

In order to retain broodstock for later use, thermal cycling was pursued as a method to stimulate spawning. This method had proved successful for inducing spawning in ripe oysters but proved more difficult with those oysters conditioned in the hatchery. The pursuit of other methods of attaining gametes requires exploration in order to determine if the failure of thermal cycling was due to the gametes not being 'ready' for spawning or the conditioning procedures rendering the method ineffective. The use of stimulants, such as serotonin, or non-lethal methods of stripping require further investigation if the reuse of broodstock is desired. However, conversations at the time with people experienced in bivalve spawning in New Zealand suggested thermal stimulation to be consistently effective when the subjects were ripe.

Strip spawning is a commonly used technique in the hatchery production of Pacific oysters (Helm *et al.* 2004, Cannuel & Beninger 2005) and would have provided a manner with which the success of conditioning could be better assessed through the success of fertilisation, larval rearing and settlement. Indeed, for those batches collected in October and December the need for conditioning was debateable and stripping, as was finally done with the December batch, should probably have been pursued immediately following the first failure to stimulate spawning. The stripping of gametes was used only in the December batch (Table 2.4) when thermal cycling had proved ineffective and proved effective in producing larvae. However, the collapse of the larval cultures suggested that the gametes were no longer in good condition which may have been as a result of the time spent in the conditioning system or that these oysters had spawned out more than had been assessed prior to arrival at the hatchery.

The first of the conditioned batches (February Table 2.4) to be spawned were raised in 25 – 26 °C with each rearing vessel containing a submersible heater to maintain consistent temperature. When these larval cultures collapsed samples of the dead larvae were sent to Dr Mike Hine (NIWA Wellington) who identified the presence of a Herpes-like virus. Herpes-like virus has been reported in *C. gigas* larvae and spat in New Zealand (Hine *et al.* 1992) and France (Renault *et al.* 2000, 2001) and the aetiology confirmed (Arzul *et al.* 2001). The herpes-like oyster virus (Ostreid Herpesvirus OSHV-1; Minson *et al.* 2000) is present throughout seawater (Vigneron *et al.* 2004) but has also been reported in bivalve gonad, suggesting the possibility of vertical migration (Arzul *et al.* 2002).

Barbosa-Solomieu *et al.* (2005) using a series of crosses between infected and uninfected broodstock found vertical migration of the virus via the eggs. However, mortality amongst larvae is highest when sperm has been contributed from an infected male (46 % larval mortality) compared to eggs from an infected female (19 % larval mortality). Hence infectivity of eggs may not be indicative of larval mortality but may provide the larvae with some kind of virus resistance, which in turn suggests a selective breeding program could improve larval production.

Elston (1997) has reported a threshold temperature for infectivity of 25 – 26 °C. Consequently, maintaining cultures below 25 °C should minimise the chance of further outbreaks. However, previous research suggests optimal temperature for Pacific oyster larval cultures is higher than 25 °C (28 °C, Helm & Millican 1977, 27 °C, Curtin 1979) although more recently Utting and Spencer (1991) suggested 24 – 26 °C and Helm *et al.* (2004) suggested 25 °C. Given the presence of oyster herpes virus in New Zealand it is likely that suboptimal larval culture temperatures will have to be used in the absence of alternative control measures.

The oysters which produced the infected larvae suffered a disease outbreak and died shortly after the spawning which suggests they may have been in an immuno-compromised state. Oyster herpes virus has not yet been associated with adult mortalities so it was thought unlikely that it contributed to the deaths. Indeed the lesions on the body and gill of these oysters were found to contain *Vibrio*-like bacteria suggesting a bacterial infection. However, further identification of the bacteria was not achieved and it is unknown whether they were the primary infective agent. It is possible that a combination of factors led to these oysters being susceptible to infection once they had been stressed by the process of spawning.

Pacific oysters, such as the February and April batches, are in a nutritionally depleted state after the summer spawning period (Ren *et al.* 2003 and Chapter 3 below) having spawned out a significant portion of their body mass (Dinamani 1987). Given the post-spawn state it is likely that those gametes spawned in the February batch were of poor quality, possibly undergoing reabsorption, and consequently the larvae would be susceptible to infection. Although Lipovsky (1984) reported that D-shell development doesn't predict larval success, the observation of improper cell division and large numbers not reaching D-shell stage (February 2, Table 2.4) was probably indicative of poor gamete quality and the

likelihood of difficulties through larval development. The persistence of the Herpes-like virus in the hatchery (despite complete cleaning with hypochlorite and/or Oxine) or in broodstock collected from Kerikeri Inlet may be related to other larval failures (Table 2.4) despite lowering larval rearing temperatures below the 25 – 26 °C threshold (Elston 1997).

It is unlikely that any larval culture failures were due to a lack of food as the feeding of microalgae at a ration of 125,000 cells ml^{-1} *C. calcitrans* and 50,000 cells ml^{-1} *I. galbana* was higher than what Strathman *et al.* (1993) described as a high or near satiating concentration of 30,000 cells ml^{-1} or Ponis *et al.* (2003) used at 100,000 cells ml^{-1}. Indeed, Moran and Manahan (2004) found that starvation merely extended the length of the larval phase rather than significantly increasing mortality for up to 15 days of post-spawning starvation. Moreover, those batches that were successfully taken to settlement, completed the larval rearing period in 16 - 18 days post-fertilisation. Therefore, if ration is a factor in larval failures it is due to changes in the quality of microalgae produced rather than quantity. This possibility is discussed further below.

2.4.3 Food quality

Stress can be induced in bivalves through manipulation of environmental variables such as available food, and temperature (Shpigel *et al.* 1992, Delaporte *et al.* 2003). Food quality could be a source of stress for both broodstock and larval cultures in the hatchery. Procedures for algal culture followed standard methods that have been well described and reported as effective in the literature (e.g. Guillard 1975). Variations, such as those described by Thompson *et al.* (1996) or Leonardos and Lucas (2000), to modify nutritional characteristics were not explored in an effort to keep microalgal culture standardised and as simple as possible. However, microalgal culture procedures contain areas where contamination or variation could occur between batches, which may be significant at the 15 litre culture level, if the stage of growth cycle varies at harvest. For example, microalgal nutrient composition changes with culture age (Fernández-Reiriz *et al.* 1989), thus the nutrient composition of the diet could be varied despite maintaining a constant ration in terms of cell number.

Bacterial contamination of microalgal cultures can be identified when foaming occurs in the culture. However, foaming occurs when the bacterial infection is in the exponential growth phase and bacterial contamination of the microalgae at the time of harvest may be unidentified. Such contamination could create a change in the nutritional quality of the

microalgae but, potentially more significant, is the introduction of large numbers of a single species of bacteria which could change the bacterial ecology of the receiving environment. In this work the use of techniques to maintain axenic cultures meant the risk of a bacterial bloom was increased as a single contamination could provide bacteria with an environment uninhibited by conspecific competition. In this situation, even usually benign bacterial species can create harmful conditions. The minimisation of bacteria throughout the hatchery with the use of filters and UV treatment may have increased the risk of harmful contamination. Current thinking in hatchery technology is trending more towards the use of non-axenic cultures to maintain control over bacterial populations in microalgae (Benzie *et al.* 2002) while conditioning, larval culture, etc is conducted in seawater minimally treated to control, rather than eliminate, bacteria. The control of bacteria and potential use of pro-biotic bacteria is an area which warrants further investigation if hatchery production is to be pursued.

The acceptability of the microalgal species supplied to the broodstock was investigated by assessing the microalgal clearance rate of adult oysters. Clearance rate in this experiment was used to describe the removal of particles from suspension but not predict the volume of water being processed, which assumes 100 % retention of particles and constant pumping rate (Coughlan 1969). As this work was conducted using oysters being conditioned calibration to meat mass was not possible although all the oysters were between 80 and 100 mm shell length. The loss of whole weight and length data further compromised this work, as did the supply of microalgae which was that left over after the provision of daily rations within the hatchery. Despite the drawbacks the clearance rate experiments indicated that, on their own, each species was cleared from suspension in relatively high proportions. The clearance rate data suggested the existence of optimal microalgal concentrations which is in agreement with results for *C. gigas* (Barillé *et al.* 1997), *C. virginica* (Epifanio & Ewart 1977) and *Mytilus edulis* (Winter 1978). The optimal food concentrations appeared to be variable depending upon microalgal species and this may relate to the shape and size of the microalgae (Bougrier *et al.* 1997). However, as clearance rate declines with declining concentration (Epifanio & Ewart 1977) it is likely that the clearance rate, calculated after 6 hours with no algal replenishment, underestimates the actual clearance rate at a given concentration. A reduced sampling interval and/or replacement of removed algae (such as described by Winter 1978 or Ren *et al.* 2000) would improve these results.

The clearance rate compared to seston concentration indicates that microalgal concentrations (ranging from 0.4 to 6.2 mg l^{-1}) in this study are low compared to studies using natural seston (Barillé *et al.* 1997). This suggests that the oysters have the capacity for higher loads of particles. It has been demonstrated in bivalves that clearance rate, filtration rate and retention efficiency are influenced by the composition of the seston both in terms of the microalgal species available and the presence of inorganic particles (Bayne *et al.* 1987, Urban & Kirchman 1992, Barille *et al.* 1993, Bougrier *et al.* 1997). Thus, optimal feeding densities must be determined using different mixes of algal species and inorganic particles. Optimal feed blends for oysters will thus be a compromise between the proportions desired to provide optimal nutrition and the proportions that stimulate the highest clearance and ingestion rates.

2.4.4 Physical hatchery and methodology

The construction of the hatchery relied upon the description of Curtin (1979) as the MAF hatchery that had operated successfully in the New Zealand context, had been designed to "be done as cheaply as possible" (p. 3) and the author was readily available for consultation. The final pilot-scale hatchery construction varied from the description of Curtin (1979) due to 1) space considerations (floor area 74 m^2 compared to 48 m^2 for the current hatchery), 2) the requirement for seawater to be trucked in and 3) incorporation of some newer technology. Other reference resources (Laing 1991, Utting & Spencer 1991) tended to refer to larger scale operations with flow through seawater access. Consequently their utility related more to the techniques they described. However, where possible, technology such as the use of plastic bags for algal culture was incorporated or adapted.

In the hatchery, water quality was the most likely inducer of stress. Water quality was monitored in terms of dissolved oxygen, nitrates, chlorine and, on two occasions, heavy metals. All of these levels were found to be acceptable at the time of testing using the guidelines described by Wickens and Helm (1981). However, these tests did not measure low level pollutants, such as tributilyin tin or chromated copper arsenate (Weis & Weis 1992), which can bioaccumulate or can be lethal at trace levels. Helm *et al.* (2004) explicitly state that the drawing of water from near commercial docks or marinas is not recommended, yet that was the only practical access to seawater available to the tanker which delivered seawater to the hatchery. Attempts to mitigate potential problems from poor seawater quality included continuous aeration, filtration to 1 μm or less, and UV

treatment. None of these were likely to have an impact on many of the potential trace pollutants and a better source of seawater would be desirable.

If the hatchery production of Pacific oysters and/or the development of a selective breeding program is to be pursued increased scale and modernisation of techniques used in this work is necessary. The Port Stephens Fisheries Centre (New South Wales, Australia) operated a hatchery for the Sydney rock oyster breeding program using techniques similar to those used in this study, including the importation of seawater. After continual problems with aspects of hatchery production a review was conducted and the results became available in 2002 (Benzie *et al.* 2002). Key to their conclusions on the hatchery technology were 1) technology was out of date with regard to commercial systems, 2) focus on hygiene was leading to unstable microbial communities which may be significant in larval rearing and setting problems, and 3) a shift to non-axenic microalgal cultures should be made. For the study presented in this chapter these findings of the Port Stephens Fisheries Centre review indicate that developing close links with successful hatcheries, including staff exchanges for training and technological advancement, would be a necessary initiative for any continuation of hatchery production.

2.4.5 Conclusions

In conclusion, the work completed in the hatchery showed that the basic processes of microalgal production, spawning, larval rearing and settlement could be accomplished with the available facilities and techniques. Conditioning proved to be problematic but improvements in capacity so more broodstock and microalgal production can be achieved may improve this. In particular, the ability to more closely monitor condition so effectiveness can be better determined would improve success. Further investigation of microalgal feeding by broodstock would help optimise feeding but more work is required on determining the adequacy of diet in terms of essential nutrients to maximise fecundity. The use of artificial foods and/or additional non-algal nutrient sources, such as cornflour and microencapsulated formulations, may improve conditioning procedures by reducing the production cost associated with microalgae and enhancing nutrient accessibility through specific nutrient formulation (Langdon & Bolton 1984, Langdon & Siegfried 1984, Urban & Langdon 1984, Ingle & Ingle 1996).

Investigation of the benefit of bacteria in terms of providing balanced microbial communities and/or as a nutrient source is also required. While microalgae are almost certainly the primary source of food for adult, larval and juvenile oysters (Dame et al 1992, Douillet & Langdon 1993) there is a potential for bacteria to provide nutritional benefit. Generally the fraction of the seston less than 10 μm is available to oysters in hatcheries as the flow through seawater is seldom filtered to remove bacterial sized particles. However, as the ultraplankton (less than 5 μm) is the most abundant potential food source for filter feeding bivalves (Le Gall *et al.* 1997) its loss may be important, requiring modifications to procedures for effective hatchery operation. Aspects of the potential importance of this small size fraction are investigated in the following chapters.

Chapter 3.0
Seasonal Variation in the Retention of Picoplankton by *Crassostrea gigas*.

3.1 Introduction

The conditioning of broodstock in the hatchery was not successful and a number of possible reasons for this were discussed in Chapter 2. One factor that may have contributed to the conditioning failure was the absence of small size fraction (< 5 μm) food particles and this was chosen for further investigation. Microalgae are likely to provide the majority of the nutritive particles for oysters in the wild. The manner with which bivalves capture, select and process nutritive items from the plankton has been the subject of scientific (Martin 1928, Bernard 1974, Jørgensen 1996), commercial (Epifanio 1979, Utting & Millican 1997, Caers *et al.* 2000) and ecosystem/environmental based research (Dame 1993, Grant & Bacher 1998, Hawkins *et al.* 1998a, Hyun *et al.* 2001), yet there is relatively little consensus between these investigations.

Conflicting hypotheses of bivalve feeding dynamics and mechanics remain despite attempts by several authors to establish unified principles (Winter 1978, Jørgensen 1990, Bayne 1998, Ward *et al.* 1993, 1998). As discussed in section 1.4, "The underlying effector mechanisms [of particle capture and selection] are cilia and mucus" (p1198; Beninger 2000) acting in combination. The great flexibility bivalves display in order to maximise their efficiency of nutrient acquisition and interspecies differences, has made it difficult to elucidate how the processes and mechanisms operate. Selective feeding processes have been shown to operate on the ctenidia (Ward *et al.* 1997, 2003), labial palps (Milke & Ward 2003) and in the gut (Shumway *et al.* 1985, Brillant & MacDonald 2000, 2003). Field research has also confirmed that bivalves receive nutritional benefits from selective feeding in response to changing seston quality and quantity (Barillé *et al.* 1993, 1997, Hawkins *et al.* 1998b, Cognie *et al.* 2001, Bayne 2002, Honkoop *et al.* 2003). However, field research has predominantly dealt with gross measures of food selection,

such as net organic selection efficiency (described in Hawkins *et al.* 1998a), rather than specific food species or type selection.

Table 3.1 Published retention efficiencies of various food items by oysters. Retention efficiency is ± 1 standard error where available. Particle sizes are expressed as equivalent spherical diameter.

Species	Food Type/Species	Particle Size (µm)	Retention Efficiency (%) of Particles	Reference
Ostrea edulis	*Isochrysis galbana*	5	65.9 (±11.2)	Wilson (1983)
	Seawater + *Monochrysis lutheri* + *Dunaliella marina* + *Tetraselmis suecica*	2	40	Møhlenberg & Riisgård (1978)
		>4	100	
Crassostrea virginica	Unknown	2	25	Winter (1978)
		3	60	
		4	80	
		5	80	
		6	95	
		7	95	
		10	100	
Crassostrea gigas	Seawater + *Skeletonema costatum*	3	70.37(±10.56)	Barillé *et al.* (1993)
		5.18	89.02(±4.45)	
		10.35	98.5(±2.12)	
	Seawater + *Tetraselmis* sp. + *Thalassiosira* sp.	3	48.17(±11.11)	
		5.18	77.62(±5.43)	
		10.35	100.27(±3.11)	
	Natural seawater	2.46	42.6(±7.9)	Ropert & Goulettquer (2000)
		5.03	60.6(±8.3)	
		9.95	80.51(±3.5)	
	Phytoplankton	2.46	18.32(±19.1)	
		5.03	25.66(±25.6)	
		9.95	56.45(±20.7)	

Generally, oysters will retain particles greater than 5 µm in diameter with maximum efficiency (Charles *et al.* 1999, Dupuy *et al.* 2000a) and microalgae are the dominant component in terms of carbon and nitrogen supplied (Dupuy *et al.* 2000b). Retention efficiency has been shown to increase with increasing particle size (Table 3.1) but size is clearly not the only factor influencing efficiency. The studies of Barillé *et al.* (1993) and Ropert and Goulettquer (2000) suggest food type, and/or composition, influences retention of particles of any given size. This is supported by the work of Riera and Richard (1996) that suggests environmental availability drives selective feeding preferences. They observed that estuarine populations of *Crassostrea gigas* derived most of their carbon from terrestrial, detrital sources, whereas river mouth populations utilised benthic microalgae, suggesting they were able to preferentially ingest and/or assimilate particular food sources "among diverse sources within the total organic matter pool" (p. 347). Riera *et al.* (2002) have also observed that preferential utilisation of different particulate organic matter size

classes resulted in relatively stable levels of $\delta^{13}C$ in *C. gigas* populations over the course of a year. This, in turn, suggests the feeding mechanism in *C. gigas* is adjusted for the purpose of maintaining stable levels of certain compounds year round.

There are clear seasonal changes in the biochemical composition of bivalve somatic and gonadal tissue (Whyte & Englar 1982, Ruiz *et al*. 1992, Mason & Nell 1995, Li *et al.* 2000). Mason and Nell (1995) found that the gametogenic cycle related to changes in both the physical and the biochemical composition of the Pacific oyster. Similarly, Li *et al.* (2000) found biochemical changes were linked to oocyte development in Pacific oyster gonads. These results support research indicating that there are critical nutritional requirements for bivalves to initiate gametogenesis and produce gametes containing the critical levels of biochemical constituents that lead to successful fertilisation and development (Lannan *et al.* 1980, Gallager & Mann 1986, Caers *et al.* 2002). For example, Seguineau *et al.* (2001) found oysters preferentially absorbing thiamine and riboflavin in spring, of which most was retained in the eggs. Noting the disappearance of riboflavin following spawning, they suggested that it accumulated exclusively in the eggs, and was essential for fertilisation and/or larval development (Seguineau *et al.* 2001). Similarly, Uriarte *et al.* (2004) found that both larval growth and survival improved when the diet, during gametogenesis, was rich in lipid and protein so that the gametes were also rich in these components. Jonsson *et al.* (1999) claimed that omega-3 polyunsaturated fatty acid C18:3, C18:4, C22:6 levels in larvae may explain settlement success as much as settlement substrate type.

Seasonal demands for specific nutrients, such as those described above, might be expected to create changes in nutrient acquisition strategies or mechanisms. Indeed, seasonal adjustments have been found in the digestive activities of some species of bivalve. Ibarrola *et al.* (1998a) for example, concluded that, in the cockle *Cerastoderma edule,* "seasonal variation of enzyme activities would reflect a process of continuous adjustment to environmental food availability. Further shaping of the seasonal cycles may be influenced by requirements of specific biochemical components and past feeding history." (p. 25). Other researchers, such as Chávez-Villalba *et al.* 2002b, have noted the effect of geographic origin on the success of broodstock conditioning under controlled conditions, although the cause of the variation is unclear.

Retention efficiency has been demonstrated to be variable according to: (a) bivalve species, (Møhlenberg & Riisgård 1978, Charles *et al.* 1999) (b) plankton species (Murphree & Tamplin 1991); (c) season (Morton 1977); and (d) current nutritional status of the bivalve (Ibarrola *et al.* 1998b). It has also been shown by numerous authors that bivalves, including oysters, can utilise the size fraction less than 5 µm (Haven & Morales-Alamo 1970, Møhlenberg & Riisgård 1978, Wisely & Reid 1978, Prieur 1981, Ropert & Goulletquer 2000, Nakamura 2001). However, no research has assessed the contribution of the abundant ultraplankton (< 5 µm) to bivalve diet on a seasonal, or *in situ,* basis. Although Newell and Shumway (1993) suggested that "variable retention efficiency in the 2 - 5 µm range may be an important aspect of mussel/particle interactions in the coastal zone" (p. 94), the utilisation in response to nutritional demands may be more informative in terms of both bivalve nutrition and temporal plankton ecology.

Microalgae and bacteria are generally considered to contain larger quantities of readily digestible molecules and essential micronutrients (Philips 1984, Seiderer & Newell 1985) than other potential food items. The picoplankton[7] can be the largest component of the particulate organic carbon and nitrogen available to suspension feeders (Fogg 1986, Weisse 1993, Campbell *et al.* 1997, LeGall *et al.* 1997). Identification and investigation of picoplankton populations has only occurred in recent years with the advent of sensitive methods of detection, such as flow cytometry and molecular biological techniques (Le Bouteiller & Blanchot 1992, Sieraki *et al.* 1995, Burton 1996, Marie *et al.* 1997, Field *et al.* 1997, Moon-van der Straay *et al.* 2001) although studies of bivalve suspension feeding have examined the retention of picoplankton sized particles since at least the 1950's (e.g. Jørgensen & Goldberg 1953). Flow cytometry has allowed large data sets to be quickly and accurately obtained covering aspects such as temporal and spatial variation in abundance, cell cycle, pigment content and species distributions of populations, especially in the pico-sized fraction (Marie *et al.* 2000, 2005). This information has progressively enhanced the knowledge of the importance of the picoplankton in oceanic, coastal and estuarine ecosystems (Campbell *et al.* 1997, Moreira-Turcq *et al.* 2001, Sin & Wetzel 2002, Safi & Gibbs 2003, Worden *et al.* 2004). As such, the contribution of picoplankton to filter feeders may have been overlooked, especially as information on the picoplankton ecology of coastal and estuarine waters is limited.

[7] Picoplankton are defined as planktonic cells which will pass through a 2 µm filter pore (Sieburth *et al.* 1978). However, with acceptance that cells may pass through despite being of a larger size, the definition became, more recently, cells less than 2–3 µm (Li 1998, Simon *et al.* 1994, Moon-van der Staay *et al.* 2000). For the purposes of this work, picoplankton are defined as the planktonic fraction less than 3 µm

Some authors (Le Gall *et al.* 1997, Loret *et al.* 2000, Dupuy *et al.* 2000a) have proposed that heterotrophic protists could provide a link between picoplanktonic carbon and suspension feeders, whereas others (Charles *et al.* 1999) suggest that free bacteria directly contribute a small proportion of the energy requirements. None, however, have considered the potential importance of the fraction supplied in terms of specific nutrient requirements and whether there are seasonal variations in retention that relate to these requirements. The work presented in this chapter was designed to investigate this aspect of Pacific oyster feeding. This study was divided into three aims:

1. To determine, *in situ*, the temporal variation in the retention efficiency of picoplankton by Pacific oysters in Kerikeri Inlet;
2. To determine the temporal changes in the physical and biochemical composition of Pacific oysters in Kerikeri Inlet;
3. To investigate the relationship between temporal variations in oyster condition, their retention of picoplankton and seasonal changes in environmental parameters.

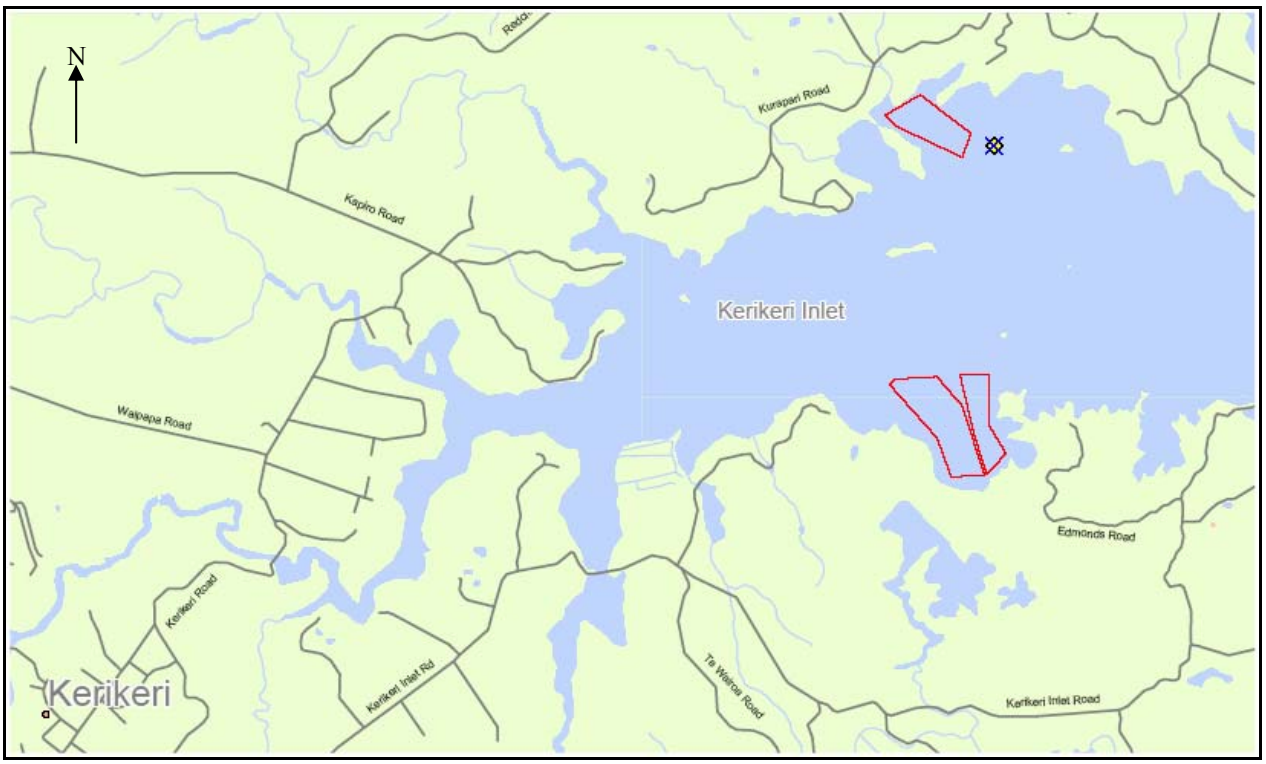


Figure 3.1 The location of the Bay Oysters Ltd/Sanford Ltd oyster farming leases (red boundaries) in Kerikeri Inlet. **X** marks the location where the barge was situated for the sample collections. Marine farm leases were defined as; on the northern side, 'Rangitane' (Lease numbers 139 & 182) and, on the southern side, 'Inlet road' (Lease numbers 17 & 18). The location of Kerikeri Inlet in north-eastern New Zealand is shown in Figure 2.2. Map courtesy of Frontier Mapping NZ Ltd and Sanford Ltd.

3.2 Materials and Methods

All chemicals were 'AnalaR' grade, BDH Laboratory Supplies (Poole, England) unless otherwise stated.

3.2.1 Sample collection

3.2.1.1 *In situ* sampling of feeding currents

All sample collection was undertaken with the support of the Sanford Ltd and Bay Oysters Ltd marine farming operations in Kerikeri Inlet (Figure 3.1). This support included the supply of a barge, from which the feeding study was conducted, and the provision of the oysters (*Crassostrea gigas*). The oysters consisted of a random selection taken from within marine farming leases 17, 18, 139 and 183. Sample oysters were collected as part of normal harvest regime on the low tide before the sampling period.

To determine the efficiency with which picoplankton were retained by oysters *in situ,* inhalant and exhalant currents of feeding oysters were sampled. The location of the feeding current was identified using Fluorescein dye (1:10 w/v in dH_2O) to visualise the current flow around and through feeding oysters. The feeding currents of oysters on growing racks and in flow-through seawater tanks were observed to develop a thorough knowledge of variation in current locations. This process was also used to develop the methodology for inhalant and exhalant feeding current sampling.

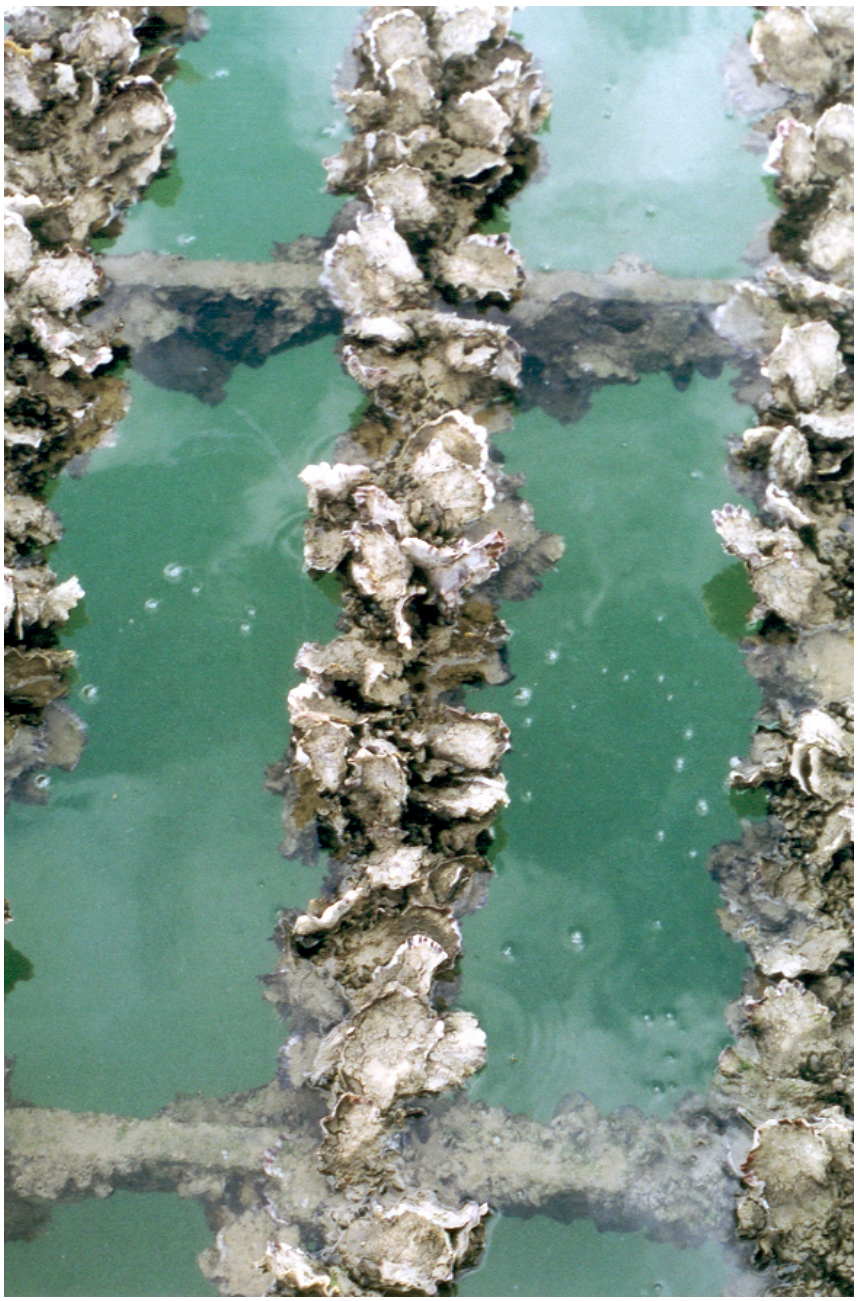


Figure 3.2 Oysters on a wooden growing stick showing the density and mud accumulation. These were impediments to the sampling of feeding currents of oysters whilst on the growing racks.

Pilot-scale sample collection showed sampling to be impractical using oysters still attached to growing structures, due primarily to poor visibility which resulted in the disturbance of the oysters, sediment and the growing racks (Figure 3.2). A stable environment for both the oysters and the researchers was created by deploying the setup for *in situ* sampling described below (and Figure 3.3) on a barge. The barge was located at the sampling site indicated in Figure 3.1, for each sampling occasion.

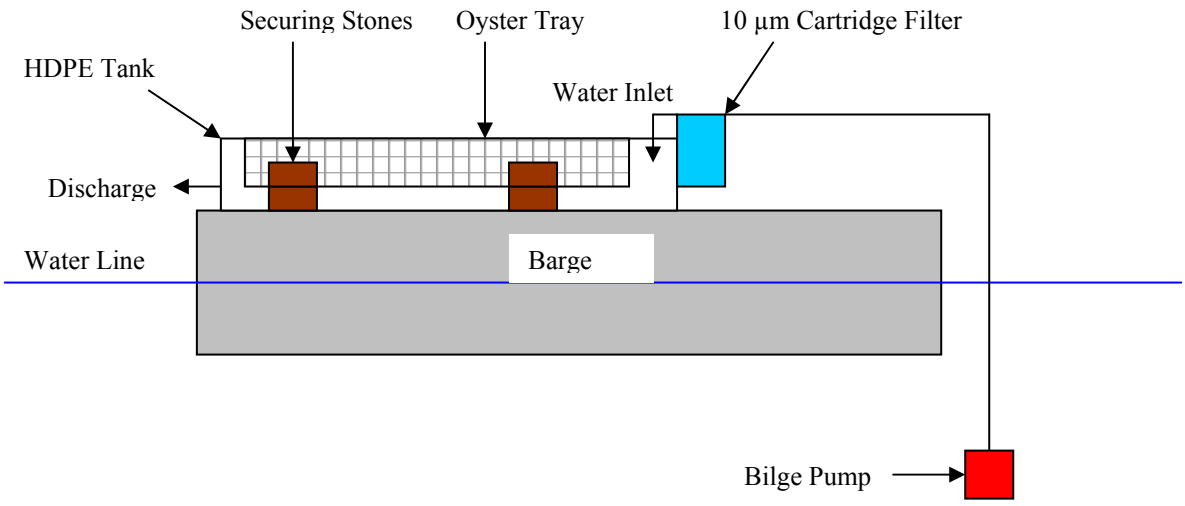


Figure 3.3 Diagram of the flow-through tank setup on a barge for the purpose of sampling feeding oysters. The water intake was located at a depth of 1.5 m below the waterline. Diagram is not to scale.

A 12 volt submersible bilge pump (Rule Industries; max. 1895 l h^{-1}) provided a continuous supply of seawater from 1.5 m below the waterline (the approximate depth of the oyster growing racks at high tide). The seawater was pumped (via 5 m of 25 mm clear, non-toxic hose) to a 250 mm filter housing which contained a nominal 10 µm pore size spun fibre cartridge (Taylor Purification Ltd). Water was filtered to remove the larger silt particles that interfere with flow cytometric analysis. However, filtration with a nominal 10 µm filter was not expected to reduce the target picoplankton population of individuals sized less than 3 µm. Flow rate (20 l min^{-1}) through the large tank (1200 x 700 x 250 mm) was controlled by a 20 mm outlet drain fitted with a PVC ball valve. Under these conditions the water in the large tank was completely changed every 10.5 minutes.

An oyster tray (Chelwood Industries; 950 x 450 x 200 mm) was secured with paving stones in the flow-through tank (see Figure 3.4). Oysters (36) were placed in the tray with the dorsal edge uppermost to allow sampling of the exhalant current with minimal disturbance. Once immersed the oysters were allowed to acclimate, for at least 30 minutes, or until actively pumping water. Active pumping was determined by observation of shell gape.

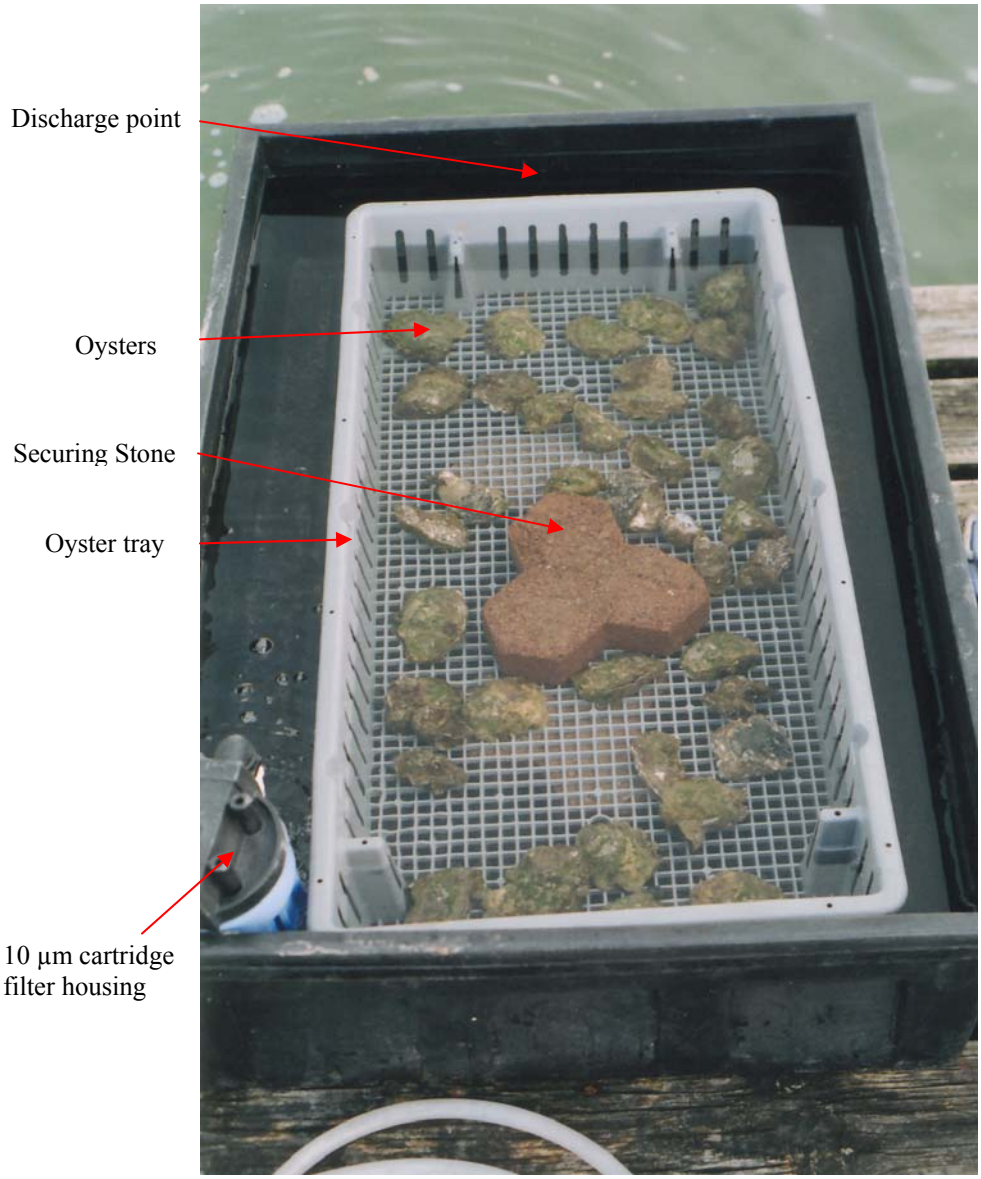


Figure 3.4 The flow-through tank system with three dozen oysters distributed in the oyster tray. The paving stones securing the oyster tray and the filter housing at the sea water inlet are visible.

Sampling of inhalant and exhalant feeding currents was carried out ± 2 hours of high tide in a manner similar to that described by Pile and Young (1999). Eight oysters were selected from those actively feeding, and each oyster was sampled five times from the inhalant current and five times from the exhalant current. Samples were taken using separate, colour coded and numbered Becton-Dickinson 5 cc Luer-Lok™ syringes, fitted with a 19 gauge B-D Precision Glide stainless steel needle (1.00 x 38.00 mm). For exhalent samples, the needle was held between the mantle edges as close as possible to the centre of the outflow current (Figure 3.5, A) whereas inhalant samples were collected more

generally from the area within 10 mm of the incurrent (Figure 3.5, B). Each sample (3 - 4 ml) was drawn with a slow, smooth action.

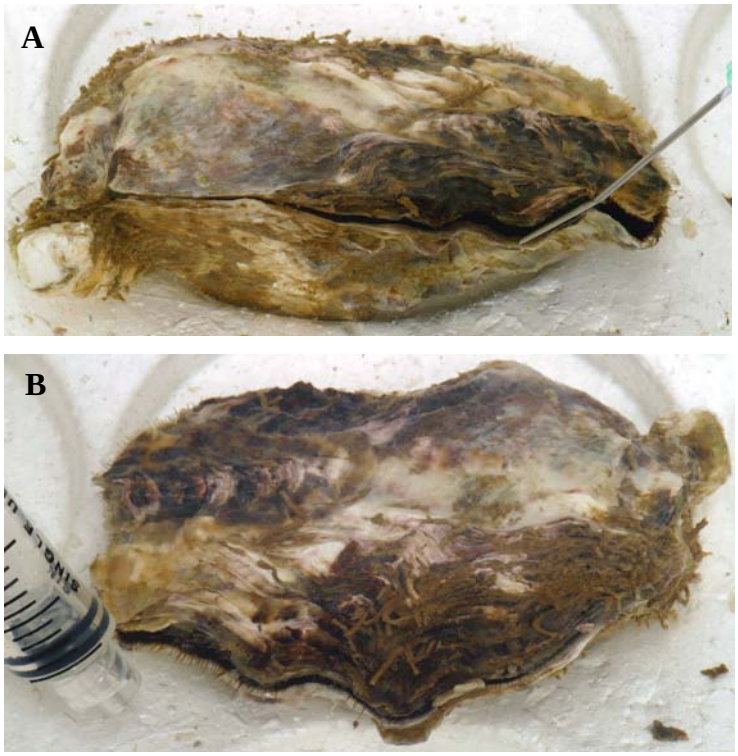


Figure 3.5 Sampling of water from the exhalant (A) and inhalant (B) feeding currents of a Pacific oyster (*Crassostrea gigas*). (Photo credit I. MacDonald)

A portion (1.8 ml) of each sample was transferred to a pre-labelled, screw cap, cryovial (Sarstedt), and 10 % (w/v) paraformaldehyde (0.2 ml, Sigma P6148 in phosphate buffered saline [Sigma P3688], pH 7.5; Marie *et al.* 1999a) added. Paraformaldehyde is the preferred preservative as it aids cell permeabilization, fluorescence maintenance (of dye) and does not modify size characteristics (Troussellier *et al.* 1995).

The capped cryovial was inverted 2 - 3 times, then placed on ice in a covered chilly bin to minimise light degradation. Cryovials were transferred to liquid nitrogen as soon as possible and transported to the laboratory where they were stored at -80 ^{o}C (Vaulot *et al.* 1989, Marie *et al.* 1999a). Stored samples were analysed as soon as possible as samples degrade after approximately 12 months at -80 ^{o}C (Marie *et al.* 1999a).

3.2.1.2 Microalgal data.

Microalgal cell counts were supplied by local oyster farmers (Kerikeri Delivery Centre Ltd) who commission the Cawthron Institute to carry out counts of microalgae for routine monitoring of harmful algal bloom events. Samples for this work were taken in Kerikeri Inlet (approximately 1000 m SE of our sampling site) on the high tide, usually mid-week every week. For the purpose of this work microalgae were defined as autotrophic eukaryotes between 10 and 200 µm. 55 microalgae species (Appendix 3.1) were monitored and include, *Chaetoceros* spp., *Gymnodinium* spp., *Gyrodinium* spp., *Laudenia* spp., *Lingulodinium polyedrum* and *Nitschia* spp., which have been previously identified as dominant microalgae in coastal waters during blooms around the NE New Zealand coast (Chang *et al.* 2003). The supplied data covered the years 1998 to 2001 with partial coverage of 2002. Therefore, microalgal abundance for the purpose of analysis was calculated as the average of all counts for each month between the years 1998 to 2001.

The Kerikeri Delivery Centre Ltd also supplied temperature data collected at the time of sampling, although this dataset was incomplete for the years 2001 and 2002.

3.2.1.3 Oyster sample collection for condition indexing

The eight oysters sampled for *in situ* picoplankton retention analysis were placed in individually numbered bags. A further four oysters, randomly selected from the tank, were placed in labelled plastic bags, retained on ice, then frozen (-36 ^{o}C), until condition index analyses (Section 3.2.2.3) were performed.

3.2.1.4 Additional data

Environmental data was collected by third parties and is presented here with their permission. The Northland Regional Council provided rainfall data collected at their Kiakaha monitoring site (35° 11’ S, 173° 57’ E) in the Kerikeri Inlet catchment.

Sea surface temperature was derived from the Leigh Marine Laboratory, University of Auckland daily monitoring at Cape Rodney due to the incomplete Kerikeri Delivery Centre Ltd dataset. Linear regression showed a close relationship between the Leigh data and the available sea surface temperature data for Kerikeri Inlet ($r^2 = 0.94$) from January 2001 to December 2002.

3.2.2 Sample analysis

3.2.2.1 Picoplankton identification and enumeration

Flow cytometry was utilised to identify and enumerate the picoplankton populations following the protocol of Marie *et al.* (1999a). Analysis was done using a Becton-Dickinson FACSCalibur™ flow cytometer equipped with a 15 mW, 488 nm air cooled argon-ion laser and a standard filter setup (488 nm bandpass filter for forward scatter, photomultiplier for side (right angle) scatter and fluorescence photomultipliers with bandpass filters at 530 nm, 585 nm, and 661 nm). All parameters were recorded on a 4 decade logarithmic scale in list mode files of Flow Cytometry Standard (FCS) 2.0 format using the BD CellQuest™ 3.3 acquisition and analysis software.

For each month (n = 13 months) 50 samples (equating to 5 of the 8 oysters sampled) were analysed. The extra samples were retained as backup should further analysis be required. Each sample was thawed at room temperature with minimum light exposure. For each sample two sub-samples (*a* and *b* below) were required and, once thawed, the sample was mixed by repeated pipeting prior to each sub-sample being drawn.

Yellow-green beads (1 µm FluoSpheres (Molecular Probes cat # F-8823) diluted in 0.1 µm filtered sea water to a concentration of 5.6×10^5 spheres ml^{-1}, were added to every sub-sample as an internal reference and for determining the sample volume.

The two sub-samples were analysed as follows:
a) **Picoeukaryote** and **cyanobacteria** populations were identified by the fluorescent properties of their photopigments (chlorophyll a in picoeukaryotes and phycoerythrin in cyanobacteria) under excitation at 488 nm. Right angle light scatter properties were also used as a proxy for cell size relative to the standard beads.

Samples (450 µl) were dispensed into 9 ml polystyrene Falcon tubes (Cat # 54-10001-00) and bead solution (10 µl) added. Each sample was mixed by vortexing immediately prior to being run on the cytometer at the high flow rate (60 µl min^{-1}). Light scatter and fluorescence parameters were captured for each cell until a total of 1000 beads had been counted or a run time of 120 seconds had elapsed;

b) **Heterotrophic bacteria** were identified with the aid of the fluorescent nucleic acid stain SYBR Green I (Molecular Probes cat # S-7567) which has a strong affinity for double stranded DNA. Samples were diluted 1:1 (v/v) with 0.1µm filtered seawater, in a 9 ml polystyrene Falcon tube. FluoSpheres (20 µl) and SYBR Green I (5 µl; diluted 1:10,000 in 1 M potassium citrate buffer) were also added to the tube. Tubes were incubated in darkness for 30 minutes, at room temperature, to allow penetration of the stain. Analysis on the flow cytometer was done, at the low flow rate (12 µl min^{-1}). Dilution was necessary to keep the event rate on the cytometer below 1000 cells s^{-1}, as Marie *et al.* (1999b) reported a significant decline in counting efficiency when the event rate exceeded 1000 events s^{-1}. Light scatter and fluorescence parameters were acquired until a total of 500 beads had been counted or 180 seconds had elapsed.

Data from each sample was processed with the open source WinMDI software allowing enumeration of each population based upon their fluorescent and light scatter properties. Side scatter was used to indicate cell size (relative to the reference beads) to assist in defining populations because the use of sheath fluid (Isoton II, Beckman Coulter), which may differ in salinity and hence refractive index, from the sample can distort forward light scatter which is normally used for cell sizing (Cucci & Sieracki 2001). Numerical data was exported from WinMDI to Microsoft Excel where it was converted to population densities.

Adding beads at a known concentration allowed an estimate of sample volume to be made. Cell counts could then be converted into densities (cells ml^{-1}) for more meaningful analysis. Data quality was assessed and points omitted where errors, such as sample degradation, failure to add or detect beads, or data file corruption, were detected.

Discrimination of the populations was validated by using a FACS Vantage, dual beam flow cytometer, to sort the populations into separate samples. Sorted samples were then observed at 1000 x magnification on a Leica DMRE epifluorescent microscope illuminating in a range from 470-490 nm. Images from the microscope were captured with a Leica DC500 digital camera using Leica IM1000 software.

3.2.2.2 Biomass estimation.

To make comparisons between populations, the biomass was calculated from carbon conversion factors of 1.393 pg C $cell^{-1}$ for picoeukaryotes (Grégori *et al.* 2001), 250 fg C

cell^{-1} for cyanobacteria (Campbell *et al.* 1994) and 30 fg C cell^{-1} for heterotrophic bacteria (Fukuda *et al.* 1998). For the microalgal data biovolumes (μm^3) were calculated using the median of reported dimensions and closest geometric shape. Biovolumes were then converted to carbon using equation 1 for diatoms, and equation 2 for dinoflagellates (Menden-Deuer & Lessard 2000).

pg C cell^{-1} = 0.288 x vol$^{0.811}$ Equation 1

pg C cell^{-1} = 0.760 x vol$^{0.819}$ Equation 2

where vol = biovolume (μm^3).

3.2.2.3 Oyster condition index analyses

For each month that *in situ* feeding samples were taken, 12 oysters were retained for condition index analysis (3.2.1.2 above). Each oyster was scrubbed clean of fouling material then external dimensions (mm) and whole weight (g) recorded. The oyster was then opened, drained (5 min), then the meat was removed and placed into a pre-weighed aluminium foil tray. The wet weight of the shell and meat were recorded. Both meat and shell were then dried for 24 hours at 80 ^{o}C and the respective dry weights were recorded. The dried meat was ground into a powder using a Framo mill (20,000 rpm, Franz Morat KG, GmbH & Co.). The resulting powder was stored in a desiccator, over silica gel, awaiting biochemical analysis.

The **condition index** (CI) of each oyster was determined using the formula described by Lawrence and Scott (1982) where;

CI = (dry meat weight (g) x 100) / internal shell cavity capacity (g)

Internal shell cavity capacity was calculated by subtracting the dry weight of the valves from the weight of the intact live oyster (Lawrence & Scott 1982).

Constituent fraction indices (CFI's) were determined for the glycogen, lipid and protein components. To make the results comparable with those of other researchers the methods used by Mason and Nell (1995) were followed as described below. The use of CFI's rather than percentage composition allows for the comparison of oysters of different size and "to assess seasonal variation independent of shell growth" (p. 875, Mason & Nell 1995; see also Whyte & Englar (1982) and Crosby & Gale (1990)). CFI's for each component were determined using the formula of Whyte and Englar (1982);

CFI = (% constituent x CI) / 100

The analysis of constituents, described below, was carried out on each oyster (n=12) retained for each month (n = 12 (June 2001 sample missing)).

A homogenate of 300 mg dry meat in 15 ml distilled water was prepared for each oyster sample and used for the glycogen and protein analyses.

a) **Glycogen** was analysed using Mason and Nell's modification of the enzymatic method developed by Keppler and Decker (1984). An aliquot (1 ml) of homogenate was transferred to a 2 ml Eppendorf tube and perchloric acid (1 ml, 0.6 M) added. This solution was vortexed and centrifuged (1 min, 10,000 *g*). Aliquots (3 x 50 µl) were then transferred into 1.5 ml Eppendorf centrifuge tubes and potassium hydrogen carbonate (25 µl, 1 M) and Glucoamylase solution [500 µl, Sigma A7420 (10 U ml^{-1} in sodium acetate buffer, pH 4.8)] were added. Tubes were incubated with shaking (15 min, 55 ^{o}C) in a Contherm incubator. After incubation, perchloric acid (250 µl, 0.6 M) was added and the tubes were centrifuged (10 min 10,000 *g*). The supernatant (50 µl) was transferred to a glass cuvette (1 cm path length) and TEA buffer [895 µl, (0.3 M Triethanolamine, 4 mM Magnesium sulphate, pH 4.8)], ATP/NADP/G6P-DH solution [100 µl, (ATP 1 mM, NADP 0.9 mM, Glucose-6-phosphate dehydrogenase 0.7 U ml^{-1})] and glucoamylase solution (5 µl) were added. After incubation (5 min room temperature) the absorbance was recorded at 339 nm (A_{339}). Hexokinase suspension G6P-DH (5 µl, 280 U ml^{-1}, Sigma H8629) was then added and the mix was incubated for 10 minutes at room temperature. Absorbance at 339 nm was recorded (A_{339}').

Glycogen (G; g l^{-1}) in the cuvette was calculated with the formula

$$G\ (g\ l^{-1}) = ((V \times MW) / (E \times d \times v \times 1000)) \times (A_{339}' - A_{339})$$

where V = Final volume (ml)

MW = Molecular weight of glycogen (162.1)

E = absorption coefficient at 339 nm (6.3)

d = Light path (1 cm)

v = sample volume (ml)

This value was then converted to a percentage of the dry meat weight and the median of the three values for each sample was used for all further analysis.

b) **Protein** was measured using the method of Mason and Nell (1995, from Layne 1957). The remaining 14 ml of the prepared homogenate was filtered (Whatman, Grade 5) to eliminate turbidity and absorbance of the filtrate was recorded at 260 nm and 280 nm. Protein (P) content (mg ml^{-1}) of the filtrate was determined by the equation;

$$P\ (mg\ ml^{-1}) = 1.55A_{280} - 0.76A_{260}$$

Where P is protein, A_{280} is absorbance at 280 nm and A_{260} is absorbance at 260 nm. This was converted to a percentage of the dry meat weight.

c) **Lipid** was measured using the modifications of Bligh and Dyer's (1959) method by Mason and Nell (1995). The limited amounts of dry oyster meat in individual samples meant that meat from multiple oysters within each month had to be combined. Dry meat (1 g) was homogenised in 100 ml of 1:2:0.8 Chloroform:Methanol:dH_2O and filtered (Whatman No. 1 paper filter). Chloroform (50 ml) and dH_2O (50 ml) were added to the filtrate and this solution was evaporated in a steam bath until dry. The dry material was rinsed into a pre-weighed beaker with chloroform (100 ml) and the chloroform was evaporated to dryness. The weight of residue represented the weight of total lipid in the dry meat and was converted to a percentage of dry meat weight. Where composite samples were used, CI (for the determination of CFI) was calculated by averaging the CI of all contributing oysters.

3.2.3 Statistical analysis

3.2.3.1 *In situ* feeding data

Analysis of variance (ANOVA, JMP 5.0) was used to test for statistically significant differences in the ambient cell densities, of each picoplankton population. ANOVA was nested as (oyster (month (season))) where season is summer (December – March) or not.

Retention efficiency (R.E.) for each oyster was calculated using the formula

$$R.E.\ (\%) = (Inh - Exh) / Inh) \times 100$$

Where Inh is the mean cell density (ml^{-1}) of the inhalant samples and Exh is the mean cell density (ml^{-1}) of the exhalant samples. This enabled comparisons of picoplankton retention between oysters.

Nested ANOVA was used to test for statistically significant differences ($\alpha = 0.05$) in R.E. between oysters, months and season (where season is summer or not). Post-hoc Tukey analysis was used to identify the source of significant differences at the month and oyster level.

3.2.3.2 Oyster condition index data

ANOVA was also used to determine the presence of significant differences between months for the constituent fraction and condition indices. Post-hoc Tukey analysis was used to determine where significant differences occurred between specific months.

3.3 Results

3.3.1 Sea surface temperature and rainfall

Sea surface temperature data reflect seasonal patterns with the warmest temperatures occurring over the summer months (December – March) and coolest in the winter months (June - September) covering a range from 14.0 to 20.7 °C over the course of the study (Figure 3.6). Rainfall data showed a less seasonal pattern, averaging 174 mm month^{-1}, with monthly totals only occasionally dropping below 100 mm (4 occasions) or exceeding 200 mm (5 occasions). Total rainfall for the five day period prior to each sampling only exceeded 50 mm on two occasions (September 2001 and July 2002). The storm events of May/June 2002, where rainfall exceeded 250 mm in each month, precluded fieldwork in those months.

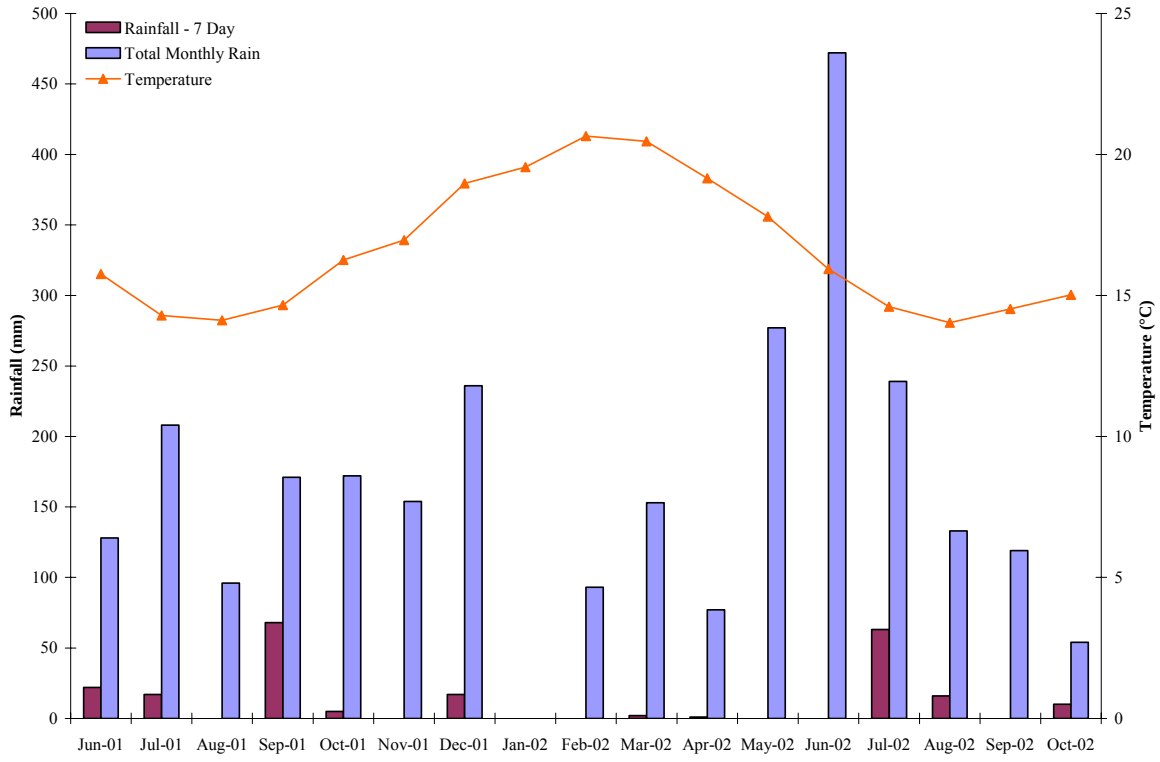


Figure 3.6 Rainfall in Kerikeri Inlet as total (mm) for the month and for the 7 days prior to sampling. Temperature data is sea surface temperature (°C) recorded at the Leigh Marine Laboratory, Cape Rodney. No rainfall data was available for January 2002.

3.3.2 Temporal ecology of picoplankton populations

Three broad picoplankton populations (picoeukaryotes, cyanobacteria and heterotrophic bacteria) were identified for enumeration by flow cytometry (Figure 3.7). This agreed with the results of other researchers (Troussellier *et al.* 1995, Li 1998, Moreira-Turcq & Martin 1998, Marie *et al.* 1999a, Li & Dickie 2001). Within certain months it was possible to discern other populations, or separation within populations, that may have been species differences, variation in "eco-type" or cell cycle. For the purpose of this study these populations were classified as cyanobacteria (Cy1, Cy2, Cy3) due to the presence of a strong orange fluorescence (phycoerythrin) signal relative to the picoeukaryote population (Figure 3.8).

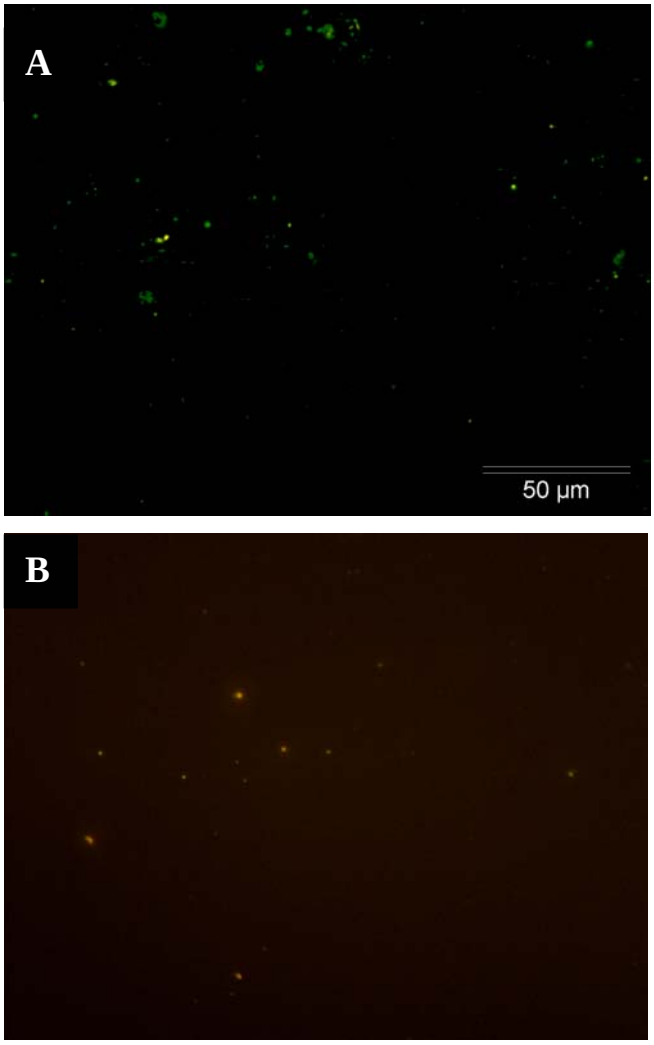


Figure 3.7 Photomicrographs of seawater samples with (A, 630 x magnification) and without (B, 630 x magnification) the addition of the nucleic acid stain SYBR Green I. Samples were illuminated with blue light to show the resultant fluorescence of either chlorophyll a (red), phycoerythrin (orange) or SYBR Green I DNA stain (green). Scale is equivalent for both.

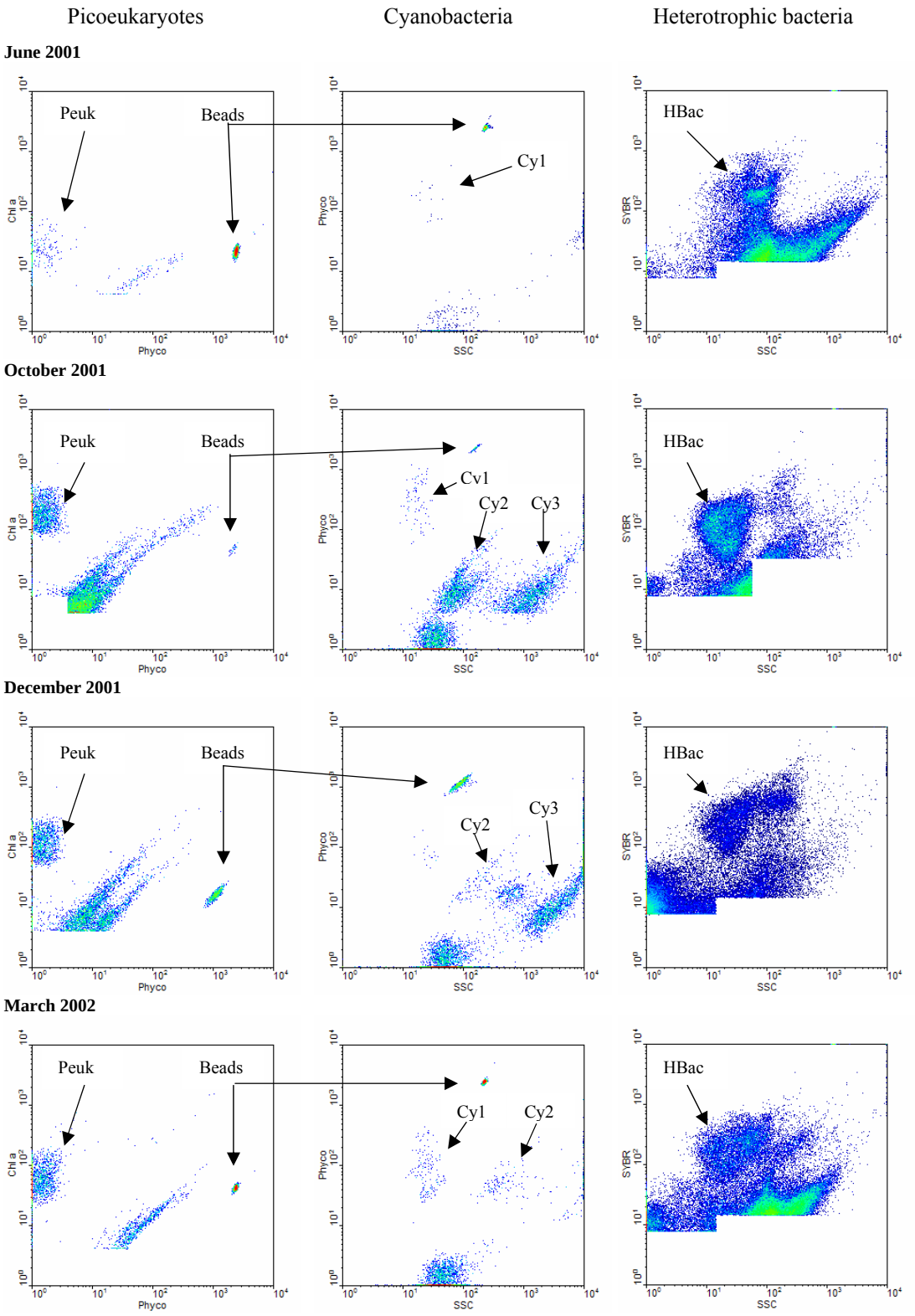


Figure 3.8 Representative density plots of picoeukaryote (Peuk), cyanobacteria (Cy) and heterotrophic bacteria (Hbac) populations showing seasonal variation. Axes are log scale, arbitrary units for right angle light scatter (SSC), and fluorescence intensity of Chlorophyll *a* (Chl a), Phycoerythrin (Phyco) and SYBR Green I DNA stain (SYBR) at 488 nm excitation. Beads are 1 µm FluoSpheres.

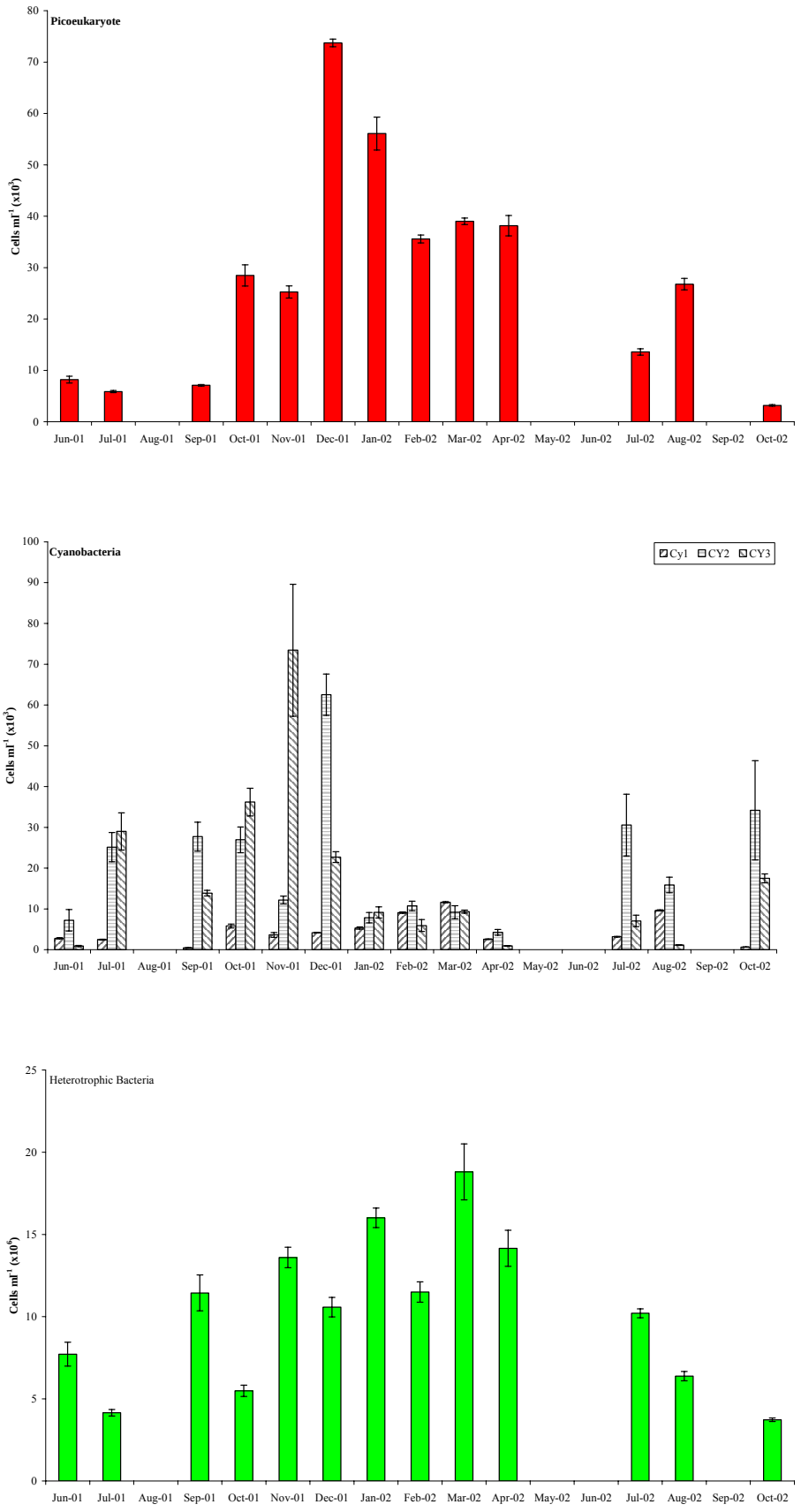


Figure 3.9 Monthly mean ambient cell concentrations (cells ml^{-1} ± 1 s.e.) of picoplankton populations (picoeukaryotes, cyanobacteria and heterotrophic bacteria) from June 2001 to October 2002. No data is available for Aug 01, May 02, Jun 02 and Sep 02.

Cy3 was the only population not to have a maximum abundance in the summer months of December through to March. Cy 2 peaked in December ($62.5 \pm 5 \times 10^3$ cells ml^{-1}) as did the picoeukaryotes ($73.7 \pm 0.7 \times 10^3$ cells ml^{-1}) while Cy3 peaked ($73.4 \pm 16 \times 10^3$ cells ml^{-1}) in November (Figure 3.9). In contrast, the heterotrophic bacteria reached a peak abundance of $18.8 \pm 1.7 \times 10^6$ cells ml^{-1} in March. Nested ANOVA showed significantly ($P < 0.01$) greater abundances in the summer months compared to the rest of the year for picoeukaryotes, Cy1 and heterotrophic bacteria. Cy3, in contrast, had significantly ($P < 0.01$) lower abundances during the summer months.

The heterotrophic bacteria contributed the greatest number of cells (cells ml^{-1}) to the picoplankton in any month. Picoeukaryote abundances were, in most instances, the next highest, although the cyanobacteria provided greater abundances in the winter months (Figure 3.9). The picoplankton contributed between 129 and 626 ng C ml^{-1} in estimated carbon biomass (Figure 3.11). Heterotrophic bacteria were the dominant contributor of carbon ranging from 112 ± 3 ng C ml^{-1} (October 2002) to 564 ± 51 ng C ml^{-1} (March 2002) while picoeukaryotes contributed between 4 ± 0.2 ng C ml^{-1} (October 2002) and 103 ± 1 ng C ml^{-1} (December 2001).

3.3.3 Microalgal abundance

The microalgal abundances for Kerikeri Inlet, in contrast to the picoplankton, showed a spring (September/October) bloom, which was dominated by *Chaetoceros* spp. and *Eucampia* spp., followed by a lesser bloom of *Chaetoceros* spp. in January (Figure 3.10). On average the total abundances of the monitored microalgae were lower than the total abundances of the picoplankton. However, as cell numbers are not representative of biomass, conversions to carbon equivalents were made.

The total microalgal biomass ranged from 11.8 to 173 µg C l^{-1} which accounted for between 3 and 57 % of the total estimated biomass (microalgae + picoplankton). Generally heterotrophic bacteria accounted for most of the estimated carbon biomass with only October 2001 and October 2002 being dominated by microalgal carbon (Figure 3.11). Picoeukaryotes contributed over 20 % of the estimated biomass in December 2001 but generally this amounted to no more than 10 %. Similarly the cyanobacteria populations never contributed more than 4 % to the estimated carbon biomass.

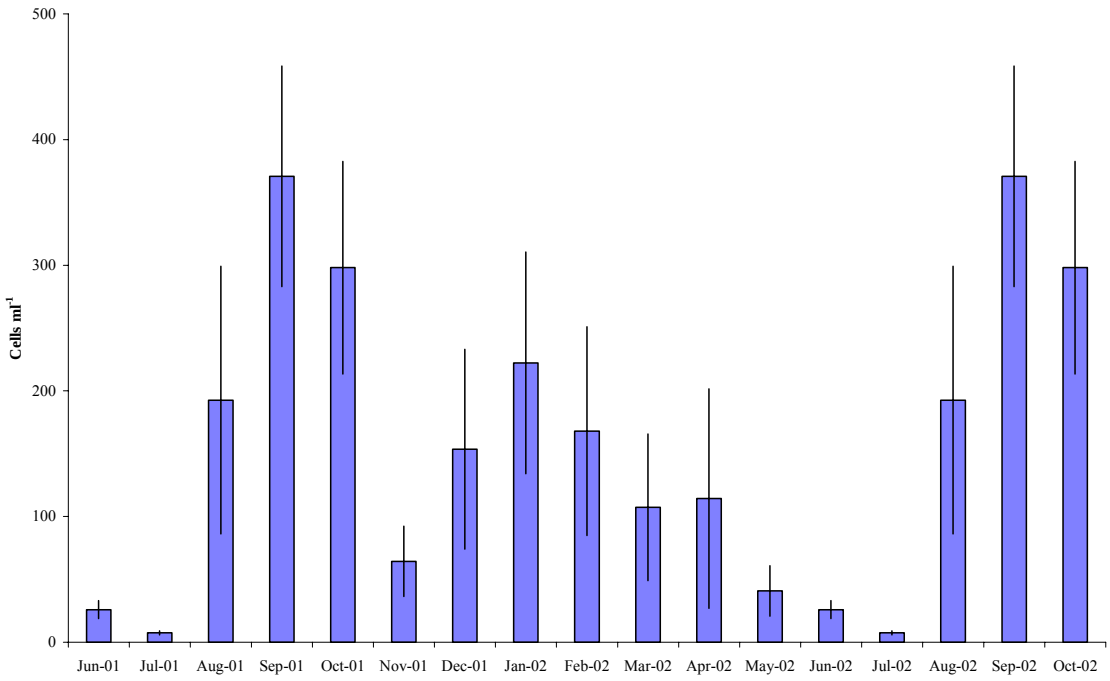


Figure 3.10 Total microalgal cell numbers (cells ml^{-1} ± 1 s.e.) averaged for each month from the Kerikeri Delivery Centre Ltd microalgal monitoring data for years 1998 – 2001.

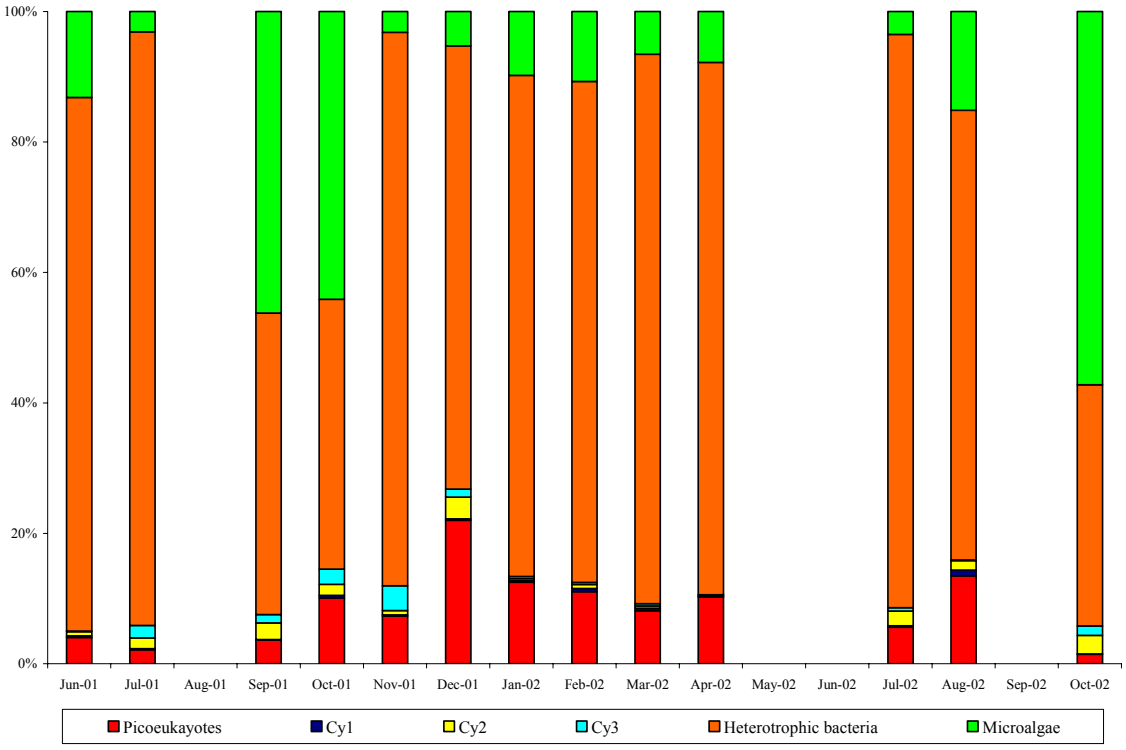


Figure 3.11 Proportion of monthly estimated total carbon attributed to microalgae (monthly average from years 1998-2001), picoeukaryotes, cyanobacteria and heterotrophic bacteria. No data is available for Aug 01, May 02, Jun 02 and Sep 02.

3.3.4 Identification of feeding currents

Inhalant and exhalant feeding currents are shown in Figure 3.6 using Fluorescein dye. Like the descriptions of Quayle (1969) and Walne (1979) it can be seen that current enters on the ventro-posterior margin and exits along the dorsal margin within the vicinity of the adductor muscle. Thus, in and out currents are clearly delineated in *C. gigas* minimising the chance of incorrectly sampling currents or simultaneously sampling both.

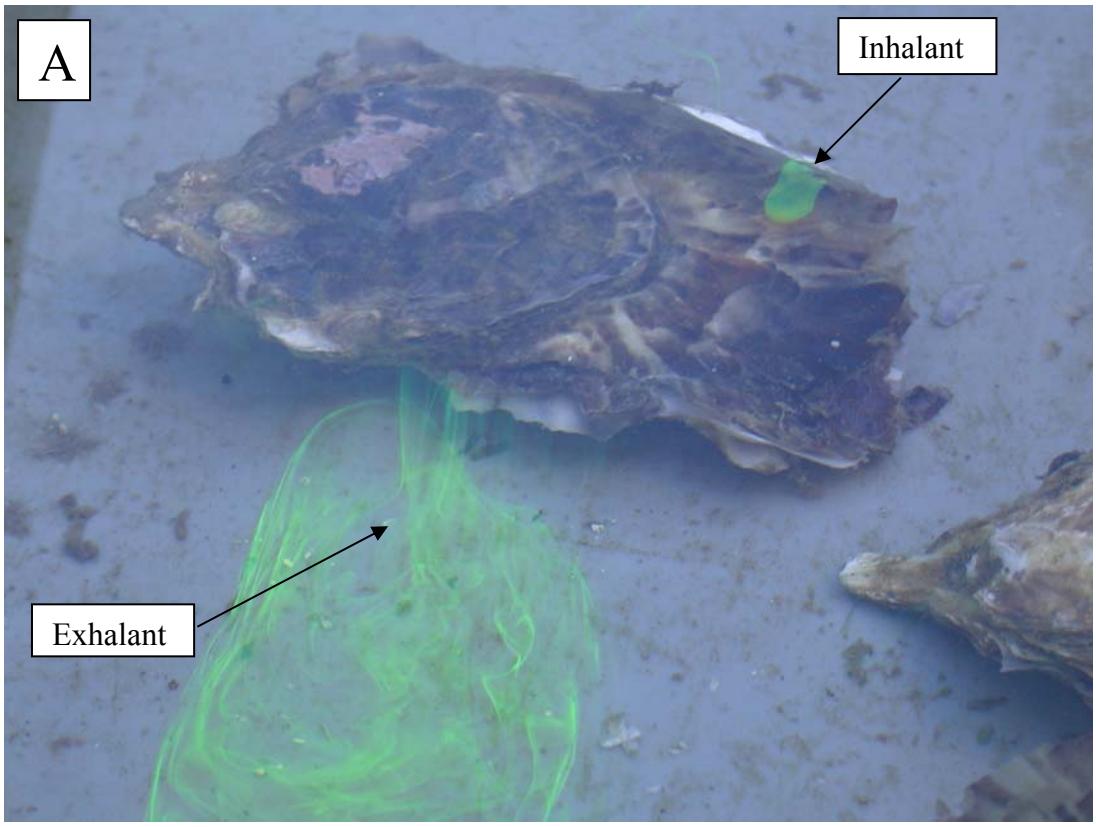


Figure 3.12 Feeding currents visualised with Fluorescein dye. (Photo credit: T. Bootten)

3.3.5 Retention efficiency of picoplankton populations

The efficiency with which picoplanktonic populations were retained by oysters (Figure 3.13) varied with both species and season. The picoeukaryotes were generally the least retained, with average monthly retention efficiency (R.E.) ranging from 1.9 ± 0.7 % to 12.3 ± 3.8 %. The heterotrophic bacteria had the next lowest average monthly R.E. (ranging from 2.3 ± 0.9 % to 38.2 ± 4.5 %) although they accounted for the greatest number of cells retained. The Cy2 cyanobacteria population achieved the highest R.E. at 42.0 ± 4.4 % in February. The Cy3 population attained the highest R.E. outside of the summer months with 38.1 ± 4.8 % in July 2002. Cy1 were retained with consistently low efficiencies attaining a maximum R.E. of 13.6 ± 4 % in October 2001.

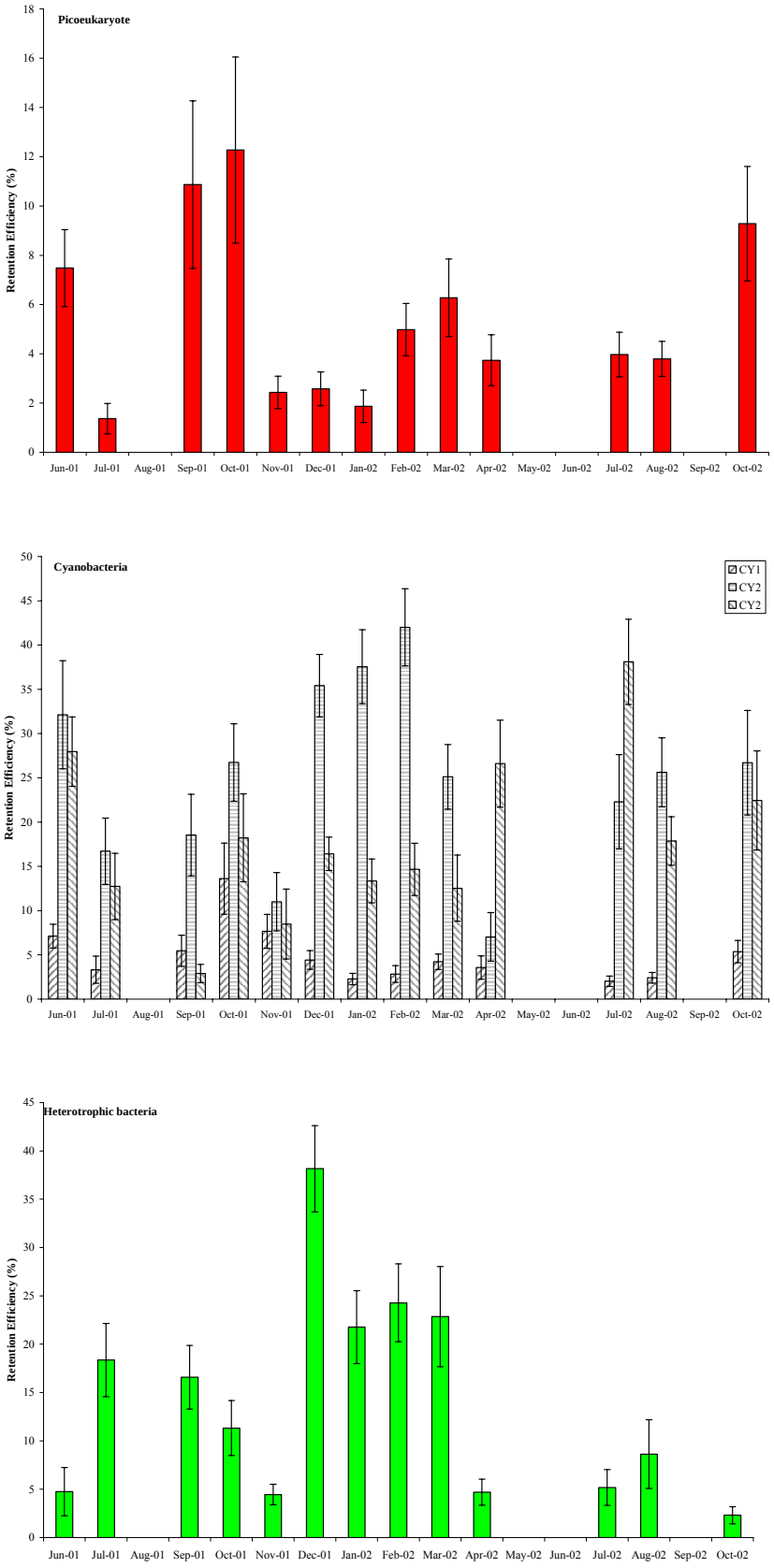


Figure 3.13 Monthly mean retention efficiency (% ± 1 s.e.) for picoeukaryotes, cyanobacteria and heterotrophic bacteria. Summer months are December to March; no data is available for Aug 01, May 02, Jun 02 and Sep 02.

Nested ANOVA showed retention efficiencies were significantly ($P < 0.01$) higher in the summer months (December – March) compared to the other months for Cy2, Cy3 and heterotrophic bacteria. Statistically significant differences between the retention efficiencies of different months were resolved using post-hoc Tukey analysis and are appended (Appendix 3.2). Analysis of the average numerical difference between each ambient and exhalant sample of each month did not resolve any significant differences using ANOVA nested as (oyster (month (season))).

3.3.6 Oyster condition

Declines in the condition index occurred over the summer months (Figure 3.12) when the major spawning events occur. Therefore, the condition index was generally inversely related to sea surface temperature. The months of March and October 2002 had the smallest oysters with average shell lengths of 77.9 ± 2.4 mm and 77.1 ± 5.1 mm respectively, compared to an overall average shell length of 97.4 ± 1.4mm. This size difference may explain the relatively large differences between the condition index values for these months and those of surrounding or corresponding months.

The constituent fraction indices (protein, lipid, glycogen) all showed seasonal trends where the levels were lowest during the summer months (Figure 3.13). The sharp decrease (December - January) and increase (May) in the glycogen level reflects the timing of spawning and the initiation of gametogenesis. The October spawn was confirmed by the Kerikeri farmers (Brown pers. comm. 2001[8]) and the presence of considerable overcatch by January.

[8] Simon Brown, Managing Director, Bay Oysters Ltd. Personal comments made in response to authors questions.

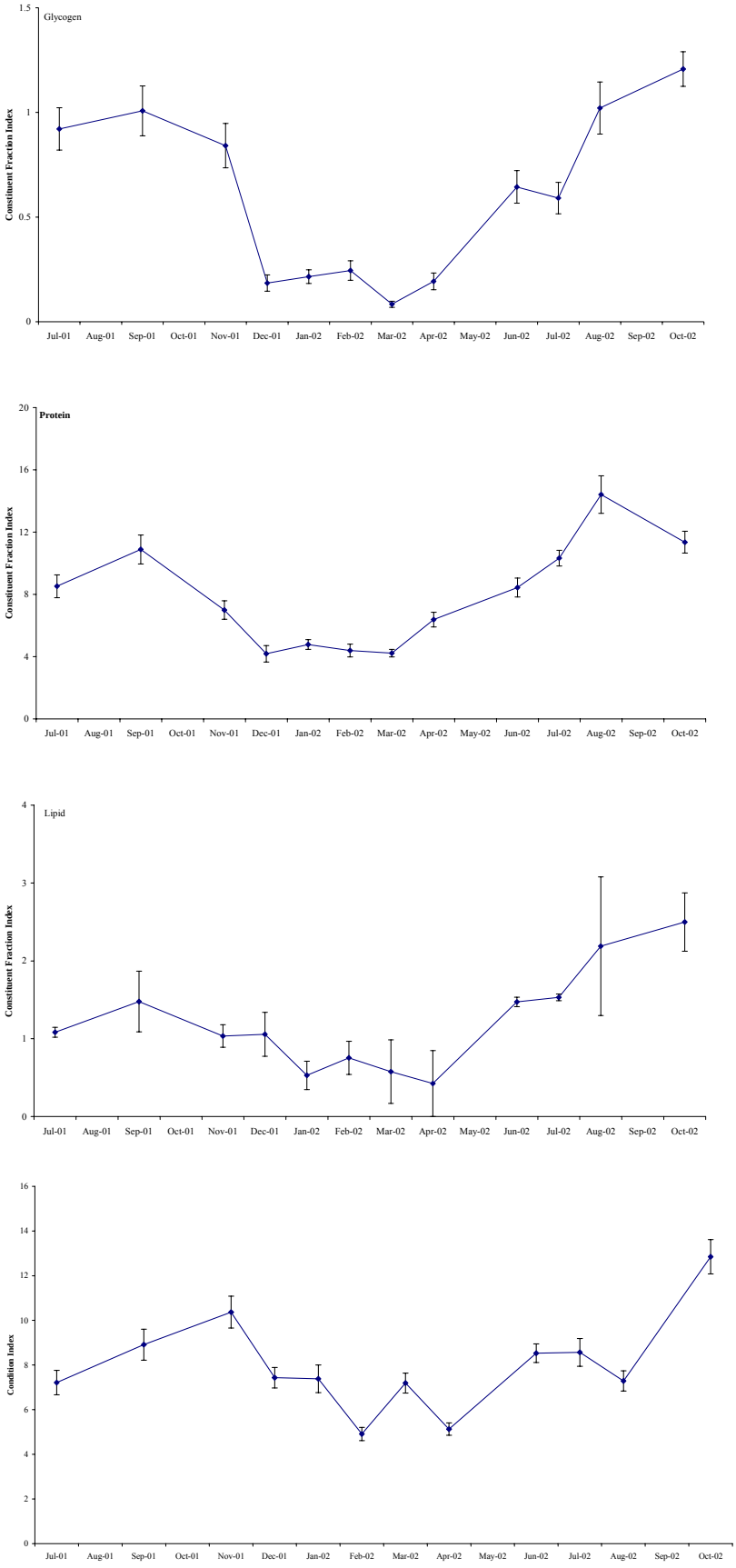


Figure 3.14 Average monthly (n = 12) levels of glycogen, protein, and lipid, expressed as constituent fraction indices (CFI, ± 1 s.e.) and biometric condition index (CI ± 1 s.e.).

3.3.7 Condition index and retention efficiency

The relationship between the condition index and the efficiency of picoplankton retention was unclear (Figure 3.15). Linear regression returned r^2 values of less than 0.1 for comparisons where the retention efficiency, condition index, shell length, live weight, and constituent fraction indices could be matched for individual oysters. The slope of fitted lines was generally negative although this was more evident for the glycogen and protein indices.

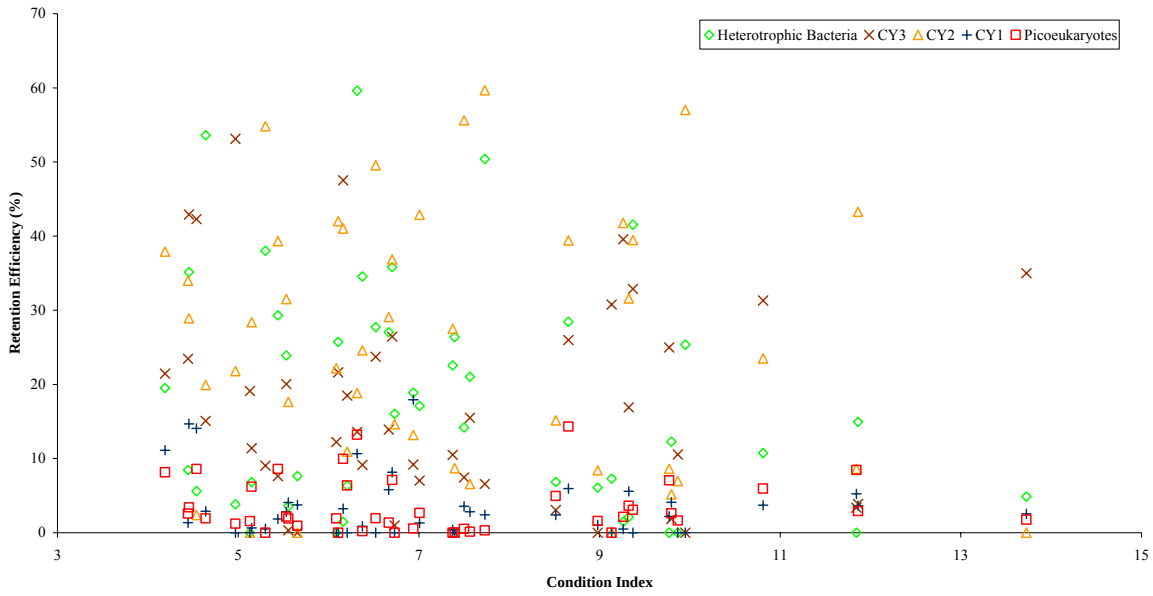
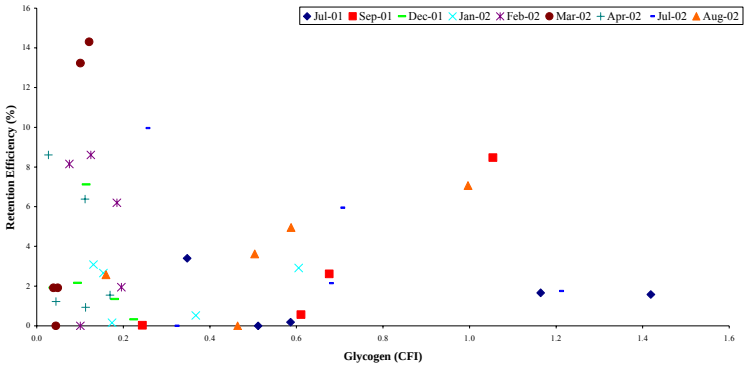
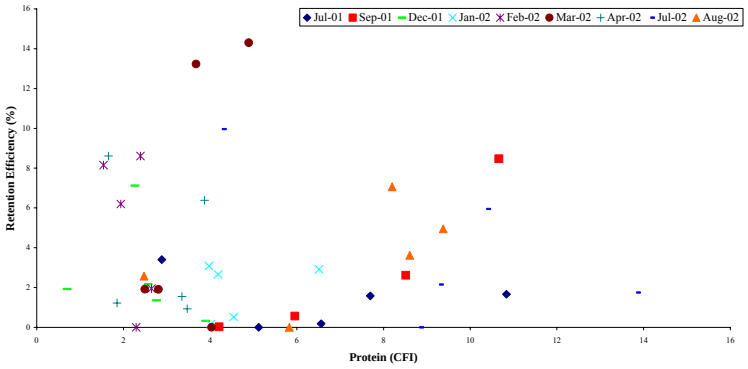


Figure 3.15 Retention efficiency of picoeukaryotes, cyanobacteria and heterotrophic bacteria in relation to the condition index of the individual oysters sampled.

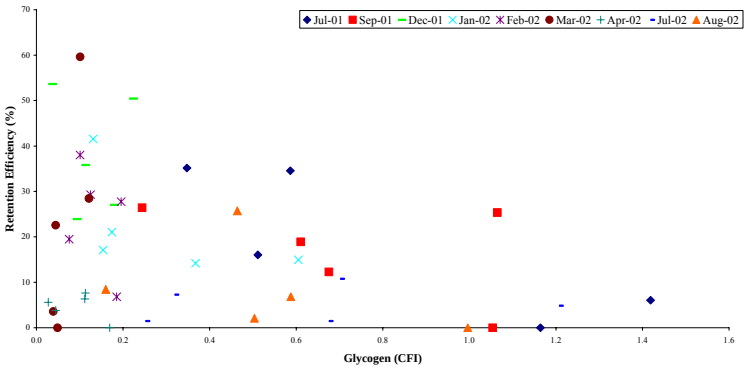
Where possible, the constituent fraction indices for glycogen and protein were paired with the retention efficiencies of individual oysters (Figure 3.16). The relationship was then analysed by month using linear regression. In general the relationships were weak with negative aspects excepting for the picoeukaryotes where two months (September 2001 and August 2002) showed a positive relationship between R.E. and CFI ($r^2 > 0.8$). The cyanobacterial population Cy2 and the heterotrophic bacteria population showed a stronger negative relationship between R.E. and CFI in the month of July 2001 ($r^2 > 0.6$).



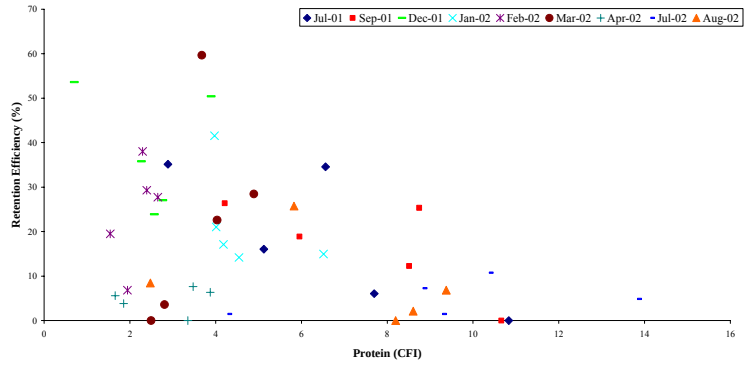
Picoeukaryotes



Picoeukaryotes



Heterotrophic Bacteria



Heterotrophic Bacteria

Figure 3.16 Retention efficiency (%) of picoeukaryotes or heterotrophic bacteria as a function of glycogen and protein constituent fraction indices for individual oysters. Data points have been colour coded for months.

3.4 Discussion

3.4.1 Picoplankton ecology

Bivalves, including Pacific oysters have been shown to retain particles less than 5 µm with reduced efficiency. Therefore, for picoplankton to be a substantial nutrient resource they would have to occur in abundance. In Kerikeri Inlet five picoplankton populations were identified with maximum abundances ranging from $11.6 \pm 0.2 \times 10^3$ cells ml^{-1} (Cy1) to $1.9 \pm 0.2 \times 10^7$ cells ml^{-1} (heterotrophic bacteria). The microalgal populations, by comparison, only reached a maximum of 371 cells ml^{-1}.

These densities of picoplankton populations are comparable to those previously reported. Li and Dickie (2001) reported densities, in a coastal Nova Scotia (Canada) basin, ranging from $> 10^3$ to $< 10^7$ cells ml^{-1} for heterotrophic bacteria and for *Synechococcus*-type cyanobacteria from 6×10^3 to 4×10^4 cells ml^{-1}, over a seven year period. Similarly, Marie *et al.* (1997) reported densities of 5.2×10^5 cells ml^{-1} (heterotrophic bacteria) and 2×10^4 cells ml^{-1} (*Synechococcus*-type cyanobacteria) in coastal Mediterranean waters. Picoeukaryote densities have been reported by Grégori *et al.* (2001) at 3×10^4 cells ml^{-1} in the Bay of Marseilles compared to a maximum of $7 \pm 0.07 \times 10^4$ cells ml^{-1} in Kerikeri Inlet. In estuarine waters, Moriera-Turcq *et al.* (2001) reported combined picoeukaryote and *Synechococcus*-type cyanobacteria densities ranging from 2.8 to 42×10^3 cells ml^{-1} in Krka estuary, 5 to 37×10^3 cells ml^{-1} in the Rhone Delta, and 1 to 50×10^3 cells ml^{-1} in the Lena-Laptev system. These ranges are lower than those found in Kerikeri Inlet but this may reflect the time of sample collection, as the lower end of the ranges found in Kerikeri Inlet provide combined picoeukaryote + cyanobacteria abundances close to those reported by Moriera-Turcq *et al.* (2001).

Temporal variation in the picoplankton populations was variable between species although picoeukaryotes, Cy1, Cy2 and heterotrophic bacteria populations all reached peak abundances in summer (December – March), whereas Cy3 peaked earlier in the spring. However, Cy2 and Cy3 populations were generally more abundant in winter with their lowest densities occurring in summer. Murrell and Lores (2004) and Li and Dickie (2001) have previously reported a link between *Synechococcus*-type cyanobacteria and temperature with maximum abundances occurring during the warmest temperatures. This is different to the Cy2 and Cy3 populations but, agrees with the Cy1 population.

Therefore, Cy1 may be a *Synechococcus*-type cyanobacteria population as commonly described in the literature, and consequently from coastal waters. The flow cytometry signature of Cy1, when plotted as side scatter versus phycoerythrin fluorescence (Figure 3.8), is similar to that published by Marie *et al.* (1999a) for oceanic *Synechococcus*-type cyanobacteria. In contrast Cy2 and Cy3 are probably larger cells with less phycoerythrin and may represent variation within a separate population of cyanobacteria, possibly native to estuarine or fresh waters.

Li and Dickie (2001) have reported consistent seasonal cycles of heterotrophic bacteria abundance with peak abundances occurring in summer, coincident with peak temperatures. In Kerikeri Inlet, the cycle is offset with peak abundance occurring in late summer (March). This may be an artefact of the sampling periodicity with heterotrophic bacteria, like all the picoplankton populations, likely to rapidly respond to changing physical conditions (Fogg 1986). A closer sampling interval might allow the population dynamics to be more accurately determined, however this was impractical in this study.

Rainfall could not be correlated to population changes in this study. This may reflect the immediacy of response to the changing environment. It is likely that the flushing and resuspension effects of freshwater inputs will influence picoplankton dynamics but more research is required including a greater diversity of temporal and spatial scales. The literature on picoplankton dynamics in estuarine and coastal waters is sparse. Further studies on fine scale changes in picoplankton populations and the relationship with physical influences are required.

Further research on picoplankton in NE New Zealand coastal and estuarine waters requires improved definition of populations. This requires a combination of techniques, including flow cytometry, cell sorting, electron microscopy and molecular studies. Such information would improve assessments of the contribution of picoplankton populations to gross measures of biomass, such as carbon and nitrogen contribution, as well as allowing assessments of population composition. Population composition may affect the functioning of a population within larger cycles of nutrient regeneration or benthic/pelagic coupling and hence the importance to the estuarine ecosystem at a particular time.

In this study, carbon conversions were used to compare relative biomass contributions of microalgae and picoplankton populations. Previous studies have addressed carbon conversion in microalgae and, to a lesser extent, picoplankton populations. Given the variability in carbon content that can exist due to different environmental conditions (Thompson *et al.* 1991, 1992), consensus on appropriate conversion values or equations, is difficult to determine. For example, bacterioplankton have reported estimates of carbon content ranging from 7 – 260 ng C $cell^{-1}$ (Fukuda *et al.* 1998), although Nagata and Watanabe (1990) previously reported there was little difference in bacterioplankton grown under varying nutrient conditions. Coastal bacterioplankton appear to have a higher carbon content than oceanic bacterioplankton (Fukuda *et al.* 1998). Therefore, a conversion factor of 30 fg C $cell^{-1}$, (Fukuda *et al.* 1998) was used over the common 20 fg C $cell^{-1}$ conversion for oceanic waters (Campbell *et al*. 1997). Grégori *et al.* (2001) determined a conversion of 1.393 pg C $cell^{-1}$ for picoeukaryotes in coastal waters and agreed with Campbell *et al.* (1994) on the conversion factor for cyanobacteria, so those were used in this study. The conversion factors of Menden-Deuer and Lessard (2000) were chosen for the microalgae as they represented the conclusion of determinations of live culture composition and literature review, which provided an extensive dataset from which the conversion factors were derived.

The relative importance of the picoplankton, and in particular the heterotrophic bacteria, was confirmed using carbon conversion to estimate the biomass contribution (Figure 3.11). The dominance of picoplanktonic carbon has previously been reported with Campbell *et al.* (1997) finding 60 – 90 % of carbon in tropical oceanic waters was contributed by prokaryotes. The proportional contribution may be inaccurate for several reasons including the accuracy of the carbon conversion factors compared to the actual carbon content of the populations in Kerikeri Inlet. The use of flow cytometry only allowed for size estimations relative to the 1 μm beads (using right angle light scatter) which did not allow accurate determinations of biovolume for conversions, such as suggested by Kogure and Koike (1987). In Figure 3.8, for example, the position of the Cy populations relative to the beads changes, which may indicate seasonal variability in population composition and consequently size, which has been reported to range from 0.87 μm (Bertilsson *et al.* 2003) to 1.8 μm (Verity *et al.* 1992) for different cyanobacterial species. Alternatively this shift could be the consequence of small changes in the flow cytometric analysis such as salinity of the sample water. While tests, such as microscopic examination of samples from each sampling occasion, may help elucidate size shifts, the development of a

technique where two, or even three, different sized standard beads are added to provide a relative scale, may allow more accurate size analysis to be achieved and thus finding the true population median size. However, the use of the microalgal data set provided by the Kerikeri Delivery Centre Ltd may account for the greatest inaccuracy.

The microalgal data are derived from a selected subset of species (Appendix 3.1). This dataset is dominated by diatoms, which are thought to provide the basis of oyster diet (Tenore & Dunstan 1973, Riera & Richard 1996, Cognie *et al.* 2001) and have been found to account for 70 to > 95 % of total cell carbon in coastal NE New Zealand waters (Chang *et al.* 2003). Thus, the dataset should be relevant as an indicator of food available to oysters, and the presence of key bloom formers (identified by Chang *et al.* 2003) should make it generally representative of biomass trends. However, due to missing data, it was necessary to use data averaged over four years which reduced the magnitude of variation. For example, Chang *et al.* (2003) reported a range from < 5 to 400 ng C ml^{-1} in coastal water compared to 12 ± 4 to 173 ± 46 ng C ml^{-1} presented here. If the available data for 2001 is examined it can be found that estimated microalgal carbon exceeded 600 ng C ml^{-1} one week in October dropping to 42 ng C ml^{-1} the following week. Thus, the biomass contribution would have been strengthened if data could have been matched on the basis of sampling date.

3.4.2 Retention efficiency

The retention of picoplankton by Pacific oysters was variable within and between the different populations. The picoeukaryotes were retained with low efficiencies even in September/October when retention efficiency (R.E.) was highest, whereas the cyanobacteria (Cy1 and Cy2) were retained with considerably greater efficiency, especially during December – February where R.E. exceeded 30 % for Cy2 (Figure 3.13). R.E. appeared to vary independently of availability for Cy2 and Cy3. For example, R.E. increased from December to February despite abundance dropping from the December peak to low levels during the remainder of summer. This may reflect a change in species composition making these populations more palatable or easier to retain. The retention of heterotrophic bacteria also showed a seasonal pattern with R.E. significantly greater over summer compared to other months. From these results it is clear that picoplankton can be retained by Pacific oysters in Kerikeri Inlet and there appears to be a seasonal change in R.E.

R.E. was variable both within and between months. Variability within months is likely to be an artefact of the methodology, where the natural patchiness in picoplankton is accurately detected by flow cytometry. Thus, small scale variations can be detected within the time difference between taking individual samples, which may explain the occurrence of negative retention efficiencies; which were treated as zero in the analysis. Sampling of more oysters with greater numbers of samples per oyster may reduce this variability.

The location of the feeding currents, identified in Figure 3.6, agreed with the basic biology and descriptions of Quayle (1969). The current that exits anterior of the adductor muscle is likely to be related to the promyal chamber. This chamber is an identifying feature of the *Crassostrea* species (Hirase 1930, Dinamani 1971) and while its function is not well understood, it has been implicated in the ability of *C. gigas* to withstand high sediment load (Walne 1979). The mantle edge, between which the syringe needle must be placed, can be 3 - 4 mm from the point at which the anus, promyal chamber and exhalant current, discharge. This mixing of discharges may relate to the negative retention efficiencies by reflecting the excretion of picoplankton that have been concentrated in the promyal chamber and/or gut.

Variability in R.E. between oysters has previously been reported for particle sizes less than 10 μm (Table 3.1, Haven & Morales-Alamo 1966, 1970). In particular, the efficiencies reported by Ropert and Goulletquer (2000), where 2.46 μm particles were retained with 42.6 % efficiency in natural seawater but only 18.3 % in phytoplankton (natural seawater enriched with the microalgal feed species *Isochrysis galbana* and *Tetraselmis suecica*). This suggests that retention may be governed by the composition of the suspension passing through the pallial cavity. This is further supported by the results of Dupuy *et al.* (2000b) where oyster retention efficiency was reported as 'low' for particles less than 5 μm. Dupuy *et al.* (2000b) suggested that the oysters had adapted to the prevailing conditions (abundant diatoms) and were thus unable to retain picoparticles. Haven & Morales-Alamo (1970) reported that variable R.E. of particles less than 3 μm could be influenced by the rate of water flow through the experimental chamber. However, regardless of R.E. the particles less than 3 μm retained constituted the single largest fraction of natural seawater particles retained (Haven & Morales-Alamo 1970).

The concentration of particulate material has been shown to mediate retention in bivalves. Barillé *et al.* (1993), for example, describe "gill porosity being controlled by the

concentration of particulate matter" (p. 91) in *Crassostrea gigas.* Similarly, clay particles have been shown to alter retention efficiency (Sornin *et al.* 1988, Urban & Kirchman 1992) with Urban and Langdon (1984) finding "the addition of kaolinite to each of the supplemented algae/yeast diets resulted in greatly improved growth, which was comparable to that of oysters fed on a 100 % algal ration." (p. 277). In this study the concentration of inorganic particles was not assessed so the potential influence upon R.E. of picoplankton remains to be investigated. However, an assessment of potential food availability was made.

The microalgal data was used as a proxy for the concentration of what are probably the oysters primary food items. The expectation, derived from research such as that of Riera and Richard (1996) and Dupuy *et al.* (2000b), was that increasing availability of microalgae would show a decrease in picoplankton retention. During the late winter/spring period the microalgae were most abundant and, correspondingly, the retention efficiencies of picoplankton were low. The highest retention efficiencies occurred during the summer period when microalgal abundances were more variable. Neither source appeared to provide much as a food resource during the June/July period, which is probably related to the low light conditions around the winter solstice (21 June) subduing autotrophic production. Cooler temperatures and changes in the nature of inputs into the estuary from terrestrial sources may have further influenced the size and structure of autotrophic and heterotrophic plankters at this time.

The endoscopy videos published by Ward *et al.* (2000) show the dynamics of flow and retention on the plicate ctenidia of *C. virginica* and *C. gigas*. These videos suggest that variation in flow occurs very close to the ctenidia and can be variable within a short time period. The complex flow patterns visualised by Ward *et al.* (2000) also suggest that smaller particles could be more readily entrained within flows, such as that toward the primary filaments at the base of each plicae. Thus, the plicate nature of *Crassostrea* spp. ctenidia may enable the retention of picoplankton more so than other gill types such as the non-plicate, homorhabdic *Mytilus* spp. ctenidia. Medler and Silverman (2001) demonstrated that smooth muscle contraction within bivalve ctenidia led to constriction of ostia and thus restriction of water flows through the ctenidia. In combination with ciliary action, this would allow oysters to control not only the rate of flow through the pallial cavity but also the manner in which it courses through the ctenidia. Such change would

alter the hydrodynamics around the ctenidial filaments which, in turn, would alter the retention efficiency of particles of any given size.

The hydrodynamics around the ctenidia can also be altered by other factors, such as temperature and changes in the ratio of meat to volume in the mantle cavity. The effect of temperature on clearance or filtration rate has been well documented for bivalves (e.g. Winter 1978, Haure *et al.* 1998). There is an increase in clearance rate as temperature rises until a maximum is reached (19 ^{o}C in *C. gigas*; Bougrier *et al.* 1995). At this point the rate declines with any further increases in temperature. The reasons for this appear to be related to (1) changes in viscosity in relation to ciliary action (Jørgensen 1990) and (2) the limits of thermal tolerance of the cilia (Winter 1978). This is in contrast with the retention efficiencies of picoplankton found in this study which increased as water temperatures reached and surpassed 19 ^{o}C. Potentially, boundary layer effects along the ctenidia, that could deflect picoplanktonic particles, are reduced as viscosity declines with increasing temperature. Combined with complex flow patterns through the ctenidia this may lead to greater entrainment of picoplankton within the mucus layer. However, this theory fails to explain the occurrence of greater retention efficiencies outside the summer months, such as for cyanobacteria populations (Figure 3.13).

Honkoop *et al.* (2003) demonstrated that gill size and mass was "flexible" relative to the total size and mass of oyster. If the mass of gill and/or meat changes within the pallial cavity, current dynamics will also be altered. Increasing fluid volumes will lead to greater inertial forces especially if currents are simply driven by the ctenidia. Within a controlled setting (conditioning system in section 2.2.1) it has been observed that oysters in poor condition (compared to oysters in good condition on the same diet) will frequently clear loosely aggregated particle masses from within the pallial cavity by means of rapid valve compressions (pers. obs.). This accumulation of particles within the cavity and evacuation along the ventral edge (normally the location of the incurrent), suggests low meat mass leads to sedimentation of microalgal particles within the pallial cavity (as has previously been suggested by Bernard, 1974). In this study, changes in both physical and biochemical condition were determined concurrently to investigate if any change related to picoplankton retention.

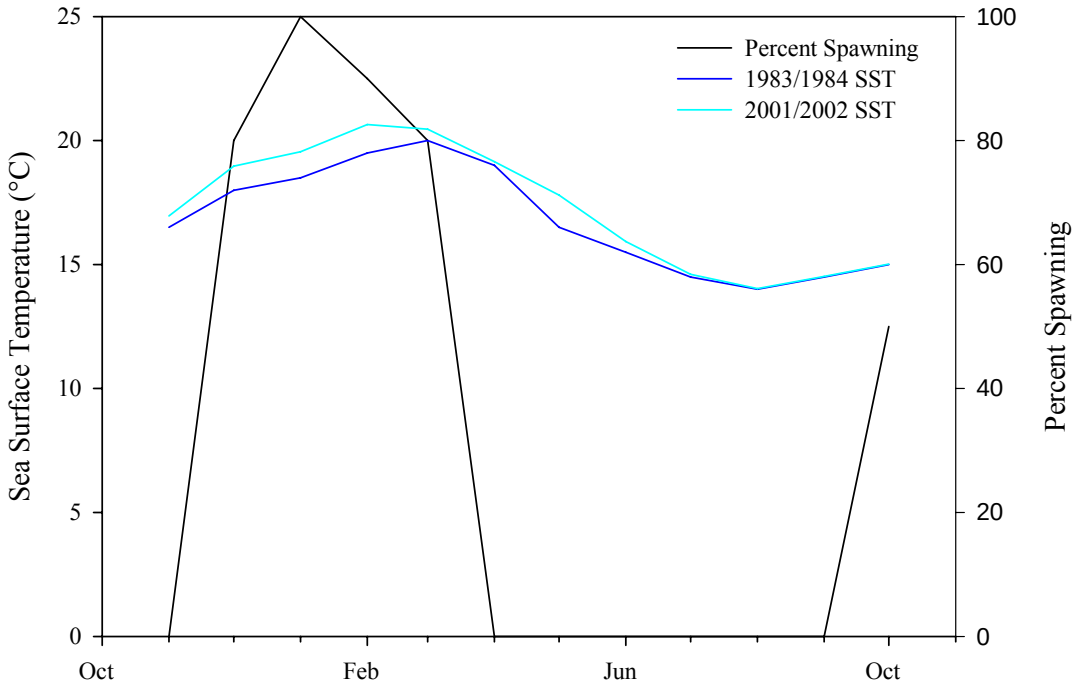


Figure 3.17 Data redrawn from Dinamani (1987) showing the correlation between sea surface temperature (SST) and the proportion of oysters either spawning or spawned. Sea surface temperature from the Leigh Marine Laboratory, used in this study (01/02 SST), has been overlaid to show the fit of annual temperature cycle between the studies.

The condition index (CI) and constituent fraction indices (CFI's) showed a clear pattern that suggested an annual cycle as has previously been reported (Askew 1972, Whyte & Englar 1982, Kaufmann *et al.* 1994, Mason & Nell 1995, Linehan *et al.* 1999, Berthelin *et al.* 2000). The primary contributor to the patterns observed is the gametogenic cycle, in particular the spawning events of spring and early summer. The extent of these changes has been highlighted by Deslous-Paoli *et al.* (1988) who attributed the reproductive effort of *C. gigas* with an energy loss of approximately 63 %. While the gonad cycle was not monitored explicitly in this study, the results of Dinamani (1987) can be mapped to the data (using the Leigh marine laboratory sea surface temperature data from the relevant years) and show a close relationship between CI, CFI's and the proportion spawning. Figure 3.17 shows the relationship described by Dinamani (1987) overlaid with the temperature data from this study. The match of the data suggests that spawning in this study could be expected to have occurred slightly earlier due to the higher spring temperatures. This fits with the observed spawnings of mid-November 2001 (Brown pers.

comm. 2002[9]). Both CI and CFI's made large declines at the time when the percent spawning/spawned could be expected to be increasing to its 100 % maximum in January.

The values for CI in this study were similar to those found by Mason and Nell (1995) for *C. gigas* in Sydney harbour. Whyte and Englar (1982) and Whyte *et al.* (1990) used a 1000x multiplier in their calculation of CI yet their CI values were still higher after correction for the multiplier. This may reflect the difference in drying technique as Whyte and Englar (1982) used freeze drying compared to oven drying in this, and the Mason and Nell (1995), study. Lawrence and Scott (1982), on whose work the CI was based, found values for *C. virginica* in the northern hemisphere winter/spring similar to that found in summer for this study. The Lawrence and Scott (1982) oysters were from temperate estuarine waters, similar to this and Mason and Nell's (1995) study thus, their results may show an interspecies difference.

Comparing the CI and CFI seasonal trends to those of picoplankton retention shows an inverse relationship. This may suggest that following spawning oysters become nutritionally stressed and, consequently, retain more picoplankton to enhance nutrient acquisition. However, as CI relates meat mass to the valve cavity volume, it is also possible that physical changes within the oysters mean they were better able to retain the picoplankton, rather than responding specifically to their changing nutritional needs. If oysters do control retention in response to nutritional needs this could be explained as the need for specific micronutrients, such as riboflavin (Seguineau *et al.* 2001) which bacteria have relative high and easily accessible quantities (Philips 1984). However, this would not explain the above average retention efficiencies of heterotrophic bacteria in September when CI and CFI's were at their highest.

The retention of particles does not necessarily mean that the particles will contribute to the nutrition of the oyster. Shumway *et al.* (1985) for example, showed particles that were cleared from the water could be rejected via the pseudofaeces or not absorbed in the gut passing in the faeces untouched. To determine the rejection of retained picoplankton was beyond the scope of this work but is recommended for future research of picoplankton utilisation by bivalves. Similarly, determination of the filtration rate is recommended for future studies to allow a more accurate determination of the potential contribution of the

[9] Simon Brown, Managing Director, Bay Oysters Ltd. Personal comments made in response to authors questions.

picoplankton populations retained. Realising these limitations however, it was possible to attempt to quantify the potential contribution of the retained picoplankton to oyster nutrition.

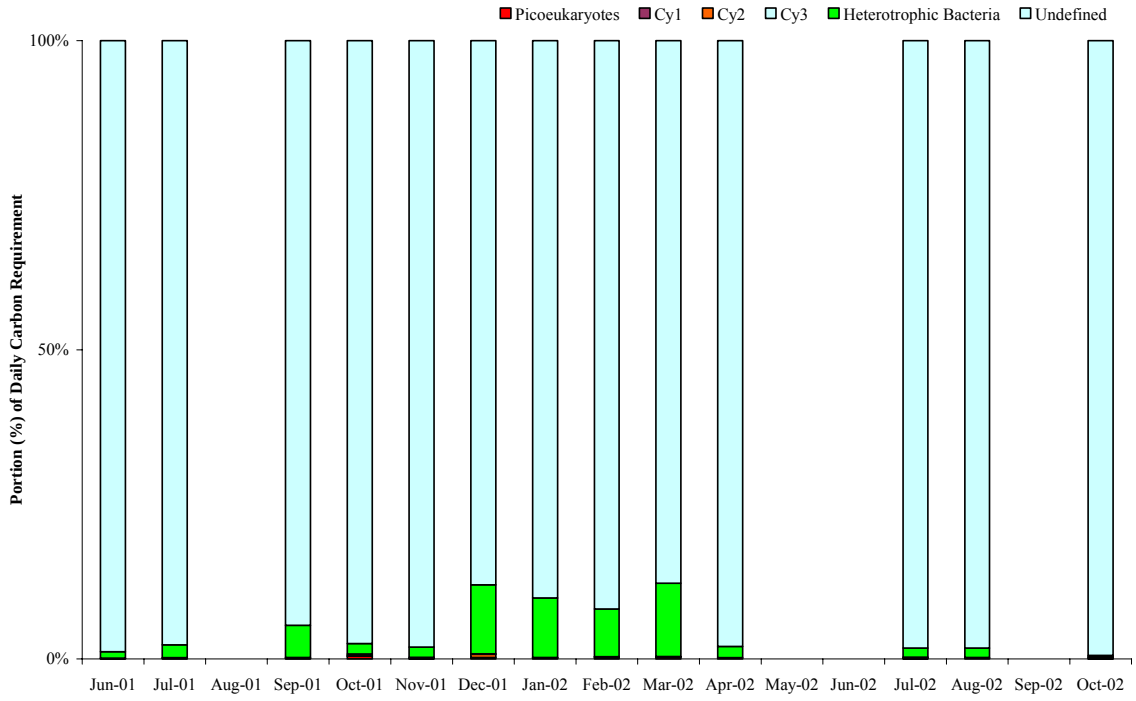


Figure 3.18 Estimated contribution of picoplankton populations to the carbon requirement of Pacific oysters. Carbon values have been calculated using published values where Picoeukaryotes = 1393 fg C cell^{-1} (Grégori *et al.* 2001); cyanobacteria (Cy1, Cy2, Cy3) = 250 fg C cell^{-1} (Campbell *et al.* 1994); Heterotrophic bacteria = 30 fg C cell^{-1} (Fukuda *et al.* 1998). Oyster carbon requirement is calculated from Crosby *et al.* 1990 (see text). Contribution to the carbon requirement has been calculated using the filtration rate of Ropert and Goulletquer (2000) and assuming R.E. remains static over a 24 hour period.

Assuming that the retained portions are utilised fully, some basic determinations of their contribution can be made if, (1) the predicted carbon requirements of oysters (Crosby *et al.* 1990), (2) the rate at which oysters filter (Crosby *et al.* 1990, Ropert & Goulletquer 2000), and (3) the standard carbon equivalents of picoplankton species (Fukuda *et al.* 1998, Grégori *et al.* 2001, Campbell *et al.* 1994) are available. Crosby *et al.* (1990) estimated a minimum carbon requirement for *C. virginica* of 2.64 x 10^{-3} mg C h^{-1} mg dry meat^{-1}, or 63.4 x 10^{-3} mg C day^{-1} mg dry meat^{-1}. Ropert and Goulletquer (2000) presented a standardised filtration rate of 2.43 l h^{-1} g dry meat^{-1} or 58.3 l day^{-1} g dry meat^{-1} for *C. gigas* and standard carbon equivalents for picoplankton biomass have already been calculated. Using this information and the retention efficiencies found in this study the nutritional

contribution, in terms of carbon, can be calculated for each picoplankton population. As can be seen from Figure 3.18 the picoplankton makes the largest proportional contribution in the summer months with the heterotrophic bacteria accounting for the majority of the contribution. In March the heterotrophic bacteria provided 12 % of the estimated oyster carbon requirement. However, if the carbon conversion value for heterotrophic bacteria is closer to 50 fg $cell^{-1}$, as suggested used by Crosby *et al.* (1990), then the contribution over the summer months would range from 12.8 (± 0.1) % in February to 19.8 (± 0.4) % in March. An evaluation of the appropriateness of carbon conversion factors to NE New Zealand estuarine waters is required to enable more accurate estimations. Similarly the potential for biovolume, and consequently size and carbon content, to vary on a seasonal basis requires further analysis as this may also be influencing the retention efficiencies.

Carbon contribution is a gross measure of nutritional worth and does not consider other potentially important constituents. Neither does it show the accessibility of the carbon for the oyster. While some cells are susceptible to enzymatic degradation, others require mechanical or chemical treatments to allow access to digestible nutrients. Bacteria have even been implicated in this process of degradation in bivalves (Brock 1989) and some authors claim that 40 % of primary production passes through bacteria on the way to secondary consumers (Ducklow 1983). Seiderer *et al.* (1984) attempted to quantify the nitrogen contribution from the bacteriolytic activity of the style in the mussel *Choromytilus meriodionalis* and concluded that up to 100 % of the nitrogen requirements could be met by bacteria. Using their C:N ration of 3.7:1 for bacteria, the filtration rate of 2.43 l h^{-1} (Ropert & Goulletquer 2000) and the retention data presented it was possible to determine that, in March, *C. gigas* were retaining 84.7 µg N h^{-1} from the heterotrophic bacteria. The nitrogen requirement for *C. gigas* - or any other *Crassostrea* spp. – has not been published therefore the significance of this is uncertain. However, Seiderer *et al.* (1984) reported nitrogen requirements of 102 µg h^{-1} and 153 µg h^{-1} for 80 and 100 mm shell length individuals, respectively. If *C. gigas* has similar requirements (average shell length for the March sample was 78 mm) then the contribution of bacterial derived nitrogen could be very significant, even if only 57 % of the bacteria could be successfully lysed (Seiderer *et al.* 1984)

The temporal changes in retention efficiency are a notable finding of this study. This may mean that previous research can only be compared amongst oysters of the same physiological state or having endured the same environmental history. The implications of

temporally variable retention, however, stretch beyond academic considerations. For industry the implications are important in helping to understand some of the factors driving oyster condition and survival (particularly spat laid-out in the summer months) and when developing protocols for closed circuit sea water systems for hatchery developments such as, secure facilities for "improved" broodstock (Wikfors 2004).

Protocols for the conditioning of broodstock have been developed in hatcheries that utilise flow-through (unlimited exchange) seawater systems. The feeding ration in such systems is thus a supplement to that food not filtered from the water. This ration is usually based on the equation published by Utting and Spencer (1991) where 6 % of the initial dry meat weight of the oysters is feed in dry weight of microalgae on a daily basis. Even with careful selection of microalgal species, when used in a recirculating seawater system, the reduced diversity and lack of seasonal variation in pico-sized particles may make this ration inadequate for the purpose of conditioning or even just maintaining oysters. The initial condition of the oyster, its feeding history and water temperature may all interact to determine when picoplankton (or components of it) are required to achieve the desired level of nutrition for the oyster.

Of the picoplankton populations retained the heterotrophic bacteria appear to have the largest potential contribution. However, it is clear from previous research that retained bacteria are not necessarily digested and may, in fact, assist in the acquisition of nutrients by digesting food particles into nutrients more readily assimilated by the oyster. The following chapter describes investigations into the occurrence and possible role of bacteria in the oyster gut.

Chapter 4.0
Investigation of the Bacterial Microflora of the Oyster Gut

4.1 Introduction

The most significant portion of the picoplankton retained by feeding oysters was the heterotrophic bacteria (Chapter 3). The heterotrophic bacteria are comprised of a diverse array of organisms and have been estimated to be responsible for fifty to sixty percent of total oceanic carbon cycling (Newell & Linley 1984). Microorganisms, such as microalgae and bacteria, have generally been "considered of better nutritive value because they contain larger quantities of readily digestible molecules and essential micronutrients" (p. 577, Charles *et al.* 1999). Heterotrophic bacteria have long been considered important in food cycles (ZoBell & Feltham 1938) and in particular, are thought to provide essential nutrients, such as B-complex vitamins or other growth factors, lacking in microalgae (Phillips 1984, Douillet & Langdon 1993). Moreover, ZoBell and Feltham (1938) have reported that mussels (*Mytilus californianus*) fed only bacteria for several months continued to grow, and Kharlamenko *et al.* (2001) has reported a "notable contribution of benthic bacteria" (p. 115) to the diet of the infaunal bivalve *Ruditapes philippinarum*.

Bacteria, however, do not supply what would generally be considered a complete diet for bivalves. In particular, marine bacteria provide little of the 20:5n-3 and 22:6n-3 polyunsaturated fatty acids (Perry *et al.* 1979, Phillips 1984, Brown *et al.* 1996) considered essential for bivalves (Soudant *et al.* 1997, Jonnson *et al.* 1999, Caers *et al.* 2002). Moreover, in contrast to ZoBell and Feltham's (1938) research with mussels, studies of Pacific oysters (*Crassostrea gigas*) have shown that larval cultures and adult oysters could not be maintained solely on a food supply of isolated bacterial strains (Douillet & Langdon 1993). However, bacterioplankton, supplied alongside microalgae, appear to be beneficial to oyster growth. Bacteria contain protein and amino acid profiles similar to those of *C. gigas* larvae and also provide large quantities of nucleic acids (4 - 6 % in bacteria compared to 1 - 3 % in microalgae) (Brown *et al.* 1997). The advantages of feeding bacteria to bivalves have been demonstrated by research such as Martin and Mengus (1977) and Douillet and Langdon (1993). The latter researchers showed improved growth

and survival of *C. gigas* larval cultures containing the CA2 strain of bacteria (presumptively identified as *Alteromonas* sp.), with larvae fed the bacterial strain retaining in excess of 140 % of their estimated active carbon metabolic requirement.

The efficiency with which bivalves absorb the nutrients contained within the bacterioplankton is likely to be species specific. However, Crosby *et al.* (1990) have reported assimilation efficiencies of 52.5 % and 57.2 %, respectively, for bacterial carbon and nitrogen for *Crassostrea virginica*. Charles *et al.* (1999) suggest a "global average absorption efficiency of bacterioplankton by suspension-feeding bivalves" (p. 582) to be approximately 60.4 ± 13 % (mean ± SD). This contrasts with a global average absorption efficiency for non-green microalgae of 83.8 ± 3.3 % (Charles *et al.* 1999), or for individual species (mean (95 % confidence) of 73.6 (63.8 – 82.3) % for *Isochrysis galbana*, 72.5 (69.2 – 75.8) % for *Thalassiosira pseudonana* and 6.5 (2 - 13.2) % for *Platymonas suecica* (Romberger & Epifanio 1981).

McHenry and co-workers (McHenry *et al.* 1979, McHenry & Birkbeck 1985) have asserted that the primary function of lysozyme-like enzymes in the crystalline style and digestive gland of several bivalve species is to digest bacteria. Indeed, Kristensen (1972) suggests that α-amylase of the bivalve style is active in releasing microorganisms from sand grains and detrital particles. In the case of *Mytilus edulis,* the digestion of microorganisms is initiated extracellularly in the stomach and completed intracellularly in the digestive gland (McHenry & Birkbeck 1985).

Romero *et al.* (2002) defined two components to the bacteria associated with Chilean oysters (*Triostrea chilensis*), firstly the allochthanous bacteria that pass through the gut and, secondly, the autochthonous bacteria that are relatively permanent. Thus, the bacteria not absorbed may still be contributing to bivalve nutrition by, for example, fixing nitrogen for protein nutrition, or aiding the digestion and assimilation of microalgae (Douillet & Langdon 1993). Prieur (1982) reported that oyster larvae had a bacterial flora that predominantly produced extracellular enzymes such as proteases and lipases. Brock (1989) indicated that detritus is a negligible energy source for bivalves unless there is some bacterial mediation after finding the significant cellulolytic activity in *C. gigas* was bacteria derived. Similarly, it has been suggested that successful utilisation of the detrital resource by bivalves would require that the high C:N ratio of the detritus be balanced by the N provided by protein rich bacteria (Newell & Field 1983, Seiderer *et al.* 1984).

The presence of a commensal bacterial flora in bivalves has not been well described, although it is accepted that healthy bivalves will have bacteria in their body fluids and tissues (Olafsen *et al.* 1993). Most bacteria associated with bivalves are considered to be transient, having an incidental, rather than obligatory, relationship with their host (Simon & McQuaid 1999). The *Cristispira*, however, are specifically associated with the crystalline style of bivalves (Bernard 1970, Tall & Nauman 1981, Simon & McQuaid 1999). These large spirochaetes (0.5 - 3 µm x 30 - 180 µm) are specialised to the viscous crystalline style environment and, although knowledge of their physiology is limited, direct observation has shown them to be able to degrade carbohydrates in the style matrix (Judd 1977). Simon and McQuaid (1999) suggest the obligate relationship between the style spirochaetes and their host bivalves leads to increased quantities of carbohydrases but no greater diversity of enzymes. In contrast, Seiderer *et al.* (1987) examined *M. edulis* and noted "The presence of bacteria-like cells in the outer lamina of the crystalline style, coupled with the non-filterable nature of the lytic agent make it seem possible that these bacteria are responsible for lysis of gram-negative bacteria which are taken in with the food supply" (p. 213).

Other bacteria have been identified in bivalves including *Spirillum ostrea* (Margulis *et al.* 1991), *Pseudomonas*, *Flavobacterium-Cytophaga*, *Vibrio, Achromobacter-Alcaligenes*, (Murchelano & Brown 1968) unidentified gram negative rods and spirochaetes (Harwood & Canale-Parola 1984). In *C. gigas*, *Vibrio* spp., *Pseudomonas* spp., *Alteromonas* spp. *Acinetobacter* spp. and *Aeromonas* spp. have been isolated as dominant bacteria, using *in vitro* techniques (Colwell & Liston 1960, Kueh & Chan 1985, Olafsen *et al.* 1993), whereas *Arcobacter* spp. have been identified as a common component of *T. chilensis* gut flora using molecular techniques (Romero *et al.* 2002).

As discussed in Chapter 2 it appeared that a diet based only on microalgae may not provide sufficient nutrition to oysters. Comparing the relevant literature to the method of conditioning utilised, it appeared that additional material could be supplied from the fraction of seawater smaller than 10 µm. In Chapter 3 the retention of the picoplankton was examined. The results from this work suggested that picoplankton were retained with low efficiencies although the nutritive value of the retained particles may be important. It appeared that selection based on nutritive requirements was occurring when picoplankton were found to be retained with greater efficiency in the post-spawn, summer months where

oysters endure nutritive stress. Of the picoplankton populations monitored, the heterotrophic bacteria were retained in the largest quantities either in terms of cell number or carbon. Consequently, the aims of the work described in this chapter were to;

1) Investigate the occurrence of bacteria in the gut of the Pacific oyster;

2) Investigate the temporal variability in oyster gut microflora;

3) Investigate the characteristics of the culturable bacteria isolated from the oyster gut

4.2 Materials and Methods

4.2.1 General

Unless otherwise stated all chemicals and reagents were supplied by BDH (Poole, England) and of AnalR grade. Microbiological media were supplied by DIFCO Laboratories. Solutions and media were prepared with distilled water (dH_2O) and sterilisation was achieved by autoclaving (121 °C, 15 psi, 15 min).

4.2.2 Comparison of the culturable gut microflora of wild and hatchery oysters

Two oysters were removed from the conditioning system of the hatchery (after 8 weeks of conditioning) and held at 1 °C overnight. On the same day three oysters were taken from the Rangitane lease in Kerikeri Inlet, packed in ice and sent to Auckland. When the Kerikeri sample reached the lab each oyster was cleaned of fouling material and the top shell removed. The meat was removed and cut transversely, halfway between the adductor muscle and anterior tip, with a sterile scalpel. The gut contents were sampled by using a 200 µl autopipettor (fitted with a sterile tip) to withdraw the content from the exposed gut lumen. The extracted gut content was added to an enrichment media of sterile salt peptone water (9 ml; 1 % (w/v) Peptone, 0.3 % (w/v) NaCl (Donovan & van Netten 1995)) and held at room temperature for six hours, then overnight at 1 °C. A 1 ml sample of seawater from the conditioning system was taken with a sterile 1 ml syringe, added to sterile salt peptone water and held at room temperature for six hours, then overnight at 1 °C.

Aliquots (100 µl) of each sample were added to plates of culture media and spread with a flame sterilised glass rod spreader. Six types of culture media (Table 4.5) were used for the spread plates based upon the findings of Colwell and Liston (1960) and the recommendations of staff at the Crop and Food, Seafood Research Unit. The hatchery oyster gut and seawater samples were spread on Marine, SPCA, Basal, YSW, YDW and 2B media whereas the wild samples were spread on Marine, SPCA, Basal and YSW media. Multiple media types were used to attain as much diversity as possible but YDW and 2B plates became contaminated before use providing only enough for the hatchery, seawater and blank control plates. For each media type, at least 1 blank control and two replicate plates were inoculated from each sample.

The plates were incubated (20 °C) and the colonies were counted 24 and 48 h after inoculation. After 48 hours those colonies deemed to be representative of each plate were

described (colony size, shape and colour) then removed from the plate with a flame sterilised loop and streaked onto marine agar plates. These plates were incubated (20 °C, 48 h) then an isolated colony was removed and streaked onto a new marine agar plate. To ensure purity of isolation the colony morphology was observed and cell morphology and gram reaction were determined (Appendix 4.2). Isolates were maintained on marine agar at 15 °C as stock culture for tests to allow for taxonomic characterisation.

Isolates were tested for arginine dihydrolase activity, catalase activity, citrate utilisation, gelatin hydrolysis, fluorescent pigmentation, nitrate reduction and denitrification, oxidase activity, oxidation/fermentation catabolism, phenylalanine deaminase activity, hydrogen sulphide production, starch utilisation, and urease activity according to accepted methods described in Gerhardt *et al.* 1994. This information provided an initial biochemical profile.

This profile was further refined by using the bioMérieux api 20E identification kit. For each api20E test strip a colony was resuspended and mixed thoroughly in a 3 % (w/v) NaCl solution (5 ml). Aliquots were then added to the pre-labelled test strips as per instructions (api 20E, Cat No. 008040-6; 02/02) and the strips incubated (15 °C, 48 h). The lower temperature, and 24 hour longer incubation, was used as marine species are often intolerant of the 35 – 37 ºC temperatures recommended for incubation by the kit. Presumptive identifications were made where possible using api identification sheets (API 20E 1994) and the Bergey's manual (Holt 1994).

4.2.3 Pilot investigation of the occurrence of gut microflora in wild oysters

Bacterial culture techniques are known to isolate only a small subset of bacteria. Therefore, two pilot scale investigations of oyster gut microflora were made to assess culture independent techniques for 1) indicative molecular profiling of the microflora and 2) visually locating gut microflora.

4.2.3.1 Molecular investigation of gut microflora

Six oysters were sampled from Kerikeri Inlet on the low tide and sent to Auckland on ice. On arrival the top shell was removed by cutting the adductor muscle away from the shell. The meat was then removed from the oyster and the gut dissected away from the other tissues using flame sterilised scalpel and forceps. The dissected gut was then placed in a pre-weighed, sterile Falcon tube (50 ml), and an equal amount (w/v) of ice cold TEx10 (Table 4.1) added (Romero *et al.* 2002). The sample was then homogenised on ice with a

Polytron blender (PT10-35, Kinematica, Switzerland) at 60 % (approx. 1800 rpm) for 1 minute. The homogenate was diluted to 10 % (w/v) in TEx10/SDS buffer (Table 4.1), shaken vigorously and placed in a water bath (70 °C, 20 min). Once removed from the water bath the sample was shaken to ensure homogeneity, then aliquoted (1 ml) into Eppendorf tubes (2 ml). One tube was immediately subjected to DNA extraction (Section 4.2.4.3.1), and the remainder stored at -80 °C. 16S rDNA fragments were amplified and analysed as described in Section 4.2.4.3.

4.2.3.2 Histological investigation of gut microflora

Three oysters were sampled at half tide (3 hours after low tide) and three oysters sampled at high tide (6 hours after low tide). All of the oysters were from the Rangitane leases in Kerikeri Inlet and had been allowed to remain *in situ* (on the oyster racks) until they were sampled. Immediately after removal from the oyster rack the sampled oysters were cleaned of fouling debris in the surrounding seawater. The meat was removed by cutting the adductor muscle away from the shell using a shucking knife and was placed in individually labelled polycarbonate specimen bottles (100 ml). Fixative (paraformaldehyde; 10 % (w/v) in dH_2O) was then added in sufficient volume to cover the tissue. These samples were held for a minimum of 48 hours in fixative before processing for histological examination (Section 4.2.4.2).

4.2.4 Temporal variation in bacterial presence and diversity in the oyster gut

The pilot study was expanded into a larger study whereby simultaneous sampling was conducted for both histological and molecular investigations of bacterial flora in the oyster gut. In this investigation, 36 oysters were collected at low tide from lease 182 (Figure 3.1) and taken to the Rangitane wharf. Six oysters were immediately sampled (low tide sample, LT + 0) and the remaining thirty added to a flow through seawater tank (Section 3.2.2.1). Six actively feeding oysters were removed 2, 4 and 6 hours after being placed in the seawater tank (hereafter described as LT + 2, LT + 4, LT + 6). Sampled oysters were processed and analysed as described in sections 4.2.4.1 to 4.2.4.3.

4.2.4.1 Sample collection and processing

4.2.4.1.1 Oysters

At each sampling interval six oysters were cleaned of fouling debris in sea water downstream of the flow through tank. Three oysters were processed for histology by removing the meat and placing it in individually labelled polycarbonate specimen bottles

(100 ml). Fixative (paraformaldehyde; 10 % (w/v) in dH_2O) was then added in sufficient volume to cover the tissue and the samples held for at least 48 hours in fixative before processing for histological examination (Section 4.2.4.2).

The remaining three oysters were processed for molecular analysis by dissecting the gut away from the surrounding tissues using flame sterilised scalpel and forceps. The dissected gut was then placed in a sterile, pre-weighed Falcon tube (15 ml), tightly capped and held in ice until they could be homogenised. Homogenisation followed the method of Romero *et al.* (2002) where each sample was weighed and an equal amount of ice cold TEx10 (Table 4.1) added. The sample was then homogenised on ice with a Polytron blender (PT10-35, Kinematica, Switzerland) at 60 % (approx. 1800 rpm) for 1 minute. The homogenate was diluted to 10 % (w/v) in TEx10/SDS buffer (Table 4.1), shaken vigorously and placed in a water bath (70 °C, 20 min). Once removed from the water bath the sample was shaken to ensure homogeneity, then aliquoted (1 ml) into Eppendorf tubes (2 ml). One tube was immediately subjected to DNA extraction (Section 4.2.4.3.1), and the remainder stored at -80 °C. 16S rDNA fragments were amplified and analysed as described in Section 4.2.4.3.

Table 4.1 Solutions used in the extraction of DNA from samples.

Buffer	**Composition**	**Reference**
TEx10	0.1 M Tris 0.01 M EDTA 0.15 M NaCl pH 7.8	Romero *et al.* 2002
TEx10/SDS	1 % SDS (w/v) 0.1 M Tris 0.01 M EDTA 0.15 M NaCl pH 7.8	Romero *et al.* 2002
TNE	10 mM Tris-HCl 10 mM NaCl 10 mM EDTA pH 8.0	Woo *et al.* 1992
TNE/X-100	2 % Triton X-100 (v/v) 10 mM Tris-HCl 10 mM NaCl 10 mM EDTA pH 8.0	Woo *et al.* 1992

4.2.4.1.2 Seawater concentration

To compare the bacteria in the gut contents with that in the ambient seawater, 40 l of seawater was collected. Two 20 l plastic containers that had been pre-cleaned (chlorinated with 1 % (v/v) sodium hypochlorite (15 %; Andrews Industries) and dechlorinated with sodium thiosulphate solution (1N, 0.25 % (v/v)) were filled with seawater at the time of the last sampling and held at 4 °C until they could be concentrated.

The seawater was concentrated to obtain practical quantities of DNA for analysis. This was done using hollow fibre filtration with a molecular cut-off of 80,000 kDa (Fresenius polysulfone capillary dialyser, HF80S) (Roberts 2002). The filter was prepared by recirculating sterile elution buffer 1 (0.5 % (w/v) Tween 80, 175 mM $Na_2HPO_4.2H_2O$, 4.8 mM NaH_2PO_4) through the filter (Figure 4.1) until a small amount of buffer could be seen in the filter housing. The pump (Watson & Marlow 302F peristaltic) was then disconnected, the filter assembly aseptically packaged and held overnight at 4 °C on a tilting platform. Excess fluid was drained from the filter prior to the setup being reassembled as per Figure 4.1.

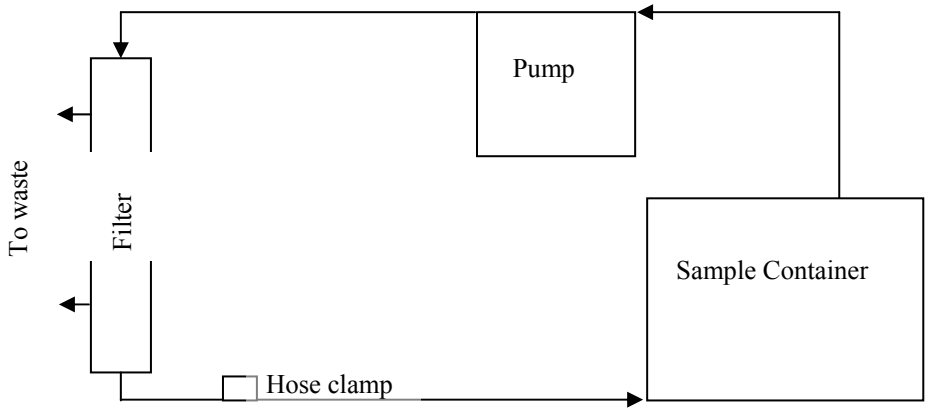


Figure 4.1 Diagram of the filter (Fresenius HF80S polysulfone capillary dialyser) and pump (Watson & Marlow 302F peristaltic) setup for the concentration of seawater samples. The hose clamp controls the flow and applies back-pressure to the filter.

Seawater samples were concentrated within 48 hours of sampling by recirculating through the filter with filtrate discharged to waste. Once the concentrated sample was reduced to approximately 500 ml, 100 ml was set aside and aliquoted into labelled sterile Falcon centrifuge tubes (50 ml). The remaining 400 ml was further concentrated with the addition

of elution buffer 2 (250 ml; 1 % Tween 80, 175 mM $Na_2HPO_4.2H_2O$, 4.8 mM NaH_2PO_4) until it was approximately 100 ml in volume. The sample was then aliquoted into labelled, sterile, Falcon centrifuge tubes (50 ml). All tubes were centrifuged (10,000 *g*, 30 min) and supernatant removed. The pellet was resuspended in sterile distilled water and the suspension aliquoted (1 ml) into labelled, sterile, Eppendorf centrifuge tubes (1.5 ml). All tubes were held at -80 °C until molecular analysis could be performed (Section 4.2.4.3).

4.2.4.1.3 Oysters sampled for their crystalline style

The crystalline style has been identified as the location of a commensal microflora (Bernard 1970, Judd 1977, Tall & Nauman 1981) of unculturable spirochaete bacteria (*Cristispira* sp.) in bivalves, including Pacific oysters. Oysters sacrificed for gamete condition analyses in the hatchery had the style removed for observation and photography at 400x magnification on a Nikon LaboPhot light microscope.

It was not possible to separate the style and gut samples in the field without risk of further contamination or excessive loss of sample. Therefore, to provide a comparison of the RFLP pattern of the style specific microflora a separate collection of thirty-six oysters was made at low tide, from the Biomarine Ltd oyster farms in Te Kapa Inlet, Mahurangi Harbour. As the style dissolves rapidly once feeding stops these oysters were cleaned of fouling material and left to feed in a flow-through seawater tank at The University of Auckland, Leigh Marine Laboratory overnight. The oysters were removed from the tank after approximately 20 hours (24 hours post-harvest) and opened by cutting the adductor muscle away from the shell.

The meat was removed from the shell and a cut made half way between the adductor muscle and anterior tip with a flame sterilised scalpel. The style was removed from the incision with flame sterilised forceps and placed in a pre-weighed, sterile, Falcon tube (50 ml). Of the 36 oysters sampled, styles were recovered from 30 and added to the same Falcon tube to provide a pooled sample. The sample was pooled as the density of *Cristispira*-like spirochaetes was expected to be low. The composite style sample was weighed and an equal amount (w/v) of cold, sterile, TEx10 buffer (Table 4.1) added. The solution was gently swirled until the styles had dissolved then diluted to a 10 % (v/v) solution with sterile TEx10/SDS (Table 4.1). The sample was then heated to 70 °C for 20 min and stored on ice until it could be aliquoted into sterile Eppendorf tubes (2 ml) and stored at -80 °C awaiting molecular analysis (Section 4.2.4.3).

4.2.4.2 Histological analysis

For histological examination of sampled oysters sections of approximately 3 mm thickness were cut by hand, with a sterile scalpel, at anterior, mid and posterior positions (Figure 4.2). The sections were placed in cassettes for embedding and blocking in paraffin wax. The paraffin blocks containing the hand sections were cut with a microtome into sections (7 µm) which were mounted on slides, dewaxed in xylene, then stained using either, Ehrlich's Haematoxylin and Eosin stain, or Gram's staining methods (Appendix 4.1). The Gram staining method was not used in the larger study as it had failed to adequately stain sections in the pilot scale study. As adjacent sections were stained with the different methods it was possible to make direct comparisons which confirmed that none of the bacteria had stained.

For every hand section at least 1 thin section was stained and all mounted sections were sealed under a coverslip. Sections were observed using a Nikon Labophot-2 compound microscope with a maximum magnification of 1000x using oil immersion. Photographs of relevant sections were made using a Leica DMRE microscope fitted with a Leica DC500 digital camera. Images were acquired with Leica IM1000 software and processed with Adobe Photoshop LE.

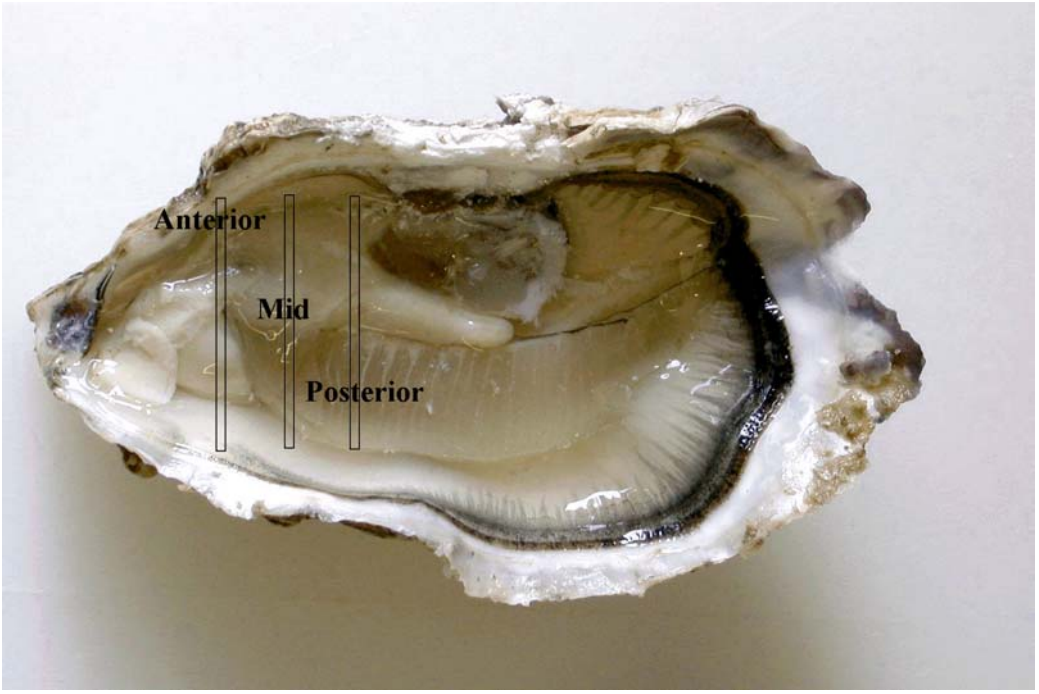


Figure 4.2 Pacific oyster with the top shell and upper mantle removed showing the location of the hand sections made for histological analysis. (Photo credit: R. Wong)

4.2.4.3 Molecular analysis

4.2.4.3.1 DNA extraction

DNA, from oyster gut homogenates, was extracted using the Phosphate, SDS, Chloroform, Bead Beater (PSC-B) method of Miller *et al*. (1999). For each sample 500 µl was added to a bead beater vial (2 ml screw cap vial containing 0.5 g of 0.1 mm and 0.5 g of 3 mm silica-zirconium beads). Phosphate buffer (300 µl, 100mM NaH_2PO_4) and SDS lysis buffer (300 µl; 100 mM NaCl, 500 mM Tris, 10 % (v/v) SDS) were then added and the tube capped and mixed by inversion. Chloroform-isoamyl alcohol (300 µl; 24:1 v/v) was added to the mixture and the vials were shaken in a FastPrep (Bio101) machine (4.5 m sec^{-1}, 40 sec). Once shaken vials were centrifuged (15,000 *g,* 5 min) and the supernatant transferred to sterile, labelled Eppendorf tube (1.5 ml).

Ammonium acetate (7 M) was added to the sample to achieve a final concentration of 2.5 M. The solution was mixed then centrifuged (15,000 *g*, 5 min) and the supernatant transferred to a new sterile, labelled Eppendorf tube (1.5 ml). Isopropanol (0.54 v/v) was then added and the sample incubated at room temperature (15 min). The sample was then centrifuged (15,000 *g*, 5 min), the supernatant discarded and the pellet washed with 70 % ethanol (1 ml). The sample was then centrifuged (10,000 *g*, 5 min) and the pellet air dried. Once dry, the pellet was resuspended in 100 µl dH_2O and stored at 4 °C.

4.2.4.3.2 PCR

Polymerase chain reaction (PCR) was used to amplify the 16S rDNA region of bacterial DNA between the forward primer PB36 and reverse primer PB38 (Table 4.2). These primers were tested for generality by submitting the sequences for a probe match of the Ribosomal Database Project website (http://rdp.cme.msu.edu/index.jsp). Matches (100 %) were found for representatives of all the bacterial phyla with the greatest numbers of matches found for the Proteobacteria, Firmicutes, Actinobacteria and Bacteriodetes.

Table 4.2 Primer sequences for amplification of 16S rDNA. Nucleotide ambiguity codes: R = Purine (A & G), Y = Pyrimidine (C & T), M = Amino (C & A), K = Keto (T & G).

Primer	Sequence (5' – 3")	Position on *E. coli* K12 16S rDNA sequence
PB36	AGRGTTTGATCMTGGCTCAG	8
PB38	GKTACCTTGTTACGACTT	1509

For each sample, extracted DNA (5 µm) was added to PCR master mix (45 µl, Table 4.3) in an Eppendorf, thin walled PCR reaction tube (200 µl). PCR was carried out using an Eppendorf Mastercycler Gradient PCR machine. The cycling conditions for the PCR were; denaturation (94 °C, 3 min), 30 cycles of [94 °C, 30 sec; 55 °C, 45 sec; 72 °C, 90 sec] and a final extension (72 °C, 7 min). Optimisation tested $MgCl_2$ concentrations of 2, 4, 6 and 8 mM and cycle number from 25 to 35 cycles by comparing PCR product quantity and quality. $MgCl_2$ concentration made no noticeable difference so was left at 2 mM. 30 cycles were used for most PCR's from raw extracts (where bacterial DNA concentration was low) however, 25 cycles were used for DNA extracts from colonies (where bacterial DNA content was high). Positive and negative controls were run alongside all samples. Positive controls were bacterial DNA extracted from an unidentified marine isolate cultured from seawater samples.

A sample (1 µl) of the resultant PCR product was analysed with a NanoDrop ND-1000 (NanoDrop Technologies) to appraise the quality (260/280 nm) and quantity (ng μl^{-1}) of DNA before other analyses were conducted.

Table 4.3 Master mix for PCR amplification of DNA extracted from oyster gut. All reagents are from Invitrogen.

Reagent	Initial concentration	Final concentration
Buffer (100 mM Tris HCl pH 8.3, 500 mM KCl)	10x	1x
$MgCl_2$	25 mM	2 mM
dNTP	25 mM	0.1 mM
PB36 primer	10 µM	0.2 µM
PB38 primer	10 µM	0.2 µM
Platinum-Taq	5 U μl^{-1}	0.75 U
H_2O	To make final volume 50 µl/reaction	

4.2.4.3.3 PAGE analysis

Polyacrylamide gel electrophoresis (PAGE) was used to visualise the PCR product from each sample. Polyacrylamide (6 %) gels (Table 4.4) were cast between glass plates. Once set, the plates were loaded in a BioRad Mini-PROTEAN® 3 Cell gel box with 1x TBE (89 mM Tris-borate, 2 mM EDTA, pH 8.0) buffer. PCR product (3 µl) was combined with loading dye (2 µl; 15 % (w/v) Ficoll 400, 0.1 % (w/v) Bromophenol Blue) and added to the wells at the top of each gel. To estimate the size of the PCR product, an aliquot (3 µl) of 1 kb plus DNA ladder (Life Technologies, 1:10 (v/v) H_2O) was combined with loading dye

(2 µl) and added to the left well of each gel. Gels were run for 40 minutes with a 100 V current (regulated with a BioRad PowerPac Basic).

Table 4.4 Composition of polyacrylamide gels.

Reagent	**Final Concentration in dH2O**
30% (w/v) Acrylamide/Bis-acrylamide (29:1) (Sigma A 3574)	6 %
20% (w/v) Ammonium persulphate (Sigma A 3678)	0.0011 %
N,N,N',N'-tetramethylethylenediamine (TEMED; Sigma 87689)	0.0012 %
20 x TBE (1.78 M Tris-borate, 40 mM EDTA, pH 8.0)	5 %

Gels were removed from the gel box, stained with 1 % ethidium bromide solution (15 min), and destained in water (15 min). Visualisation and imaging of gels was carried out by UV transillumination (Ultraviolet Products Gel Documentation System). Images of each gel were captured using Imagestore 7500 software and the images stored in tagged image file (.tif) format.

4.2.4.3.4 RFLP

The Restriction Fragment Length Polymorphism (RFLP) technique was used to assess the diversity of flora in the gut, style and seawater samples subjected to PCR. Digestions of 16S rDNA PCR product were made with *Hae* III endonuclease (10 U μl^{-1}; Invitrogen) cutting at 5'-GG↓CC-3' 3'-CC↑GG-5'. *Hae* III (2 µl), PCR product (10 µl) and reaction buffer (REact 2; 2 µl) were combing in 200 µl thin walled PCR tubes and the resulting digests were incubated overnight at 37 °C. An aliquot of digest (2 µl) was combined with loading dye (3 µl; 15 % (w/v) Ficoll 400, 0.1 % (w/v) Bromophenol Blue) and run on an agarose gel (1.5 % (w/v) in 1 x TBE). A 1 kb plus DNA ladder (3 µl; Life Technologies, 1:10 (v/v) dH_2O) was combined with loading dye (2 µl) and added to the left well of each gel. Gels were run for 60 min with a 120 V current (regulated with a BioRad PowerPac Basic). Gels were removed from the gel box, stained with 1 % ethidium bromide solution (15 min), and destained in water (15 min). Gels were visualised by UV transillumination (Ultraviolet Products Gel Documentation System) and imaged using Imagestore 7500 software. Images were stored in tagged image file (.tif) format.

4.2.5 Characterisation of the culturable bacteria from the guts of wild oysters

To investigate of the diversity of the gut microflora, culturable bacteria were isolated from gut samples and characterised as follows.

4.2.5.1 Oyster sampling

Oysters (6) were delivered, on ice, from Kerikeri Inlet approximately 18 hours after they had been harvested, on the previous low tide. The oysters were cleaned of fouling debris and the top shell removed by cutting the adductor muscle. The meat was removed from the shell and, with flame sterilised scalpel and forceps, the mantle, gill, heart, adductor muscle and as much gonad as possible were removed to leave the gut, digestive diverticular and some surrounding tissue. The dissected gut was placed in a sterile, labelled glass tube (15 ml) on ice and homogenised using a flame sterilised glass rod. The homogenate was then transferred to labelled, sterile, Eppendorf tubes (2 ml) and stored at -80 °C until sample analyses could be initiated.

4.2.5.2 Bacterial isolation and culture

For each sample, an aliquot (10 µl) was added to each of three types of agar plate (marine, starch and cellulose media; Table 4.5). The plates were incubated at 20 °C and colony counts made at 24 and 48 hours after inoculation. After 48 hours those colonies deemed to be representative of each plate were described (colony size, shape and colour) and removed with a flame sterilised loop. The colony was then streaked onto marine agar plates, incubated (20 °C, 48 h) and an isolated colony removed. To ensure a pure culture the isolated colony was re-streaked onto marine agar and the cell type and gram reaction described (Appendix 4.2).

Initial characterisation of the isolated colonies was achieved by streaking an isolated colony onto one plate of each media type (Marine, Starch, Cellulose; Table 4.5). Four isolates were grown in separate quadrants on each plate so incubation temperature was lowered to 15 °C to slow growth and incubation time reduced to 24 hours. The degree of growth on the different media was recorded. An isolated colony from the marine plate was removed and streaked into a slant tube of marine agar (5 ml). The slant tube was incubated (15 °C, 24 h) then held at 1 °C as a stock culture for future analysis.

Table 4.5 Media used for culturing, isolating and determining nutrient utilisation, of bacteria from samples of oyster gut. Composition of media is g l^{-1} in dH_2O (except YSW where 0.2 µm filtered seawater was used) with ingredients dissolved by boiling prior to sterilisation of the media. Difco catalogue numbers are included were available as (#xxxx).

Media	Media Composition	Culture and Determinative Methods Used	Uses
2B agar	1 g l^{-1} Bacto peptone 1 g l^{-1} Bacto yeast extract 1 g l^{-1} Bacto beef extract 18 g l^{-1} Bacto agar (#0140)	Spread	Culture for colony counts and description
Basal agar	5 g l^{-1} Yeast extract 8 g l^{-1} Nutrient agar (#0001) 5 g l^{-1} Bacto agar (#0140) 10 g l^{-1} Glucose	Spread	Culture for colony counts and description
Marine agar	37.4 g l^{-1} Marine Broth 2216 (#0791) 15 g l^{-1} Bacto agar (#0140)	Spread and streak	Culture for colony counts, description and isolation
Salt plate count agar (SPCA)	30 g l^{-1} Plate count agar (#0479) 10 g l^{-1} NaCl	Spread	Culture for colony counts and description
Yeast salt agar (YDW)	5 g l^{-1} Yeast extract 10 g l^{-1} glucose 10 g l^{-1} NaCl 15 g l^{-1} Bacto agar (#0140)	Spread	Culture for colony counts and description
Yeast seawater agar (YSW)	5 g l^{-1} Yeast extract 10 g l^{-1} Glucose 15 g l^{-1} Bacto agar (#0140) (uses 0.2 µm filtered seawater)	Spread	Culture for colony counts and description
Cellulose agar	37.4 g l^{-1} Marine Broth 2216 (#0791) 15 g l^{-1} Bacto agar (#0140) 5 g l^{-1} Carboxymethyl cellulose	Spread or; Streak, incubate, then flood plate with Congo Red (C.I. 22120, 0.1% w/v), after 5 minutes drain excess and record the presence or absence of stain. Absence indicates cellulose degradation.	Culture for colony counts, description and nutrient utilisation
Protein agar	18.2 g l^{-1} R2A agar (# 1826) 7.2 g l^{-1} Skim milk (Mainland Dairy)	Direct observation of the occurrence of clear zones in the agar. Clear zones indicate protein degradation.	Nutrient utilisation
Starch agar	37.4 g l^{-1} Marine Broth 2216 (#0791) 15 g l^{-1} Bacto agar (#0140) 5 g l^{-1} Soluble starch	Spread or; Streak, incubate, then flood plate with Potassium Iodide solution (0.5 % (w/v) I_2, 1 % KI). After 5 minutes drain excess stain and record the presence or absence of staining. Absence indicates starch degradation.	Culture for colony counts, description and nutrient utilisation.
T-iso agar	15 g l^{-1} Bacto agar (#0140) Dissolve in 1 l of *Isochrysis galbana* microalgal culture (3 x 10^6 cells ml^{-1}) in which cells were lysed by boiling.	Direct observation of the presence or absence of colonies.	Nutrient utilisation

4.2.5.3 Biochemical profiling of isolates

Biochemical profiling was used to begin the taxonomic identification of isolated bacteria. The isolates were streaked on four different media types (T-iso, Starch, Cellulose, Protein; Table 4.5) and their utilisation of, or growth on, the different nutrient sources was assessed (as described in Table 4.5) after incubation (15 °C, 24 h) of the plates. The number of isolates was refined after the utilisation tests and the remaining isolates were described by gram reaction and cell description.

These isolates were further described through the use of the bioMérieux api 20E identification kit. For each isolate a colony from marine plate culture was resuspended and mixed thoroughly in a 3 % (w/v) NaCl solution (5 ml). Aliquots were added to the pre-labelled test strips as per instructions (api 20E, Cat No. 008040-6; 02/02) and incubated at 15 °C for 24 hours. A lower than recommended incubation temperature was used as the bacteria may be intolerant of the recommended 35 – 37 ºC but are know to grow well on media at 15 °C. All reactions were recorded and presumptive identifications were made where possible using the api20E identification chart (API 20E, 1994).

4.2.5.4 Molecular identification of isolates

From the biochemical profiling a further subset of the isolates were selected for taxonomic identification using molecular methods. To extract DNA the selected isolates were re-streaked onto marine agar plates and incubated (15 °C). After 36 hours isolated colonies were removed from these plates for DNA extraction following the method of Woo *et al*. (1992).

4.2.5.4.1 DNA extraction from cultures

Each colony was resuspended in TNE buffer (135 µl; Table 4.1), then TNE/X-100 buffer (135 µl; Table 4.1) and lysozyme (30 µl of 5 mg ml^{-1}, ~ 50,000 U mg^{-1}, Roche) were added. Each sample was then mixed with vortexing (2200 rpm) and incubated in a water bath (37 °C, 30 minutes). An aliquot (15 µl) of Proteinase K solution (20 mg ml^{-1}, > 30 U mg^{-1}, Roche) was added and mixed by inversion. Samples were then incubated for a further two hours in a water bath (65 °C), diluted 1:5 in sterile, distilled water and aliquoted into sterile, labelled, Eppendorf tubes (2 ml) for storage at -20 °C.

Extracted DNA was amplified using the PB36/PB38 primers (Table 4.2) and PCR method described in Section 4.2.4.3.2 using 25 cycles rather than 30. The PCR product was

analysed for quality and quantity using a 1 µl aliquot on the Nanodrop ND1000 spectrophotometer. PAGE analysis (Section 4.2.4.3.3) was used to ensure the sample contained a single band of DNA (approximately 1500 bp), indicative of amplification of the 16S rDNA fragment.

4.2.5.4.2 Sequencing and identification

Once PCR products of the selected isolates were assessed to be of good enough quality and quantity they were purified using a High Pure PCR purification kit (Roche). The purified product was then submitted to The University of Auckland DNA sequencing facility. Using the PB36 primer, sequencing reactions used the ABI Prism BIG DYE Terminator Sequencing Kit on a Perkin Elmer 9700 thermal cycler. Sequencing products were separated with an ABI Prism 3100 genetic analyser. The resultant digital sequences were edited to provide the longest section without error or unknown nucleotides. This sequence was submitted for nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST) analysis (Altschul *et al.* 1997) on the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/). Isolates were then given a presumptive identification based upon the percent similarity with other sequences in the database.

4.3 Results

4.3.1 Comparison of culturable gut microflora in hatchery and farmed oysters

As the hatchery work presented in Chapter 2 progressed it was theorised that a lack of, or imbalance in, bacterial flora in the hatchery may affect conditioning of the oysters. An initial investigation was conducted by comparing the number and diversity of colonies cultured from; the seawater of the hatchery conditioning system, the guts of hatchery conditioned oysters and the guts of wild oysters. Spread plates of subsamples on different media produced colony counts that were highly variable between replicate plates, samples and location. YSW media, for example, yielded between 204 and 1300 colony forming units (cfu) 0.1 ml^{-1} of gut sample for hatchery samples, between 14 and 1184 cfu $0.1ml^{-1}$ of gut sample for farm samples, and between 39 and 93 cfu $0.1ml^{-1}$ of sample for seawater samples (Appendix 4.3). The different media provided variable results with generally fewer colonies developing on the Basal media than any other type.

Table 4.6 Presumptive identifications of isolates from samples of hatchery seawater, the gut of hatchery conditioned oysters or wild oysters. Numbers of isolates fitting the species identification are given in brackets. Colonies for isolation were selected as representatives of each plate.

Presumptive Identification		
Hatchery Isolates (n = 64 isolates)	**Hatchery Seawater Isolates** (n = 28 isolates)	**Wild Isolates** (n = 86 isolates)
Aeromonas sp.(1)	*Alcaligenes spp.*(2)	*Acinetobacter sp.* (1)
Alcaligenes spp.(12)	*Bacillus sp.*(1)	*Aeromonas spp.* (4)
Enterobacter sp.(1)	*Bacillus mycoides* (3)	*Alcaligenes spp.* (2)
Flavobacterium spp. (2)	*Chromobacterium sp.*(1)	*Bacillus sp.* (1)
Micrococcus spp. (2)	*Micrococcus sp.* (1)	*Corynebacterium spp.* (2)
Pseudomonas spp. (2)	*Shewanella spp.*(2)	*Enterobacter spp.* (3)
Shewanella sp.(1)	*Vibrio fluvialis* (2)	*Flavobacterium spp.* (2)
Vibrio fluvialis (2)		*Micrococcus spp.* (9)
Vibrio sp. (1)		*Pseudomonas diminuta* (1)
		Pseudomonas spp. (4)
		Pseudomonas fluorescens (4)
		Shewanella spp. (6)
		Vibrio alginolyticus (2)
Unidentified isolates (40)	Unidentified isolates (19)	Unidentified isolates (45)

Differences in the culturable bacterial diversity between the hatchery seawater, hatchery gut and wild gut microflora were investigated by isolating representative colonies from the plates of each respective sample. The farmed oyster samples yielded 86 isolates (41 presumptively identified), the hatchery oyster gut samples yielded 64 isolates (24 presumptively identified) and the hatchery seawater yielded 28 isolates (9 presumptively identified) (Table 4.6). All isolates were predominantly gram negative rods although 16 %

of the wild isolates were gram positive, compared to 3 % and 7 % respectively for hatchery gut and seawater samples. The diversity of the tentative identifications does not suggest a major difference in the populations of bacteria from gut or seawater samples. However, over half of the isolates were unidentified and it is likely that only a small proportion of the bacteria were cultivated, thus little insight is provided by this work.

4.3.2 Pilot scale investigation of oyster gut bacteria

Unculturable bacteria, such as *Cristispira* spp., have been specifically identified in association with oysters, and consequently other techniques are necessary to evaluate the gut microflora (Romero & Espejo 2001). A pilot investigation evaluated the use of both molecular and histological techniques for investigating the gut microflora.

4.3.2.1 Histological analysis

The three transverse sections taken from each oyster provided samples along the digestive system allowing observation of structures such as the buccal cavity, the style sac, and the digestive diverticular (Figure 4.3). *Cristispira*-like spirochaetes were most commonly identified (Figure 4.5), although often dense aggregations of rod-shaped bacteria were also found (Figure 4.9). Bacteria were most commonly found in the posterior and middle sections within the food filled, crescent shaped, lumen (Figure 4.8). In these lumen, bacteria were generally situated between the food mass and lumen wall. *Cristispira*-like spirochaetes were occasionally found in the style lumen associated with a thin layer of food particles and a pink stained substance (possibly mucoprotein) adjacent to the cilia of the lumen wall (Figure 4.7). Analysis of the presence of bacteria in the pilot-scale histological sections indicated that there was more inter-individual variation than variation between the two different tidal exposures (Table 4.7).

Table 4.7 Occurrence of bacteria in histological sections taken in the pilot-scale investigation. Sections are labelled according to sampling time of either 3 (LT+3) or 6 (LT+6) hours after low tide, with replicate number in parentheses.

Oyster	Section		
	Anterior	Middle	Posterior
LT+3 (1)		Bacteria - rods	Spirochaetes/Bacteria – rods
LT+3 (2)			Spirochaetes/Bacteria – rods
LT+3 (3)			Spirochaetes
LT+6 (1)			Spirochaetes
LT+6 (2)		Bacteria - rods	Bacteria – rods
LT+6 (3)		Bacteria - rods	

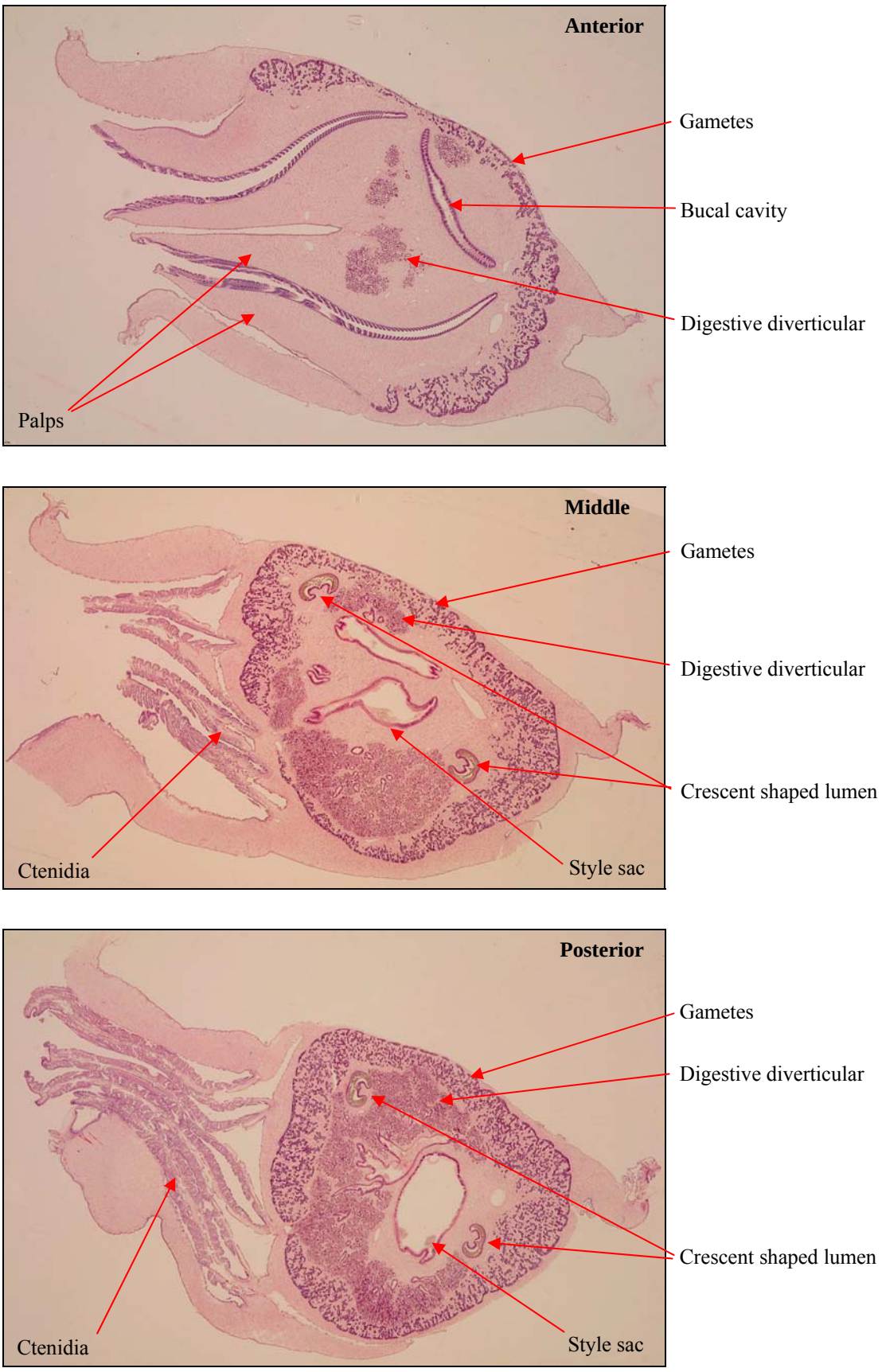


Figure 4.3 Representations of the three sections (anterior, middle and posterior) made from each oyster sampled showing the location of the gut lumen, style sac and digestive diverticular. (Photo credit I. MacDonald)

4.3.2.2 Molecular analysis

Initial assessment of molecular analysis of the oyster gut microflora used six oysters collected from Kerikeri Inlet to provide an indication of the inter-individual variability in the gut microflora. The *Hae* III digest of the amplified 16S rDNA produced a profile of between seven and nine bands for the oysters (Figure 4.8). Most of the samples share 4 bands in common with only sample 2 showing greater variation from the other samples.

In Figure 4.9 the digested style sample is included to indicate the bands probably associated with style microflora (spirochaetes). Of the clear bands the fragments that are approximately at and below the 300 bp band of the ladder and the one above the 1000 bp band appear to be most indicative of the style microflora and are reflected in the patterns of most of the replicate oysters

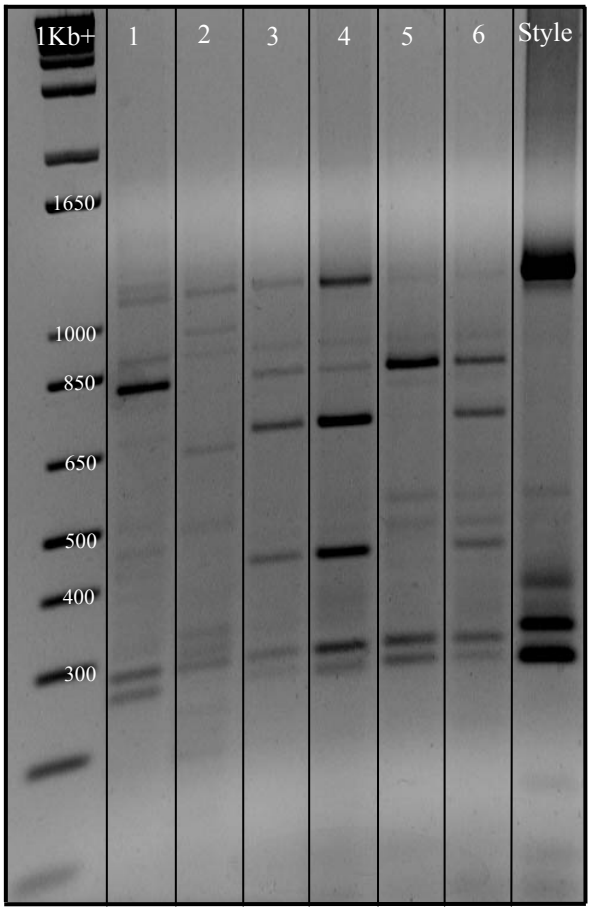


Figure 4.4 RFLP banding pattern from *Hae* III digest of 16S rDNA amplified by PCR from DNA extracted from the gut of Pacific oysters in the pilot study. DNA for lanes 1 – 6 was extracted from the gut of individual oysters collected from Kerikeri Inlet. DNA for the lane labelled Style was extracted from the crystalline style removed from 30 oysters sampled from Te Kapa Inlet, Mahurangi Harbour. Ladder on the left is 1 kb Plus (Invitrogen).

4.3.3 Temporal variation in bacterial presence and diversity in the oyster gut

From the results of the pilot-scale investigations a larger investigation of the oyster gut microflora, using histology and molecular techniques in tandem, was initiated. This work sought to investigate the temporal variability in the bacterial flora through the tidal cycle from the non-feeding period of low tide through to a maximum of six hours of immersion and, consequently, potential feeding time.

4.3.3.1 Histological analysis

The histological analysis found *Cristispira*-like spirochaetes were the only observable bacteria in any of the sections. The spirochaetes observed tended to be aggregated in larger densities than those in the pilot scale samples. *Cristispira*-like spirochaetes were found in 5 of the posterior, 4 of the anterior and 3 of the middle sections (Table 4.8) with *Cristispira*-like spirochaetes identified in all sections of one sample (LT + 6 (1)). Oysters that had been immersed for six hours (LT + 6) had *Cristispira*-like spirochaetes in all samples compared to only one section of one sample for oysters sampled at low tide (LT + 0(3)).

Table 4.8 Occurrence of bacteria in histological sections from the broader investigation. Sections are labelled LT + X (I) where X = time immersed when sampled (hours) and I = replicate number.

Oyster	Section		
	Anterior	Middle	Posterior
LT + 0 (1)			
LT + 0 (2)			
LT + 0 (3)			Spirochaetes
LT + 2 (1)	Spirochaetes	Spirochaetes	
LT + 2 (2)			
LT + 2 (3)	Spirochaetes		
LT + 4 (1)			Spirochaetes
LT + 4 (2)	Spirochaetes		Spirochaetes
LT + 4 (3)			
LT + 6 (1)	Spirochaetes	Spirochaetes	Spirochaetes
LT + 6 (2)			Spirochaetes
LT + 6 (3)		Spirochaetes	

The presence of spirochaetes in the crystalline style was confirmed by light microscopy (400x magnification) of freshly isolated styles (Figure 4.6). While spirochaetes were clearly observable in the style no other bacteria were visible at 400x magnification. Some unidentifiable debris was observed along the style but it was unclear if this was style associated or a bi-product of style removal.

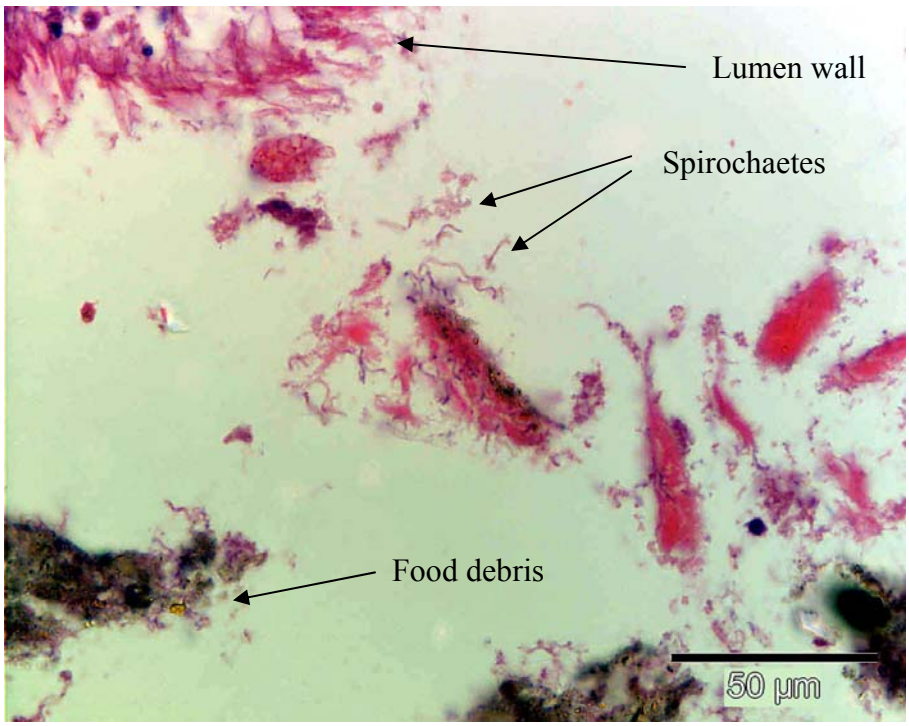


Figure 4.5 Photomicrograph of *Cristispira*-like spirochaetes in a gut lumen. Posterior section of an oyster sampled at half tide in the pilot scale study.

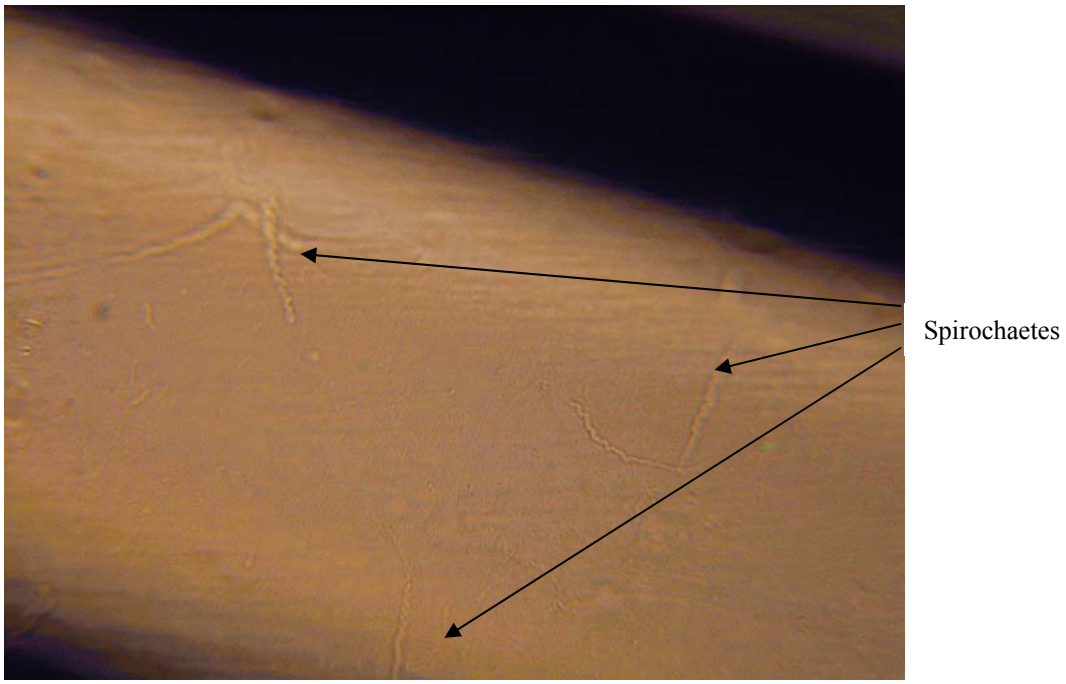


Figure 4.6 Photomicrograph of spirochaetes in the outer laminates of a freshly isolated crystalline style. Magnification is 100x; approximate diameter of style in figure is 1.5mm.

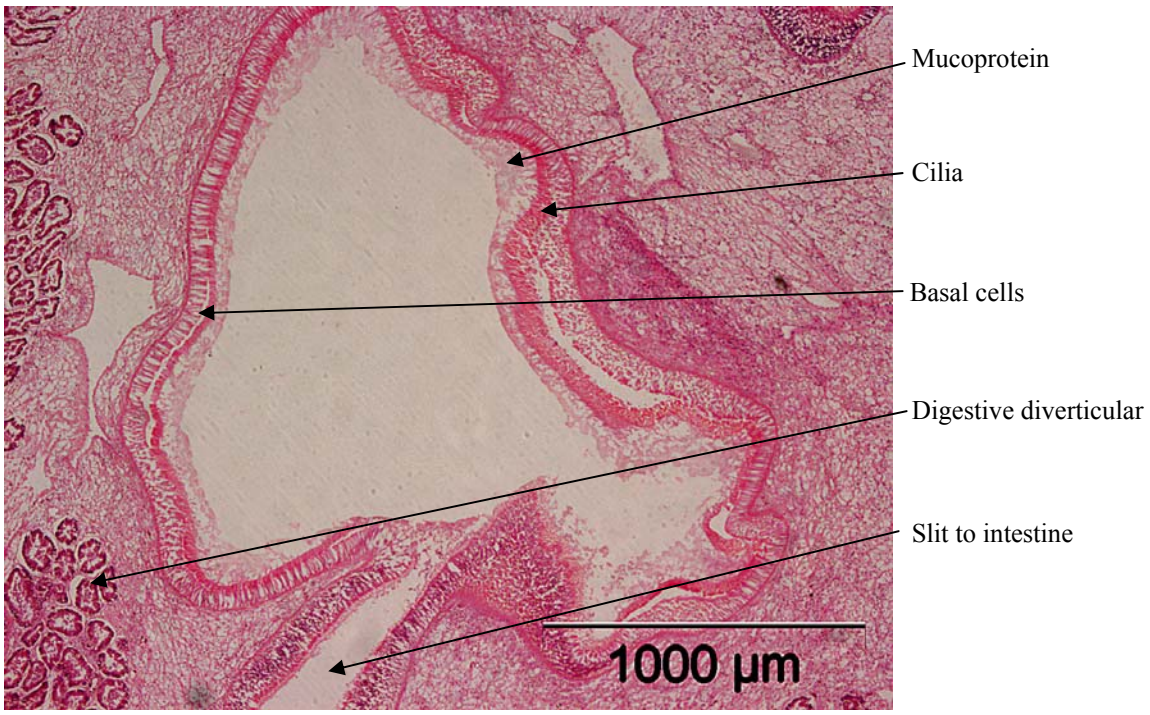


Figure 4.7 Photomicrograph of a typical section through the style lumen from a posterior section. The structure of the lumen wall and substance likely to be mucoprotein are labelled.

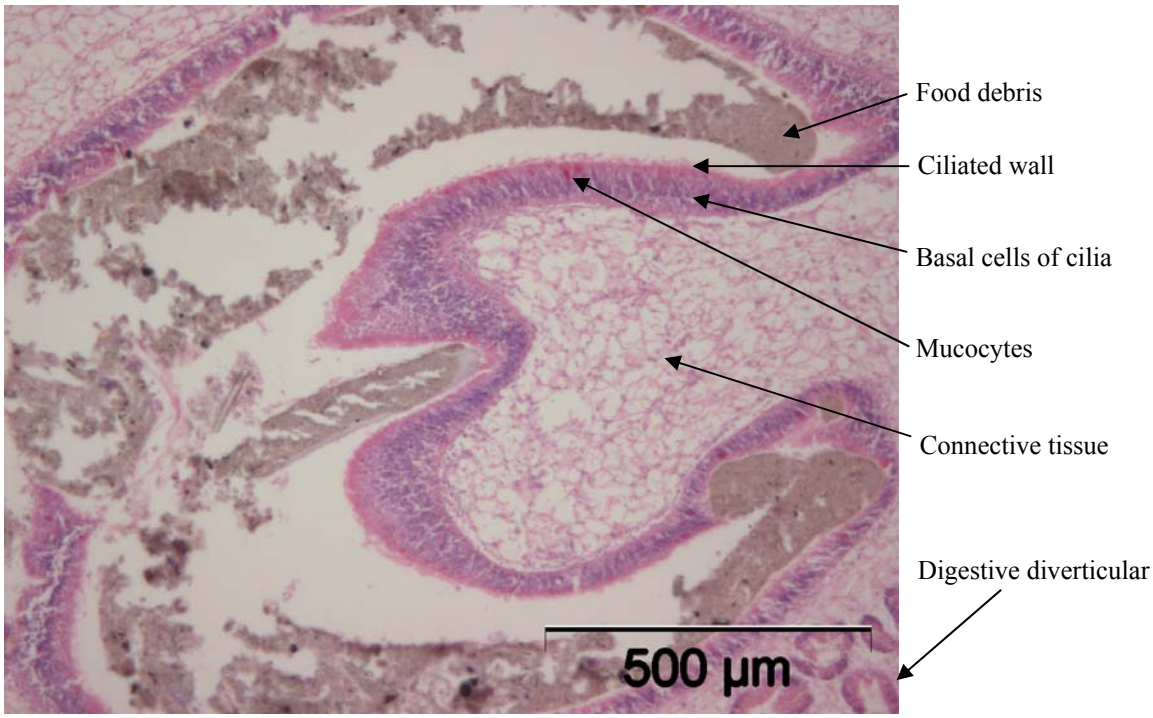


Figure 4.8 Photomicrograph of a typical "crescent shaped" lumen showing "food debris" (brown) and the ciliated lumen wall. Digestive diverticular can be seen in the lower, right corner.

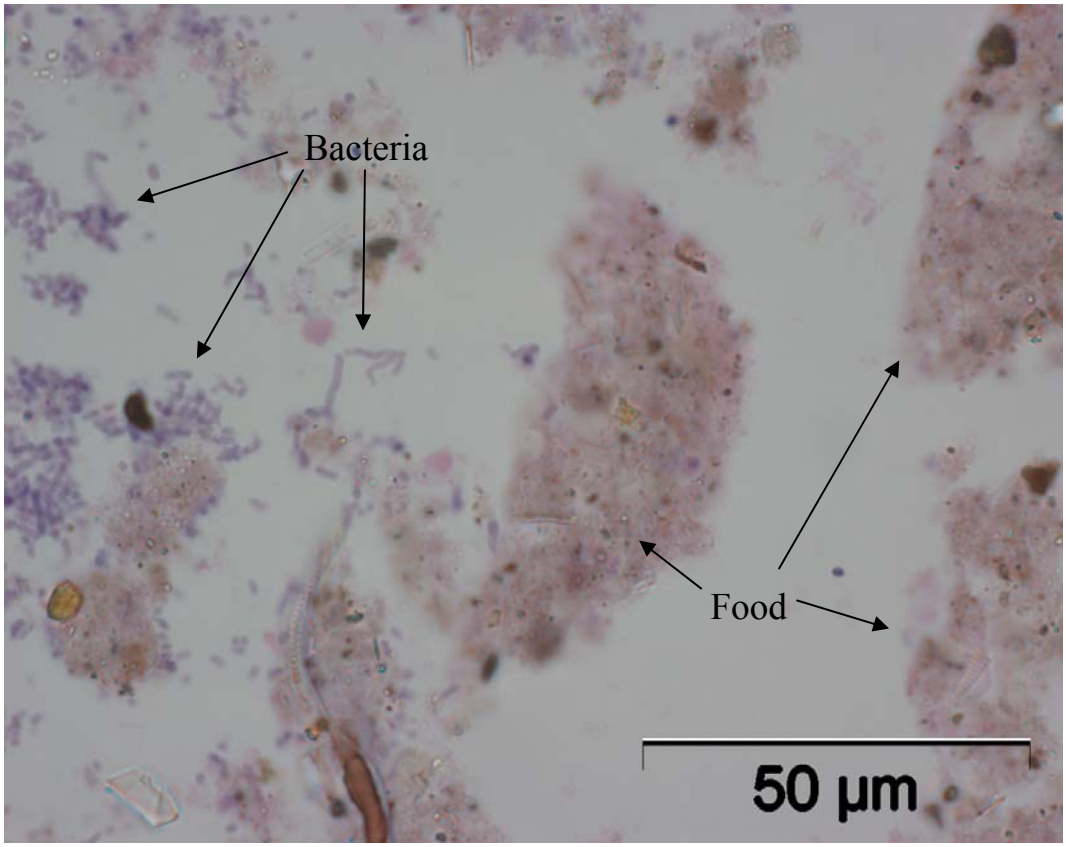


Figure 4.9 Photomicrograph of bacterial rods found amongst food debris in a gut lumen. Section from an oyster sampled at half tide in the pilot study.

4.3.3.2 Molecular analysis

The RFLP analysis of 16S rDNA fragments amplified by PCR from the gut samples taken after different tidal exposures, seawater samples and style samples (Figure 4.10) showed some similarities in banding pattern to that of the pilot scale oysters. Bands around 300 bp and 1000 bp were observed in most samples including the style. Seawater samples showed clear bands at 300 bp and 500 bp of which only that at 300 bp was similar to the oyster samples. Density of the bands appeared to vary with time, especially those similar to the style. The band just above 1000 bp, for example, is distinct at LT+0 but less so at LT+6 where it appeared to have separated into several closely associated bands. Of the bands clearly visible in the seawater samples only the band at 300 bp appeared to occur in the oyster samples suggesting that the gut microflora does not simply reflect the surrounding seawater. However, the curve of the gel at the seawater end made the comparison difficult.

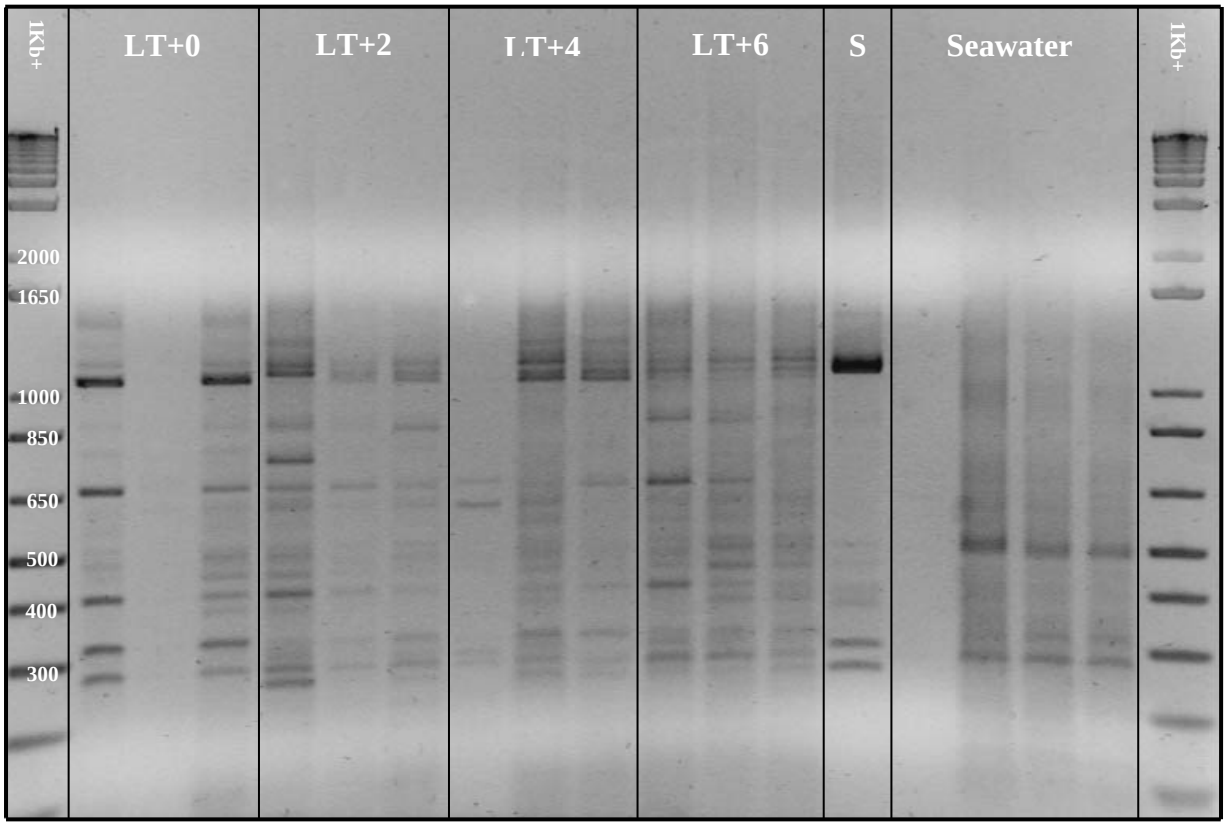


Figure 4.10 RFLP pattern from *Hae* III digestion of PCR amplified, 16S rDNA extracted from the gut of Pacific oysters. LT+0 – LT+6 = time immersed since low tide (hours) with each lane representing 1 – 3 replicate oysters; S = isolated crystalline style; Seawater = replicate samples of concentrated seawater. Ladders on each side are 1 kb Plus (Invitrogen; 1 kb+) with the bp values superimposed on the left ladder. All samples were collected on the same day in Kerikeri Inlet excepting the style which is a composite sample of 30 styles removed from oysters grown in Te Kapa Inlet, Mahurangi Harbour. The patterns have been visualised on an agarose gel stained with ethidium bromide.

4.3.4 Characterisation of the oyster gut microflora

Further investigation of the gut microbiology of farmed oysters was undertaken to characterise and identify the gut microflora. Bacteria, from samples of gut homogenate from six oysters, were cultured by spread plating on marine, starch and cellulose media. Selecting representative colonies from each plate, a total of 104 colonies were isolated. Initial characterisation of these isolates was made by recording growth on marine, starch and cellulose media. While all of the isolates grew on marine agar, only 86 showed growth on starch and 81 showed growth on cellulose (Appendix 4.4, Table A4.2). Twelve of the isolates showed no growth on either starch or cellulose while 70 of the isolates showed some growth on both media. Ninety-one isolates (13 isolates were lost during subculture) were further tested for nutrient utilisation using T-iso, starch, cellulose and protein media (Table 4.5).

The T-iso media was created to test the ability of bacterial isolates to utilise *Isochrysis galbana*, or its contents, as a food source because the degradation of microalgae may be an important feature for gut microflora. The T-iso medium allowed strong growth for fifty one of the isolates, while a further thirty three showed weak growth on this medium, and eight did not show any growth (Appendix 4.4, Table A4.2).

The ability of the isolates to degrade certain cell components was assessed using the cellulose, protein and starch media. The media used for these analyses had clear visual assessment methods to indicate nutrient utilisation (e.g. starch degradation demonstrated by failure to stain with iodine as shown in Figure 4.11). On the starch medium thirty eight isolates clearly degraded the starch and a further twelve produced lesser zones of degradation. Similarly the cellulose was clearly degraded by thirty two isolates and to a lesser extent by a further thirteen isolates. The ability to degrade protein was less common with only six strong and four weak zones of protein degradation. Incubation of the protein plates for a further 24 hours gave a further two weak responses (Appendix 4.4, Table A4.2).

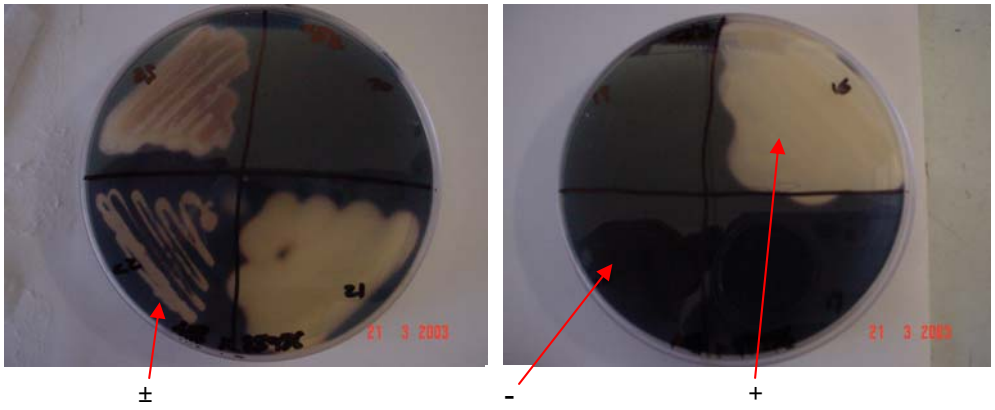


Figure 4.11 Examples of starch plates that have been stained with I_2/KI solution (Table 4.1). Clear patches indicate where starch has been degraded by the isolates. Isolates have been streaked into quadrants and the symbols refer to examples of strong (+), weak (±), and negative (-) degradation of starch.

For all of the degradation tests, only two isolates returned strong results on all media whereas a further five isolates showed either strong or weak responses on all media. Fifteen isolates showed strong degradation of both starch and cellulose while a further eleven showed either strong or weak degradation of starch and cellulose (Appendix 4.4, Table A4.2).

A representative group of thirty six isolates were selected on the basis of colony description, growth characteristics and nutrient degrading capability so as to provide a full range of characteristics (Appendix 4.4, Table A4.3). Characterisation of the metabolic capabilities of thirty six isolates, with the bioMérieux api 20E identification kit, showed a range of responses although the tests for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, indole and sorbitol activity were negative for every isolate tested (Appendix 4.4, Table A4.3). A number of tests also failed to show a definite colour change in many cases, especially the beta-galactasidase and tryptophane deaminase activity tests. However, only six isolates failed to show a response to any of the api tests with a further three only recording indefinite results.

Ten of the isolates were then selected, based upon their phenotypic characterisation (Table 4.8), for molecular identification. All of these selected isolates were gram negative rods, excepting isolate 30 which was gram positive. Isolate 30 grew on all media types and utilised starch cellulose and protein but only tested positive for tryptophane deaminase in the api tests. In contrast, isolate 58 tested positive for 8 of the api tests, showed at least some growth on different media but did not utilise starch cellulose or protein.

Table 4.9 Phenotypic characters of the 10 isolated bacteria sequenced. All were gram negative rods, except isolate 30 which was gram positive, and all had white colonies, excepting isolates 30 and 45 who displayed red pigmentation when grown on marine agar. For the media and nutrient columns Mar = marine, Sta = starch, Cell = cellulose, Pro = protein, T-iso = T-iso media (Table 4.1). + refers to growth/utilisation, - refer to no growth/utilisation and +/- is used where some evidence of growth/utilisation was present. Positive reactions for the api20E tests for activity/utilisation of; onpg = beta-galactosidase; TDa = tryptophane deaminase; Gel = gelatinase; Glu = glucose; Man = mannitol; Sac = sucrose; Mel = melibiose; Amy = amygdalin; Ara = arabinose; NO_2 = production of NO_2 from potassium nitrate; N_2 = reduction of potassium nitrate to gas.

ID	Growth on media				Utilisation of nutrients			Positive reactions with api 20E								Mucoid
	Mar	Sta	Cell	T-iso	Sta	Cell	Pro									
16	+	+/-	+/-	+	+	+	-	TDa	Gel	Man	Sac	NO_2				+
20	+	+/-	+/-	+	-	+	-	TDa	Glu	Man	Sac	Amy				+
30	+	+	+	+	+/-	+	+	TDa								-
41	+	+	+	+	+	+	-	TDa	Glu	Man	Sac	NO_2				+
45	+	+	+	+	-	+	-	TDa	Glu	Man	Sac	Amy	NO_2			+
58	+	+/-	+/-	+	-	-	-	onpg	TDa	Glu	Man	Sac	Mel	Ara	NO_2	+
61	+	+	+	+	+	+	+/-	TDa	Glu	Man	Sac	NO_2				+
64	+	+	+/-	+	+	+	-	TDa	Glu	Man	Sac	Amy	N_2			+
72	+	-	-	+/-	+	-	-	onpg	TDa	Man	Sac					-
80	+	+	+	+	+	-	-	TDa	Glu	Man	Sac	Amy	N_2			+

The 16S rDNA fragment was amplified from DNA extracted from isolated colonies of each isolate and sequenced. BLAST searches of the NCBI database showed the 16S rDNA sequences from the isolates closely matched sequences attributed to various *Vibrio* spp. excepting isolate 30 which matched *Bacillus* sp. (Table 4.9). The matches in Table

4.9 are all within six base pairs of a perfect (100 %) sequence match. However, none of the sequences used for the BLAST search exceed 1000 bp in length and half are less than 700 bp.

Information on the NCBI database indicated that of all the matched strains, only *Bacillus* sp. 19500 was derived from a terrestrial source (Heyrman & Swings 2001) while all the other species had been isolated from marine sources (Table 4.9). Of the marine species, *V. pomeroyi, V. splendidus, V. tasmaniensis* and *Vibrio* sp. PMV19 have all been associated with marine bivalves (Torkildsen *et al.* 2001, Thompson *et al.* 2003, Guisande *et al.* 2004). Four of the isolates (16, 41, 45, 61) were closely matched to *Vibrio lentus* strains. The sequences of these strains were submitted by Nishiguchi and Nair (2003) who isolated them from squid (*Sepiola atlantica*) and Macián *et al.* (2001) have isolated the same strains from oysters in the Mediterranean.

Table 4.10 Results of the Blast search (Altschul *et al.* 1997) of sequences derived from the PCR amplification of 16S rDNA from each isolate. The number and percentage of base pairs (bp) matched to the database are given as an indication of the closeness of match. Species in blue have previously been associated with marine bivalves.

Isolate number	Blast identification	Number of matched bp	Percent matched bp	Reference
16	*Vibrio lentus* strain Sat201	686/687	99	Nishiguchi & Nair (2003)
	Vibrio lentus strain Sat101	686/687	99	Nishiguchi & Nair (2003)
	Vibrio pomeroyi strain 337.98	684/687	99	Guisande *et al.* (2004)
20	*Vibrio* sp. Strain DA4	923/925	99	Tajima *et al.* (2000)
	Vibrio sp. Strain DA2	923/925	99	Tajima *et al.* (2000)
	Vibrio sp. OC25	922/925	99	Urakawa H (2000)
30	*Bacillus* sp. strain A-38	753/753	100	Fajon *et al.* (1999)
	Bacillus sp. 19500	750/753	99	Heyrman & Swings (2001)
	Bacillus sp. V4.BE.06	748/753	99	Fritz (2000)
41	*Vibrio lentus* strain Sat201	735/748	98	Nishiguchi & Nair (2003)
	Vibrio lentus strain Sat101	735/748	98	Nishiguchi & Nair (2003)
	Vibrio pomeroyi LMG 20537T	734/748	98	Thompson *et al.* (2003)
45	*Vibrio lentus* strain Sat201	653/654	99	Nishiguchi & Nair (2003)
	Vibrio lentus strain Sat101	653/654	99	Nishiguchi & Nair (2003)
	Vibrio pomeroyi strain 337.98	651/654	99	Guisande *et al.* (2004)
58	Marine bacterium isolate DPT1.1	651/651	100	Giuliano *et al.* (1997)
	Vibrio sp. R-3884	649/651	99	Thompson *et al.* (2001)
	Vibrio sp. QY101	650/651	99	Han *et al.* 2002
61	*Vibrio lentus* strain Sat201	758/759	99	Nishiguchi & Nair (2003)
	Vibrio lentus strain Sat101	758/759	99	Nishiguchi & Nair (2003)
	Vibrio sp. LMG 20539	753/759	99	Thompson *et al.* (2001)
64	*Vibrio* sp. PMV19	668/668	100	Torkildsen *et al.* (2001)
	Vibrio sp. Da4	668/668	100	Tajima *et al.* (2000)
	Vibrio sp. Da2	668/668	100	Tajima *et al.* (2000)
72	*Vibrio splendidus* strain 636	646/648	99	Guisande *et al.* (2004)
	Vibrio splendidus strain 630	645/648	99	Guisande *et al.* (2004)
	Unidentified bacterium 4c	645/648	99	Christensen *et al.* (2000)
80	*Vibrio tasmaniensis* strain 236.10	762/764	99	Guisande *et al.* (2004)
	Vibrio tasmaniensis strain 364.11	761/764	99	Guisande *et al.* (2004)
	Vibrio sp. ED4	761/764	99	Mo *et al.* (2001)

4.4 Discussion

Bacteria were associated with the gut of oysters from both the hatchery and Kerikeri Inlet. Of the culturable bacteria (Table 4.6) there was a predominance of gram-negative, rod shaped, bacteria (e.g. *Vibrio* spp, *Pseudomonas* spp., *Aeromonas* spp., *Flavobacterium* spp.). This is similar to previous descriptions of oyster-associated bacteria such as, Colwell and Liston (1960), Kueh and Chan (1985), and Hoffman (2004). Comparing bacterial counts from hatchery and wild samples proved difficult due to the large variation within samples. However, it was clear that there were fewer culturable bacteria in 1 ml of seawater than 100 µl of oyster gut from the hatchery conditioning system, especially for the Basal, both Yeast and the 2B culture media. This suggests that the oysters are concentrating the available bacteria, as has been noted by other researchers (Potasman *et al.* 2002).

Of the identified isolates only four species were found in both the gut and seawater samples. This observation can be compared to the 7 matching bacterial identifications between the hatchery and wild oyster gut samples and may indicate that selective retention, or population expansion, of bacteria occurs in the oyster gut. However, the technique of obtaining culturable bacteria may have lead to selection within those bacteria. Multiple media types were used to limit bias by widening the diversity of nutrient sources available for bacteria. This may have also reduced selection bias when colonies were picked for isolation as often different media will produce differences in colony morphology. Moreover, a large number of the isolated colonies were not identified and consequently, diversity may be considerably underestimated. While more replication, at both the oyster and plating levels, may improve the resolution of any comparison between the culturable bacteria of seawater, oyster and the oyster gut, other methods may provide better resolution.

The *Cristispira*-like spirochaetes were the most commonly found bacteria in the histological sections, although bacterial rods were numerous in the pilot scale experiment. Identification of rod shaped bacteria in the pilot scale samples, but not the large scale experiment (Table 4.10), may be due to temporal variability in bacterial occurrence as both sets of samples came from the same Kerikeri Inlet farm. The relatively low densities of *Cristispira*-like spirochaetes in all of the sections suggested that either, these bacteria are only present in low densities (in contrast to the micrographs of Judd 1977, Tall & Nauman

1981), or the use of transverse sectioning failed to identify where the spirochaetes relocate to with the dissolution of the style. The *Cristispira*-like spirochaetes were generally found in the crescent-shaped lumen (Figure 4.6) associated with food debris, rather than the style sac which was expected from previous descriptions (Dimitroff 1926, Bernard 1970, Tall & Nauman 1981). This may indicate that the spirochaetes become more mobile within the gut system when the style has dissolved.

Relocation of the spirochaetes through the oyster gut, once the style has dissolved, may be dependent upon gut fullness as sections from oysters immersed for the longest period, and consequently with the longest potential feeding period, more frequently contained spirochaetes. However, this may also be an artefact of the fixation technique whereby the mobile bacteria attempt to avoid the encroaching fixative. No evidence of gut evacuation was noted in the specimen bottles so gut content at the time of sampling is likely to be represented in the sections but may be displaced within the lumen due to fixation effects such as dehydration. Investigation of other fixatives, fixation techniques, longitudinal sectioning and concurrent observation with scanning electron microscopy may prove more valuable in establishing the distribution of bacteria contained within the gut.

Bacterial diversity not shown by the histological sections was evident in the pilot study when using PCR amplification and restriction enzyme digestion of isolated bacterial 16S rDNA fragments. The pilot study showed inter-individual variability in the RFLP patterns with shared bands found at 5 locations. In comparison to the style RFLP pattern at least two of these bands (less than the 300 bp marker) are probably indicative of the style microflora while the bands greater than 1000 bp are likely to be undigested fragments. The use of a single restriction enzyme for the RFLP analysis of the amplified bacterial DNA is likely to have underestimated the actual diversity within the samples. Acinas *et al.* (1997), for example, used the combinations of *AluI* + *RsaI* and *MboI* + *HinfI* endonucleases to digest amplified 16S rDNA for the assessment of marine bacterioplankton diversity. Similarly, Romero *et al*. (2002) used *Alu*I, *Rsa*I, and *Hae*III when investigating *Triostrea chilensis* microflora. This study would have benefited from the use of additional restriction enzymes but they were not available so the larger study was undertaken without this improvement.

Indications of a commensal gut microflora were thought to be provided by two observations in the pilot studies. Firstly, in the histological sections the bacteria were

located between the food debris and the lumen wall which was similar to the location demonstrated by Simon and McQuaid (1999) using scanning electron microscopy of the bacteria in mussels. Secondly, the similarities and dissimilarities between the RFLP patterns of individual oysters suggested that there may be some taxonomic consistency in the microflora despite variation which could relate to factors such as feeding time and physiological state prior to sampling. The larger study was thus undertaken to compare change through a feeding cycle in an attempt to refine these observations. It was expected that transient microflora would be more dominant after longer periods of feeding compared to the low tide period of no feeding as, when the style has dissolved, bacteriolytic enzymes are released throughout the gut (Morton 1977).

The histological analysis failed to identify bacteria other than spirochaetes in the sections. However, the spirochaetes were more frequently identified in sections that had had the longest feeding period, which may indicate the slower dissolution of the style, and subsequent redistribution of spirochaetes, relative to the rapidity of fixative penetration. In contrast, the RFLP patterns showed that, in comparison to seawater, both the style and oyster gut extracts (Figure 4.10) contain a biased subset of the available bacteria. The lack of bands in the seawater RFLP pattern may indicate a failure in the concentration method to retain bacteria. However, it may also indicate the dominance of a taxonomic group within the concentrate from which DNA was more easily extracted and amplified obscuring the real diversity within the seawater.

The style band pattern, while unique relative to seawater, was reflected in the band pattern of oyster samples. There was a slight difference in the positioning of the bands near the 300 bp marker which may have been due to the style sample having been collected from oysters in Te Kapa Inlet. Te Kapa inlet is approximately 160 km south of Kerikeri Inlet and may contain a spirochaete flora which is isolated enough from that in Kerikeri to allow for some genetic variation detectable in the RFLP pattern. The use of multiple restriction enzymes is recommended for any further RFLP comparisons so as to provide a greater diversity of bands.

The PB36/PB38 primers were designed to amplify a large (1500 bp) sequence of bacterial DNA which is highly conserved across taxonomic groupings. Matching against sequences in the ribosomal database showed representatives of all phyla could be identified by the primer sequences. However, Hagström *et al.* (2002), using a similarity of 97 % as the

upper limit for species delineation, found that their model indicated that high similarity did not guarantee species singularity. Similarly, Wiik *et al*. (1995) reported being unable to phylogeneticaly separate phenotypicaly distinct isolates of marine bacteria using 16S rDNA sequences. Consequently, the conservative nature of the 16S rDNA sequence may produce false positive or false negative results when used to gauge species diversity.

The RFLP pattern of oyster guts did appear to change with immersion time despite the drawbacks of using 16S rDNA sequences and a single endonuclease. In particular, the band greater than 1000 bp became less distinct with more banding greater than 1000 bp noticeable amongst the LT + 4 and LT + 6 samples. This may indicate that the relative diversity of bacteria in the gut was increasing with increasing immersion time. The pattern for the less than 1000 bp bands also changes with increasing immersion time providing greater dissimilarity. This indicates that changes in the microflora probably occur with feeding which would support the possibility of a transient microflora being flushed through the gut as feeding progresses. In contrast, the similarity between the RFLP patterns of oysters at time 0 and +2 may indicate a commensal flora which can persist, and possibly even multiply, within the gut as suggested by Colwell and Liston (1960). However, Seiderer *et al.* (1984) has suggested that only 57 % of bacteria could be successfully lysed by oysters. Hence, the band pattern could represent those bacteria contained in the gut when the oyster stopped feeding on the outgoing tide but not lysed by the release of enzymes associated with the dissolution of the style.

While the molecular techniques were able to detect the bacterial presence that was not observed in histological sections, the results did not have the required resolution to indicate anything more than the possibility that the oyster gut microbial ecology may change in relation to feeding. While the resolution may have improved with the use of multiple restriction enzymes to produce more detailed banding patterns, other techniques should also be investigated. In particular, the use of ARDRA (Amplified Ribosomal DNA Restriction Analysis; Heyndrickx *et al*. 1996), where a clone library is developed, would provide useful information on the phylogenetic profile of oyster gut microflora. Change on temporal and spatial scales can be further elucidated once a baseline profile exists from which specific primers or fluorescent nucleic acid probes can be created.

The possibility that bacteria, other than spirochaetes, may persist in the oyster gut was further investigated by isolating and phenotypically characterising culturable bacteria. The

selection of the initial assays of the isolates were based upon published observations, such as Crosby and Peele (1987), who reported a loss in cellulolytic capability in antibiotic treated oysters, Brock (1989), who noted oyster cellulolytic activity was derived from bacteria, and Crosby *et al*. (1990) who observed that detrital resources were unavailable to oysters without bacterial mediation. Indeed, it is generally assumed that animals are unable to degrade the crystalline cellulose characteristic of algae and microorganisms (fungi, yeast and/or bacteria) are required to hydrolyse cellulose to oligosaccharides and eventually glucose (Tenkanen *et al.* 2003). The initial assays showed a predominance of isolates capable of degrading cellulose and starch (Appendix 4.4, Table A4.2). Further characterisation was achieved with the use of api20E identification strips. Contrary to Colwell and Liston (1960), who noted a high incidence of bacteria able to ferment glucose, only 30 % of the isolates produced positive api20E results for glucose degradation. The results of the api20E strips were frequently unclear or negative which may suggest inappropriate selection of api strip or that the particular batch used was in some way faulty. The design of the api strips is such that poor selection is likely to make identification difficult, as opposed to reactions failing to take place or being unclear. The batch used was near the use by date and had been stored for a considerable period, mostly at 4 °C, so it is possible that degradation of some of the reagents had occurred making reactions less clear.

From the results of the phenotypic characterisation of the culturable gut bacteria 10 isolates were selected for taxonomic identification by sequencing isolated and amplified 16S rDNA. The selection of the isolates was made to cover a range of phenotypic characters although there was a bias toward those isolates which may provide a nutritional link to oysters either as transient or commensal microflora. The BLAST search identified that seven 16S sequences had previously been isolated from bivalves (Table 4.9). The most frequently matched *Vibrio lentus* strain has been isolated from, and identified as a symbiont of, the light organs of squid (Nishiguchi & Nair 2003) which suggests a possible predilection of this species to form mollusc symbioses. Without more sequence information it is impossible to confirm the taxonomic match and it is possible that the particular section of the 16S fragment most cleanly amplified in this study is common to symbiotic bacteria.

However, the identification by sequence match may be incorrect for several reasons. Firstly, the use of a single primer (PB36) for the sequencing reactions meant that half of the isolates were identified using less than 700 bp, which is less than half of the 1501 bp

amplified by the PB36/PB38 primer set. In highly conserved regions of the DNA, the larger the sequence available for comparison, the greater the chance of attaining unambiguous results as even 100 % matches may change with the submission of a greater sequence length. Sequencing from both PB36 and PB38 primers would allow a complete sequence to be compiled and submitted to the database making identifications more robust. The biochemical profiling of the isolates cast further doubt on the accuracy of the sequence based identifications. For example, isolates 16, 41, 45 and 61 were all identified *Vibrio lentus* yet their profiles varied with; only isolate 61 showing some protein degradation, only isolate 16 producing gelatinase, and only isolate 45 fermenting amygdalin. However, as noted above, other researchers have reported the occurrence of similar (> 97 % bp similarity) 16S rDNA sequences from phenotypicaly distinct isolates (Wiik *et al.* 1995). Similarly, Kersters *et al*. (2003) has noted that a single ribosomal nucleotide sequence cannot define a phyla, let alone all bacteria. Consequently, improving sequence quality so larger (> 1000 bp) sequences are available for matching and the use of a second region, such as 23S, may improve the resolution of taxonomic identification in future studies.

The presence of 16S rDNA sequences that match those of a species reported as a symbiont and the occurrence of characteristics such as mucoid colonies and degradation of cellulose, suggest at least some of the isolated bacteria could be effective as members of a commensal microflora. The possibility that a commensal flora exists is not new, with several authors previously speculating that observed bacterial flora are a normal feature of healthy bivalves (Colwell & Liston 1960, Kueh & Chan 1985, Olafsen *et al.* 1993, Romero *et al.* 2002) and many demonstrating the colonisation of the crystalline style by *Cristispira*-like spirochaetes (Dimitroff 1926, Bernard 1970, Judd 1977, Tall & Nauman 1981). Similarly, the association of these bacteria with beneficial enzymatic activities is not new with *Cristispira*-like spirochaetes, for example, being associated with extracellular carbohydrases (Judd 1977, Simon & McQuaid 1999).

The evidence of the production of amylase's, cellulases and proteinases was provided by the degrading of starch, cellulose and protein by the isolates. It is known that bacteria produce multiple enzymes for tasks such as cellulose degradation (Warren 1996) and this would be beneficial to oysters in an environment where nutrient sources may be variable, requiring an array of enzymes to access the carbon or nitrogen resources. Brock (1989) has previously noted that cellulolytic activity in oysters (*C. gigas*) was associated with bacteria and, therefore, detritus would be a negligible energy source in the absence of

bacteria. Similarly, Crosby *et al.* (1990) observed that the detrital resource was unavailable to oysters (*C. virginica*) without the mediation of cellulolytic bacteria. Protein degradation was not a common attribute among the isolates. This might be more compelling evidence of commensalism, as the oyster gut environment could be providing the protein degradation required for the release of essential amino acids from food items.

In conclusion, the work presented in this chapter has provided some evidence that oysters have an associated bacterial flora, although the exact diversity and transience, or otherwise, remains to be elucidated. The phenotypic characteristics of bacteria isolated from gut samples suggest they may have the capacity to aid in the acquisition of nutrients by the host oyster, in particular, through the degradation of the carbohydrate cellulose. The *Cristispira*-like spirochaetes appeared to be a permanent commensal flora although their relative dominance within the oyster gut may vary with the length of time the oyster has spent feeding (Figure 4.10). The relationship between the *Cristispira*-like spirochaetes and their host requires further investigation to determine whether it could be better described as parasitic or symbiotic. Certainly there appear to be complex interactions between bactivory, symbiosis, mutualism, and parasitism within the oyster gut.

Chapter 5.0
General Discussion

This thesis developed from a research effort to investigate the hatchery production of Pacific oysters (*Crassostrea gigas*), and led to an investigation that highlighted the importance of heterotrophic bacteria to the feeding physiology in Pacific oysters. The studies commenced with the pilot-scale hatchery investigations, which were initiated by members of the oyster industry who sought a secure supply of oyster spat. The initial production attempts, using 'in-season' broodstock oysters, with or without selection for morphological characteristics, were successful in producing and on-growing oysters. Whilst improvements were required to make production more efficient, the resulting growth rates of these oysters, suggested further investigation of hatchery production, including a program of selective breeding, could be justified.

A further investigation of hatchery production proceeded to condition out-of-season broodstock to achieve out-of-season spat production. This was not successful despite following established techniques (Utting & Spencer 1991). The incidence of disease and failures of larval cultures suggested the broodstock were enduring physiological stress in the conditioning system. While stress may have been induced by a number of factors *Crassostrea gigas* has been reported to be tolerant to a wide range of environmental conditions (Mann 1984). Consequently, food supply seemed a likely stressor; degrading the quality of both the broodstock and their gametogenic capacity through a lack of, or insufficient quantity of, some essential nutrients. However, previous researchers have suggested that the ration that was provided to the oysters ought to be adequate (Utting & Millican 1997) if not excessive (Helm *et al.* 2004). Therefore, clearance rate assessments were used to indicate whether the particular microalgal species provided to the oysters were appropriate. The rates and proportional clearance of each microalgal species showed that concentration could affect clearance rate (similar to Barillé *et al.* 1997) and the large proportions cleared suggest the food was at least being captured on the gill.

The limited size of the pilot-scale hatchery made replicated experimentation, to determine why the oyster conditioning was failing, difficult. Consequently, differences between the

pilot-scale hatchery and descriptions in the literature of successful conditioning of oysters (Dupuy & Rivkin 1972, Utting & Spencer 1991, Robinson 1992, Utting & Millican 1997, Chávez-Villalba *et al.* 2002a, b, Helm *et al*. 2004) were assessed to isolate potential reasons for the conditioning failure. One major difference between the pilot-scale hatchery and other systems described in the literature was that these hatcheries usually used a flow-through seawater supply for broodstock conditioning. In these hatcheries, seawater treatment ranged from none to filtration to approximately 10 µm compared to the pilot-scale hatchery where the seawater was filtered to 1 µm and treated with UV (Appendix 2.1). As the majority of the production in marine systems has been attributed to the fraction less than 10 µm (Grégori *et al*. 2001) the hatchery systems described in the literature would be providing the microalgal ration as a supplement to the potential food available in the seawater supply. This is in contrast to the pilot-scale hatchery where the fraction was mostly removed from the seawater. Therefore, the importance of this small fraction to oyster condition became the focus the following studies.

The picoplankton and its potential contribution to oyster nutrition was investigated *in situ* in Kerikeri Inlet. The retention of picoplankton by Pacific oysters is not well described with conflicting reports of efficiency but, given the quantities of available carbon, even low retention efficiencies could supply significant quantities of nutrients. Indeed, the picoplankton proved to be a large resource in Kerikeri Inlet and even at the retention efficiencies found, picoplankton (especially the heterotrophic bacteria) could make a substantial contribution to oyster nutrition.

The retention efficiency of picoplankton by oysters in Kerikeri Inlet was found to have seasonal variability. In particular, the increase in the efficiency with which the heterotrophic bacteria were retained coincided with changing nutritional demands in the oysters as they entered the depleted post-spawn state (December to March). However, efficiency of retention does not necessarily mean that the bacteria are making a direct nutritive contribution to the oyster. For example, Crosby *et al.* (1990) suggested that bacteria mediate the carbon and nitrogen flows between detrital resources and suspension feeding bivalves. Similarly, Brock (1989) has suggest that native cellulase production by *C. gigas* is low, which is similar to the findings of Simon and McQuaid (1999) in mussels, and, consequently, a resident microflora may be required to process food particles. This could be of particular importance where crystalline cellulose is predominant, such as in algae (Tenkanen *et al*. 2003). Further research to elucidate the absorption efficiency and

direct nutritive contribution of picoplankton, especially in terms of essential nutrients, such as Vitamin B complexes (Phillips 1984), is warranted to improve our understanding of oyster nutrient acquisition. However, in this study, an investigation of the bacteria present in the gut of oysters was initiated.

The bacterial microflora of the oyster gut was investigated initially by comparing the culturable bacteria from, the gut of hatchery conditioned oysters, the gut of farmed oysters and the hatchery seawater. The results suggested the microflora may be influenced by environment, but did not necessarily reflect the external microbial ecology. However, the wide variability in plate counts and relatively few replicates made comparisons between these results difficult. The isolation of culturable bacteria is necessary if the phenotype is to be characterised. However, this investigation would have benefited from the use of molecular analysis to determine if one media was growing a more diverse subset, rather than just a greater number, of bacterial colonies. This would improve future studies by identifying the best media, or group of different media, to isolate as diverse a range of the culturable bacteria as possible.

Culture independent techniques were pursued in following investigations which clearly indicated that a spirochaete microflora was present in the oyster gut. While the *Cristispira*-type spirochaetes have been associated with the crystalline style (Tall & Nauman) and are thought to provide digestive enzymes, such as carbohydrases (Judd 1977), the contribution is not thought to be substantial. For example, Simon and McQuaid (1999) failed to determine either positive or negative effects from the presence of spirochaetes in mussels. Similarly, Bernard (1970) has found that Pacific oysters without *Cristispira* sp. populations were in similar condition to oysters with *Cristispira* sp. populations. Thus, Bernard (1970) concluded that *Cristispira* sp. were "not an obligate part of the gut fauna" (p. 34). Evidence of the bacterial contribution to digestive processes in bivalves (Seiderer *et al*. 1987, Brock 1989, Crosby *et al.* 1990) may, therefore, be derived from a bacterial flora that excludes spirochaetes and may not be associated with the crystalline style.

Evidence that other bacteria occurred in the oyster gut was provided by using molecular analysis. Oyster gut samples collected at low tide provided a 16S rDNA RFLP signature of which components could be identified in the RFLP signatures of oysters collected after varying immersion/potential feeding periods of up to 6 hours. The bands in common

appeared to become more diluted, but were not eliminated, with longer feeding periods. This work requires improvement to confirm the presence of a commensal microflora, through the use of an array of restriction enzymes, such as used by Acinas *et al.* (1997) and Romero *et al.* (2002), to improve the definition of RFLP signatures. Expanding from improved RFLP analysis the investigation could be extended to use the cloning based ARDRA technique to confirm the possibility that a commensal bacterial flora exists and to what extent it is maintained.

Phenotypic characterisation of the gut microflora was further pursued by isolating culturable bacteria from the gut of wild oysters. A large proportion of the isolated bacteria were found to be able to degrade cellulose and/or starch, attributes which have previously been suggested as beneficial for host bivalves (Brock 1989, Crosby *et al*. 1990, Tenkanen *et al*. 2003). Selective identifications of these isolates using 16S rDNA sequence analysis showed a dominance of *Vibrio* spp. previously associated with molluscs, including as confirmed symbionts (*Vibrio lentus*, Nishiguchi & Nair 2003). Consequently, the bacteria cultured from the oyster gut may have been providing an enzymatic diversity which was integral to the ability of oysters to utilise particles collected from suspension. Moreover, these bacteria may be particularly important in an environment where nutrient sources can vary considerably, both daily and seasonally. However, further research is required to determine if a beneficial connection exists between the bacteria and the oyster. For practical application this research would, necessarily, be restricted to culturable bacteria but the possibility that mixed bacterial assemblages or certain bacteria-microalgal associations may be more effective than the addition of a single species or strain, requires careful consideration. Consequently, the benefit of bacteria (in isolation or as part of a greater assemblage of food particles) to oysters must be assessed at different life stages using relevant measures, such as growth for juvenile oysters or gamete quality in broodstock.

The research presented in this thesis indicates that Pacific oysters may employ a range of mechanisms which improve their ability to access the available nutrient resources. It seems unlikely that oysters (or any bivalves) make cognitive decisions about these mechanisms but they are the product of feedback loops, responding to both seston quality and quantity. Certainly, feeding strategies and digestive responses in bivalves have been shown to respond to maximise energetic gain from the available food (Hawkins & Bayne 1984, 1985, Iglesias *et al*. 1996, Ibarrola *et al* 2000, Navarro *et al*. 2003) and particles are

known to be sorted and selected on the ctenidia, palps and in the gut (Shumway *et al*. 1985, Ward *et al*. 1997, Milke & Ward 2003). However, assessments of seston quality should perhaps be related more to the nutrients acquired rather than the physical properties of each accepted particle. Particularly as the sorting mechanisms appear to be disrupted or confounded by apparently simple alterations, such as seston composition (Urban & Kirchman 1992) and cell shape (Bougrier *et al*. 1997). Nutrient acquisition may, therefore, feedback to retention efficiency by physical change in the oyster. For example, when oysters begin to retain picoplankton at the highest levels, spawning has begun and ratios within the oyster, such as meat mass to shell volume, or gill size to meat mass, have altered with the loss of the considerable mass of gametes (Quayle 1988). These changes may, in turn, induce changes in the pallial cavity which enable different retention efficiencies to be achieved. For example, Honkoop *et al.* (2003) suggested changing ratios of meat mass to gill mass in *C. gigas* indicated an enhanced capacity to capture food when body mass was lighter. Thus, when oysters are most in need of nutrient inputs, their internal configuration has altered to improve the range of items they can effectively retain, including the large picoplanktonic resource.

The retention of large quantities of heterotrophic bacteria may provide the oyster with more than just the direct nutritive resource of each bacterial cell. Bivalves are reported to digest bacteria with an efficiency of less than 60 % (Seiderer *et al*. 1984). Therefore, the remaining bacteria in the gut may be degrading, for example, algal and/or detrital cellulose to provide the added nutritive input of the resulting secondary metabolites, as proposed by Crosby *et al*. (1990). It could also be speculated that when the oysters stop feeding, while tidally exposed, residual and/or resident bacterial flora continue the digestive processes of the gut contents and, hence, provide a resource of readily digestible nutrients available when feeding resumes. Investigation of this possibility, using oyster gut extracts collected at a range of periods of non-feeding, would require a careful monitoring of degradation end-products, such as oligosaccharides. However, given sample size requirements, inter-individual variability may be difficult to define without the development of some specific markers. Therefore, consideration should be given to constructing a model stomach system, as has been done in ruminant research (France *et al.* 1993).

In this study, no significant relationship was found between particle retention efficiency and oyster size or condition index (Figure 3.11). Moreover, the monthly *in situ* assessments of picoplankton retention showed that, in winter, retention efficiencies could

be similar to those in summer. This suggests that retention of picoplankton may adjust in response to stimuli operating on scales finer than those resolved by the sampling used in this research, such as microalgal species composition and/or individual species abundance. Clearly, finer scale temporal repetition of these experiments would provide some insight, as would increasing the size class examined. Other assessments of seston composition, such as size fractionated lipid and carbohydrate content, may also add interpretive potential to these studies. The association of bacterial retention to the composition of the retained seston may also be informative. For example, when the ingested seston has a large proportion of cellulose, that would otherwise be inefficiently digested, does the retention of bacteria increase or is there an association between bacteria and the ingested particles before they are even retained by the oyster?

The application of the results of this investigation to hatchery technology requires further research. While it is likely that cultured microalgae provide the majority of the food required by oysters, the addition of bacteria to the food supply may be beneficial. Previous research has shown the benefit that a single bacterial strain may have when added to the feed (Douillet & Langdon 1993), and there is a trend toward the use of non-axenic microalgal cultures in hatcheries (Benzie *et al*. 2002). However, the most effective composition of any bacteria provided for oyster feeding has not yet been elucidated. The isolation of bacteria, which may directly supply essential nutrients or mediate an efficient supply of nutrients, requires investigation. This knowledge is particularly important where isolated hatchery systems using recirculating seawater are intended (e.g. Wikfors *et al.* 2004) as such a system would lack bacteria which may be necessary for the persistence of a commensal microflora or the supply of certain essential micronutrients, especially in the early stages of gametogenesis.

Application of bacteria to larval cultures was beyond the scope of this research but other researchers have demonstrated the potential of bacteria provided as a sole food source or in association with microalgae (Martin & Mengus 1977, Douillet & Langdon 1993). If a resident population of gut bacteria exists, then the time of recruitment may influence mortality of either larvae or spat. Certainly, the larvae encounter similar potential food items in the planktonic stage and early recruitment of a symbiotic gut flora might endow nutritional benefit. Trialling of low density supplementation of larval food with bacterial assemblages could be informative, especially if beneficial isolates could be derived from research such as that suggested above.

Further investigation of the stimulation of small particle retention would be of interest to oyster farming operations. When oysters are retaining particles in the small size fraction, they are also susceptible to opportunistic invasion by bacterial pathogens. These pathogens may be more harmful to the consumers than the oyster, such as in the case of human enteric viruses (Roberts 2002). However, if times when the oysters are susceptible to retaining and/or accumulating pathogens can be identified, enhanced monitoring and preventative measures could be undertaken to mitigate the impact on farm operations.

Oyster feeding physiology and the mechanisms of particle capture, selection and digestion are still not fully understood. The research presented here has extended knowledge of the complex relationships that exist between oysters and the seston on which they feed. However, it has also indicated gaps in our understanding of oyster feeding and conditioning and the role of picoplankton in oyster nutrition. Variable retention of certain components of the seston suggests that oysters have a capacity for selective retention of particles at fine scales. The mechanisms by which selectivity is controlled are unclear, but it appears to enable the oyster to utilise the bacterial resource. Both transitory and resident bacterial populations may exist in the oyster gut providing a potentially rich resource of nutrients, either through direct digestion or indirect enhancement of enzymatic capability and consequent nutrient release. This research has demonstrated that feeding physiology should be considered in relation to both environmental and endogenous influences on oysters. This has previously been highlighted by Bayne (1998) in introducing the TROPHEE workshop which sought to interface bivalve physiology and ecology to allow predictive modelling, particularly for carrying capacity determinations. Given the growing economic importance of Pacific oysters, and more generally the aquaculture of lower trophic level species, the current additions to the knowledge base provide directions for future research in order to improve species management.

Appendices

Appendix 2.1 Hatchery Details

The pilot scale hatchery described briefly in Section 2.2.1 is more fully described here. The design was based upon the description of Curtin (1979) with modifications mostly due to the size and location of the available space.

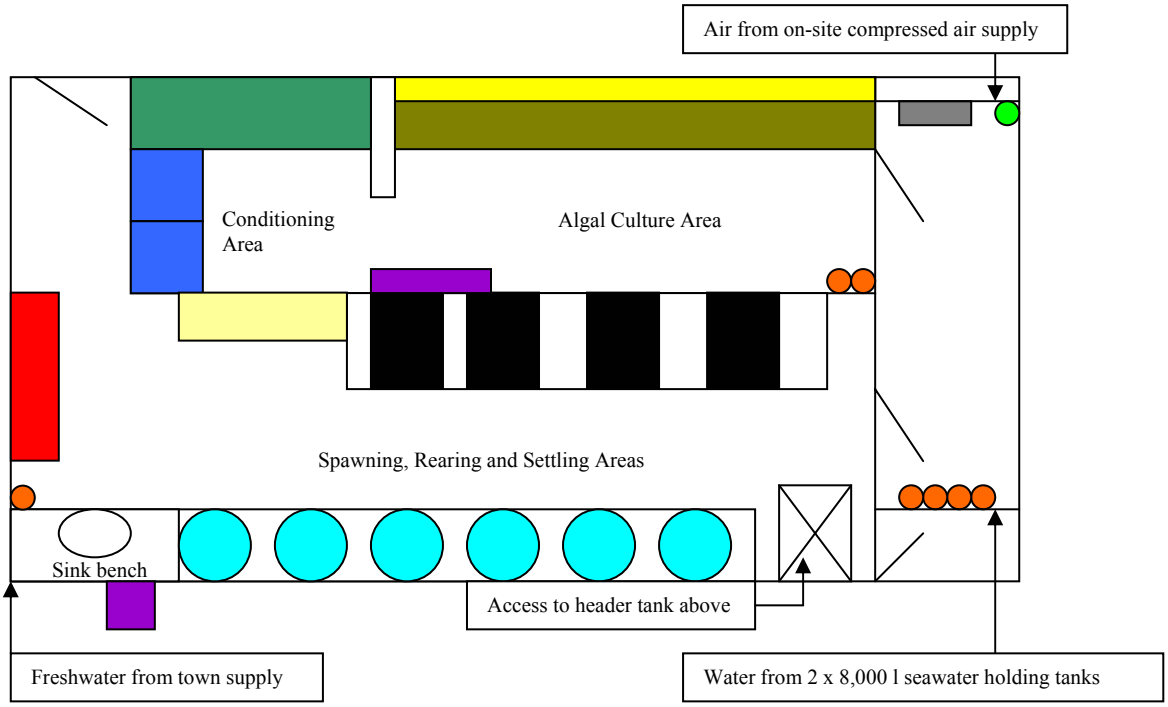


Figure A2.1 Floor plan of pilot scale hatchery at the Mt Albert Research Centre. = conditioning system; = workbench and storage; = Algal culture racks; = Light bank; = Air conditioning units; = Air supply filtration and mixing; = CO_2 supply; = Water filters; = Larval rearing tanks; = Settling tanks; = dry bench; = Spawning bench. The overall floor area is approximately 8 m long by 6 m wide.

The algal culture and conditioning room (Figure A2.1) was constructed of steel clad polystyrene refrigeration panel in which the temperature was regulated to 18 ± 0.5 ^{o}C by an air conditioning unit retro fitted with a Carel ir32 temperature controller. The primary purpose of this room was to culture microalgae in a bulk, batch culture system (32 x 15 l bags) similar to the method described by Laing (1991). Light for microalgal growth was supplied from paired fluorescent tubes (30 W Gro-Lux, Sylvania) aligned horizontally, every 250 mm up the wall behind the bag structure. A light:dark cycle of 20:4 was used

for all the algal cultures. Dark periods have previously been found to be beneficial to microalgal cultures (Brand & Guillard 1981, Caron *et al.* 1988) and allow recovery from pH change (induced by photosynthesis and not fully buffered by the CO_2 enrichment) which can reduce growth rates (Goldman *et al.* 1982). Those cultures that were aerated had a continuous supply of 0.2 µm filtered dry air, enriched with CO_2 (1 % final concentration).

A small partition defined the oyster conditioning area at the rear of the algal culture room. Conditioning took place in a recirculating seawater system consisting of a sump (500 l) from which water was pumped into six plastic bins (45 l) which, in turn, discharged back into the sump. Within each bin, oysters were held on grates 25 mm above the bottom. The inlet water for each bin was discharged through a perforated, PVC pipe that lay under the grate to maximise water circulation. The water depth was maintained at approximately 350 mm in each bin, by an overflow-type outlet pipe. Bags of coral in the sump acted as a substrate for biofiltration that primarily controlled nitrogen levels. All of the conditioning bins were continuously aerated with 0.2 µm filtered, dry air.

The larger room (Figure A2.1) was maintained at 24 ± 2 ^{o}C. This was a multipurpose area for the spawning, fertilisation, larval rearing and settling of the oysters. Figure 2.2 shows how these functions were separated within the room which helped to facilitate transfers from one stage to another. The spawning area consisted of 6 plastic tanks (40 l) that were temperature controlled. The larval rearing equipment consisted of 120 l or 200 l cylindrical plastic tanks. These tanks were lidded to help control temperature, evaporation and aerial contamination. Each tank could be drained through a tap near the base to enable water changes. The settling tanks were 1200 x 700 x 250 mm to fit plastic slats (1000 x 40 x 3 mm), as supplied by Bay Oysters, to be used as the settlement substrate for oyster larvae. These tanks were also fitted with a drain tap near the base. All of the tanks were continuously aerated when in use and 200 W submersible heaters were available for additional heating as required.

Seawater for the hatchery was delivered by tanker truck from a collection site at Wynyard Wharf, Auckland. The seawater was stored in two 8,000 l tanks and pumped, as required, into the hatchery header tank (3,000 l). All of the seawater was filtered, upon entry into the hatchery, through a disk filter (pore size 200 µm), followed by a series of cartridge filters (pore size 20 µm, 10 µm, 1 µm; Taylor Purification Ltd). Once in the header tank,

the seawater was continuously circulated through a UV sterilizer unit (35 W, Steriflow 369) at approximately 250 l h^{-1}. The header tank, situated above the larval rearing and settling area, was heated by a 300 W, thermostatically regulated (Carel ir32), submersible element, to 24 ± 1 ^{o}C. From the header tank the seawater was distributed to outlets throughout the hatchery. Non-toxic hose (25 mm diameter) could be attached to any outlet using standard garden hose clip attachments (Nylex, Gardenia). The seawater used for microalgal culture was further filtered through 0.45 µm and 0.2 µm cartridge filters (as suggested by Lewis *et al.* 1988) to remove potentially contaminating particles.

Appendix 2.2 Composition of Guillard's F/2 Microalgal Culture Medium

Table A2.1 Composition of the stock solutions used in the F/2 culture medium. The final F/2 culture medium contained, for every litre of seawater, sodium nitrate (1 ml), sodium orthophosphate (1 ml), trace metal solution (1 ml) and vitamin solution (1 ml). Sodium silicate solution (1 ml) was added if diatoms were being cultured

Stock Solution	Chemicals	Concentration in Stock Solution
Sodium nitrate	$NaNO_3$	75 g l^{-1}
Sodium orthophosphate	NaH_2PO_4	5 g l^{-1}
Sodium silicate	Na_2SiO_3	60 g l^{-1}
Trace metals	$FeCl_3$	3.15 g l^{-1}
	Na_2EDTA	4.36 g l^{-1}
	$CuSO_4$	9.8 mg l^{-1}
	Na_2MoO_4	6.3 mg l^{-1}
	$ZnSO_4$	22 mg l^{-1}
	$CoCl_2$	10 mg l^{-1}
	$MnCl_2$	180 mg l^{-1}
Vitamins	Vitamin B_{12}	1.0 mg l^{-1}
	Biotin	1 mg l^{-1}
	Thiamine HCl	200 mg l^{-1}

Appendix 2.3 Procedures for the Culture of Microalgae

As described in 2.2.3 above four different stages of microalgal culture were maintained for each species used in the hatchery. The inviolate and working lines were in static flask cultures, the starter lines in aerated flasks and the bulk line in bags. The following equipment and techniques were employed to maintain each of these lines.

Foil caps: A square of aluminium foil was folded in half and in half again to give a square of foil 4 layers thick which could then be moulded over the mouth of the flask. Sizes varied depending upon flask volume.

Cotton bungs: For the static cultures a length of tubular gauze (USL Medical Ltd) was knotted at one end, a piece of non-absorbent cotton wool (approx 3 cm^2) was stuffed into the stocking and the open end knotted to contain the cotton wool. Excess stocking was trimmed to leave a small tab that allowed easy, aseptic, handling of the bung in and out of the flask. These bungs were reused for approximately six month periods.

For the aerated flask culture bungs were constructed by taking a rectangle of non-absorbent cotton wool (approx 20 x 10 cm), folding it in half lengthways and rolling it into a cylinder. The cylinder was fitted into the flask with the folded end down to minimise the loss of cotton pieces into the culture. These bungs were discarded when the culture was used.

250 ml and 500 ml conical flasks (static cultures): Clean flasks were prepared with filtered (0.45 µm nominal cartridge filter) seawater (40 % capacity), F/2 nutrients, a bung of non-absorbent cotton wool (Jacob Cowen & sons Ltd), and capped with aluminium foil. Flasks were autoclaved (15 min @ 15 psi, 121 °C) to sterilise them before use.

3 l conical flasks (aerated cultures): Clean flasks were filled (1.5 l) with filtered (0.45 µm nominal cartridge filter) seawater and F/2 nutrients. A bung of non-absorbent cotton wool and foil cap were added and the flasks autoclaved (30 min @ 15 psi, 121 °C) to sterilise.

15 l clear plastic bags: Unused bags had the open end sealed shut prior to them being suspended on racks in the algal culture area. A 1 cm cut was made in the top edge of each bag with an ethanol sterilised scalpel blade. F/2 nutrients were added with a funnel (rinsed in ethanol immediately prior to use) and filtered (0.45 µm nominal cartridge filter) seawater added with a non-toxic hose (cleaned in ethanol immediately prior to use). Air supply was added to the bags by fitting a sterile 200 µl pipette tip to an air hose and inserting the tip into the bag at its base.

Transfer of cultures: Once sterilised, flasks were cooled to room temperature and inoculated from a donor culture. Transfers between all conical flasks took place in a laminar flow hood (where all surfaces were wiped with 90 % ethanol prior to use and the unit had been operating for at least 10 minutes prior to use). Hands and forearms were cleaned prior to any work in the laminar flow hood. The procedure for a transfer between flasks was;

1) Remove foil caps from donor and recipient flasks,
2) Hold one flask in each hand either side of the Bunsen flame within the laminar flow hood.
3) Remove the bung of the donor flask with the opposing hand and flame the neck
4) Repeat with the recipient flask
5) Transfer the appropriate amount of donor culture into the recipient flask without them touching
6) Flame the neck and replace the bung on the recipient flask
7) Flame the neck and replace the bung on the donor flask
8) Loosely refit the foil caps

As 3 l flasks could not be flamed removal of the bung was done as quickly and carefully as possible within the hood. When the bung was returned to the 3 l flasks a sterile glass pipette with a cotton filter was added to act as an aeration tube when air supply was attached in the algal culture room.

Bag cultures were inoculated from 3 l flasks using a sterile funnel. Any excess culture from 3 l flasks was discarded or fed to the oysters.

Appendix 3.1 Microalgal Species, their Biovolume and Carbon Content

Table A3.1 Microalgal species enumerated by the Cawthron Institute for the Kerikeri Delivery Centre Ltd. Carbon content is given for all species. Where carbon content values were not available biovolume (μm^3) was calculated using the closest geometric shape (Hillebrand *et al.* 1999) and median of available dimensions (Cawthron Institute). Carbon content was then calculated from biovolume using the equations of Menden-Deuer and Lessard (2000), (see 3.2.2.2).

Species	Biovolume (μm^3)	Carbon Content (pg $cell^{-1}$)
Alexandrium margalefii	32,663	3,783
Alexandrium minutum	6,300	983
Alexandrium ostenfeldii	12,900	1,768
Alexandrium pseudogonyaulax	65,400	6,680
Alexandrium tamerense	-	3,000
Alexandrium concavum	65,450	6,684
Alexandrium fraterculus	14,137	1,905
Amphidinium carterae	258	72
Biduluphia spp	5,300	853
Cachonina spp	3,078	547
Ceratium spp	47,435	5,135
Chaetoceros convolutus	3,315	206
Chaetoceros spp	33,510	1,347
Chattonella marina	14,137	669
Chrysochromulina spp	384	36
Coolia monotis	22,450	973
Dictyocha speculum fibula	3,216	567
Dinophysis acuminata	43,600	4,792
Dinophysis acuta	154,000	13,471
Dinophysis spp	-	4,800
Eucampia sp	23,425	1,007
Fibrocapsa japonica	9,503	1,376
Gambierdiscus toxicus	38,790	1,517
Gonyaulax spp	16,800	2,195
Guinnardia spp	12,768	616
Gymnodinium cf breve	13,442	1,828
Gymnodinium mikimotoi	4,032	682
Gymnodinium spp	954	209
Gyrodinium	45,158	4,932
Herterosiigma akashiwo	1,400	287
Laudenia spp	56,000	2,043
Leptocylindricus spp	523	46
Linqulodinium polyedrum	54,360	5,741
Melosira spp	39,782	4,446
Navicula spp	274	27
Nitzschia spp	141	16
Odontella spp	12,783	1,754
Ostreopsis spp	20,039	888
Paralia spp	74,800	7,457
Peridinium spp	19,704	2,501
Phaeocysystis spp	87	29
Pleurosigma spp	122	14
Polykrikos schwartzii	98	32
Prorocentrum cf gracile	1,533	309
Prorocentrum lima	21,100	2,645

Species	Biovolume (µm^3)	Carbon Content (pg cell^{-1})
Prorocentrum spp	-	283
Protoceratium reticulatum	50,534	1,879
Protoperidinium Spp.	24,181	2,957
Prymnesium spp	796	181
Pseudo nitzschia spp	113	13
Rhizosolenia spp	1,596	114
Scrippsiella spp	11,044	1,556
Skeletonema spp	312	30
Thalassionema spp	170	51
Thalassiosira spp	150	17

Appendix 3.2 Post-hoc Tukey Analysis of ANOVA of Picoplankton

Table A3.2 Significant differences between months for ambient concentrations and retention efficiencies of picoeukaryotes. Months not connected by the same letter were significantly different.

Month	Ambient Picoeukaryote									RE Picoeukaryote
Dec-01	A									A
Jan-02		B								A
Mar-02			C							A
Apr-02			C	D						A
Feb-02				D						A
Oct-01					E					A
Aug-02					E	F				A
Nov-01						F				A
Jul-02							G			A
Jun-01								H		A
Sep-01								H		A
Jul-01								H	I	A
Oct-02									I	A

Table A3.3 Significant differences between months for ambient concentrations and retention efficiencies of cyanobacterial population 1. Months not connected by the same letter were significantly different.

Month	Ambient Cy1									RE Cy1
Mar-02	A									A
Aug-02		B								A
Feb-02			C							A
Oct-01				D						A
Jan-02				D						A
Dec-01					E					A
Nov-01						F				A
Jul-02						F	G			A
Jun-01							G	H		A
Apr-02								H		A
Jul-01								H		A
Oct-02									I	A
Sep-01									I	A

Table A3.4 Significant differences between months for ambient concentrations and retention efficiencies of cyanobacterial population 2. Months not connected by the same letter were significantly different.

Month	Ambient Cy2				RE Cy2		
Dec-01	A				A	B	
Oct-02		B				B	C
Jul-02		B	C		A		
Sep-01		B	C				C
Oct-01		B	C	D	A	B	C
Jul-01		B	C	D	A	B	C
Aug-02		B	C	D	A	B	C
Nov-01		B	C	D			C
Feb-02			C	D	A	B	C
Mar-02			C	D	A	B	C
Jun-01			C	D	A	B	
Jan-02			C	D	A	B	C
Apr-02				D	A	B	C

Table A3.5 Significant differences between months for ambient concentrations and retention efficiencies of cyanobacterial population 3. Months not connected by the same letter were significantly different.

Month	Ambient Cy3								RE Cy3	
Nov-01	A								A	B
Oct-01		B							A	
Jul-01		B	C						A	
Dec-01			C	D					A	
Oct-02				D	E					B
Sep-01					E	F			A	B
Mar-02						F	G		A	
Jul-02						F	G	H	A	B
Jan-02						F	G	H	A	
Feb-02							G	H	A	
Aug-02								H	A	
Apr-02								H	A	B
Jun-01								H	A	

Table A3.6 Significant differences between months for ambient concentrations and retention efficiencies of heterotrophic bacteria. Months not connected by the same letter were significantly different.

Month	Ambient Heterotrophic Bacteria					RE Heterotrophic Bacteria				
Mar-02	A						B	C	D	
Jan-02	A					A	B	C		
Feb-02	A	B				A	B			
Nov-01	A	B	C				B	C	D	E
Apr-02	A	B	C						D	E
Jul-01	A	B	C	D			B	C	D	
Dec-01		B	C	D	E	A				
Jul-02			C	D	E		B	C	D	E
Jun-01			C	D	E					E
Aug-02			C	D	E		B	C	D	E
Oct-01				D	E				D	E
Sep-01				D	E		B	C	D	
Oct-02					E			C	D	E

Appendix 4.1 Staining Protocols for Histological Sections

Ehrlich's Haematoxylin and Eosin stain (Humason 1972)

Reagents (all chemicals are BDH 'AnalaR' grade unless stated otherwise)

Ehrlich's Haematoxylin	Haematoxylin technical (C.I. 75290)
	Ethanol (95 %)
	Glycerol
	dH_2O
	Aluminium potassium sulphate
	Glacial acetic acid
1 % Eosin	Eosin technical dye (C.I. 45380)
	dH_2O
	Calcium chloride (1 ml for every 100 ml of stain)
STWS	Sodium Bicarbonate
	Magnesium sulphate
	dH_2O
1 % acid alcohol	Ethanol
	dH_2O
	Hydrochloric acid

For thin sections on glass slides;

1. Dewax sections in xylene (100 %) (5 min)
2. Hydrate through absolute alcohol (95 %) (2 x 2 min)
3. Rinse in running tap water
4. Stain in Ehrlich's Haematoxylin (15 min)
5. Wash in running tap water, drain
6. Dip in acid alcohol (x 3)
7. Wash in running tap water
8. Immerse in STWS (1 min)
9. Wash in running tap water (10 min)
10. Stain in 1% Eosin (5 min)
11. Wash in running tap water
12. Dehydrate through 95 % absolute alcohol (3 x 6 immersions)
13. Clear in xylene (3 x 6 immersions)
14. Mount with resin and coverslip

Gram's stain (Disbrey & Rack 1970; in Humason 1972)

Reagents (all chemicals are BDH 'AnalaR' grade unless stated otherwise)

Lillies Crystal Violet	Crystal violet (C.I. 42555)
	Ethanol (95 %)
	Ammonium oxalate
	dH_2O
Lugol's iodine	Iodine
	Potassium iodide
	dH_2O
Gram's iodine	Dilute Lugol's iodine 3:1
Sodium thiosulphate	3 % (w/v) Sodium thiosulphate in dH_2O
Neutral red	1 % (w/v) Neutral red (C.I. 50040) in dH_2O

For thin sections on glass slides;

1. Dewax sections in xylene (100 %) (5 min)
2. Hydrate through 95 % absolute alcohol (2 x 2 min)
3. Rinse in running tap water
4. Stain in Crystal Violet (2 min)
5. Rinse in running tap water, drain
6. Stain in Gram's iodine (2 min)
7. Rinse in running tap water
8. Differentiate in running acetone
9. Wash in running tap water
10. Immerse in sodium thiosulphate (5 min)
11. Wash in running tap water (10 min)
12. Stain in neutral red (1 min)
13. Was in running tap water (1 min)
14. Dehydrate through 95 % absolute alcohol (3 x 6 immersions)
15. Clear in xylene (3 x 6 immersions)
14. Mount with resin and coverslip

Appendix 4.2 Gram Staining of Bacterial Isolates

1. A sample of an isolated colony is smeared onto a glass slide with a small drop of water, air dried then heat fixed by passing through a flame.
2. The slide is flooded with crystal violet stain for 60 seconds then flushed clear with tap water.
3. Grams iodine is then flooded over the slide for 60 seconds then flushed clear with tap water.
4. Ethanol is dripped over the slide to decolourise then the slide is flushed clear with tap water.
5. The slide is flooded with saffarin stain for 60 seconds then flushed with tap water.
6. After blotting dry the bacterial cells are observed by light microscopy under 1000x magnification, oil immersion.

Appendix 4.3 Colony Counts from Samples of Farmed and Hatchery Oysters

Table A4.1 Number of colonies counted on agar plates of different media types inoculated with 100 µl of dilute oyster gut content or seawater (1:10 v/v in salt peptone water). Duplicate plates for the wild samples were lost. The media types are described in Table 4.1.

Sample	Colony Forming Units					
	Marine	**SPCA**	**Basal**	**YSW**	**YDW**	**2B**
Hatchery 1	112	ND	176	204	173	172
	220	ND	92	252	47	302
Hatchery 2	460	ND	532	880	385	568
	800	ND	300	1300	444	1037
Hatchery seawater	170	ND	42	39	24	55
	184	ND	70	93	20	61
Farm 1	392	220	90	1184	ND	ND
Farm 2	ND	85	32	14	ND	ND
Farm 3	ND	336	99	296	ND	ND

ND = No data

Appendix 4.4 Results of Determinative Assays for the Characterisation of Oyster Gut Microflora

Table A4.2 Colony morphology, cell descriptions, growth and utilisation of different media for all isolates. Media are Mar = Marine agar, Sta = Starch agar, Cell = Cellulose agar, Pro = Protein agar, and are described in Table 4.1 above. Gram reactions are either positive (G+) or negative (G-). Where tests were not clear +/- has been recorded (see Figure 4.3 for an example).

Isolate No.	Media of initial isolation	Colony description at initial isolation	Growth on media			Iodine starch test	Colony description on marine agar	Gram Stain	Cell description	Nutrient utilisation assays			
			Mar	Sta	Cell					T-iso	Sta	Cell	Pro
1	Starch	Red brown	+	+	+	+	Small wet white	G-	Rod	-	-	-	+
2	Starch	White, concentric rings	+	+/-	+/-	+							
3	Starch	dark brown	+	+	+	-							
4	Starch	White	+	+/-	+/-	-							
5	Starch	small clear/white	+	-	-	-							
6	Starch	white	+	+/-	-	+							
7	Starch	clear, concentric rings	+	+	+/-	+							
8	Starch	red brown, with rings	+	+/-	+/-	-							
9	Starch	white wet	+	+	+/-	-	creamy wet white			+	-	-	-
10	Starch	clear, white centre	+	-	-	+	very small wet white	G-	Rod	-	+	-	-
11	Starch	golden brown	+	+/-	+/-	-	red/orange			+	-	-	-
12	Cellulose	Red brown, clear halo	+	+/-	+/-	-	red brown			+	-	+	-
13	Cellulose	Clear yellowish centre	+	+/-	+/-	+	small white			+/-	-	+	-
14	Cellulose	Red small	+	+/-	-	-	white concentric rings			+/-	-	+	+/-
15	Cellulose	clear	+	-	-	+	wet cream	G-	Rod	+	+/-	-	-
16	Cellulose	white	+	+/-	+/-	-	wet white	G-	Rod	+	+	+	-
17	Cellulose	Yellow centre, clear/white	+	+	+	+	brown/white			+	-	-	-
18	Cellulose	reddish golden brown	+	+	+	+	brown, clear edge			+	-	+	-
19	Marine	red brown	+	+	+	+	brown white	G-	Rod	-	-	-	-
20	Marine	white cream centre	+	+/-	+/-	-	wet cream white	G-	Rod	+	-	+	-
21	Marine	creamy white	+	+/-	-	-	wet clear			+	+	-	-
22	Marine	brown centre, white halo	+	+/-	-	-	brown	G-	Rod	-	+/-	-	-

Isolate No.	Media of initial isolation	Colony description at initial isolation	Growth on media			Iodine starch test	Colony description on marine agar	Gram Stain	Cell description	Nutrient utilisation assays			
			Mar	Sta	Cell					T-iso	Sta	Cell	Pro
23	Cellulose	red	+	+	+	+	bright orange	G-	Rod	+	+	+/-	+
24	Cellulose	creamy brown	+	+	+/-	-	creamy white			+	+/-	+/-	-
25	Cellulose	cream	+	+/-	-	-	creamy white	G-	Rod	-	+/-	+/-	-
26	Cellulose	clear	+	-	-	+	very small creamy white	G-	Rod	-	+	-	-
27	Cellulose	white spreader	+	+	+	+	white spreader			+/-	+	+	-
28	Cellulose	golden brown, white halo	+	+/-	-	+	small cream	G-	Rod	-	+/-	+	-
29	Marine	white spreader	+	+	+	+	creamy orange brown			+	+	+	-
30	Marine	red brown	+	+	+	+	bright orange	G+		+	+/-	+	+
31	Marine	cream centre, white halo	+	+/-	+/-	+	wet white	G-	Rod	+	+	+	-
32	Marine	white	+	+/-	-	-	wet cream			+	+	+	-
33	Marine	clear white	+	+/-	-	-	clear white			+	+/-	+	-
34	Marine	brown	+	+	+	+	white			+	+	+	-
35	Marine	clear	+	-	+/-	-	white			+	+	+	-
36	Marine	golden brown	+	+	+	-	white brown edge	G-	Rod	+	+	+	-
37	Marine	reddish brown, clear	+	+/-	+/-	-	orange brown	G-	Rod	+	-	+	-
38	Starch	Apricot	+	+	+	+	orange	G-	Rod	+	-	+/-	+
39	Starch	Red white halo	+	+	+	+	red brown	G-	Rod	+/-	-	-	-
40	Starch	clear brown	+	+/-	+	+	brown			+/-	-	+/-	-
41	Starch	white	+	+	+	+	white	G-	Rod	+	+	+	-
42	Starch	brown	+	+	+	-							
43	Starch	clear	+	-	-	+							
44	Starch	white concentric rings	+	-	-	+	creamy white			+/-	+/-	+/-	+/-
45	Starch	dark red	+	+	+	-	creamy white	G-	Rod	+	-	+	-
46	Starch	white concentric rings	+	+/-	+/-	+	yellow/brown white			+	+	+	-
47	Starch	golden brown	+	+	+	-	golden brown			+	-	+	-
48	Starch	cream/brown centre, clear halo	+/-	+/-	+	-							
49	Starch	red brown	+	+/-	-	-	brown			+	-	+/-	-
50	Starch	white	+	+	+	-	cream	G-	Rod	+	-	+	-
51	Starch	clear white	+	+/-	-	+							-
52	Marine	clear	+	+/-	-	+	clear brown			+	+	+	-

Isolate No.	Media of initial isolation	Colony description at initial isolation	Growth on media			Iodine starch test	Colony description on marine agar	Gram Stain	Cell description	Nutrient utilisation assays			
			Mar	Sta	Cell					T-iso	Sta	Cell	Pro
53	Marine	clear white	+	+/-	+/-	+	cream/white			+	+	+	+
54	Marine	white	+	+	+	-	small white	G-	Rod	+	-	-	-
55	Cellulose	brown/black	+	-	+/-	-	dark brown			+	-	-	-
56	Cellulose	creamy yellow	+	+/-	-	+	yellow cream			+	-	-	-
57	Cellulose	cream white	+	+	+/-	+	clear white			-	+	-	-
58	Cellulose	white cream centre	+	+/-	+/-	+	clear white	G-	Rod	+	-	-	-
59	Starch	yellow white	+	-	-	+							
60	Starch	clear/creamy centre	+	-	+/-	+	cream, clear edge	G-	Rod	+/-	-	-	-
61	Starch	white	+	+	+	+	wet, white spread	G-	Rod	+	+	+	+/-
62	Starch	reddish white	+	+	+	+	red brown			+	-	+/-	-
63	Starch	dark red	+	+	+	-	white			+	+	+	+
64	Starch	white concentric rings	+	+	+/-	-	creamy white	G-	Rod	+	+	+	-
65	Marine	clear white halo	+	+/-	-	+	clear brown	G-	Rod	+	+	-	-
66	Marine	white	+	+/-	+/-	+	brown, white edge			+	+/-	-	-
67	Marine	brownish cream	+	+	+	+	golden brown			+	-	+/-	-
68	Marine	golden brown, clear halo	+	+	+	+	red/brown clear			+	-	+/-	-
69	Cellulose	golden brown	+	+	+	+	orange brown			+/-	-	+/-	-
70	Cellulose	clear	+	+/-	+/-	-	clear yellow	G-	Rod	+/-	+	+/-	-
71	Cellulose	white	+	+/-	+/-	+	white			+/-	+/-	+	-
72	Cellulose	clear centre, white halo	+	-	-	-	cream	G-	Rod	+/-	+	-	-
73	Cellulose	light yellow	+	+	+	+	brown white			+/-	-	-	-
74	Cellulose	apricot red	+	+	+	+	clear brown			+/-	-	-	-
75	Cellulose	clear yellow	+	+/-	-	+	clear cream	G-	Rod	+/-	-	-	-
76	Cellulose	white	+	+	+	+	white	G-	Rod	+/-	+	+	+/-
77	Cellulose	cream	+	+	+	+	white			+	+	-	-
78	Cellulose	creamy white irregular	+	+	-	+	yellow			+/-	+/-	-	-
79	Marine	red apricot	+	+	+	+	red brown			+/-	-	+	-
80	Marine	creamy white	+	+	+	+	white	G-	Rod	+	+	-	-
81	Marine	clear	+	-	+/-	+	clear gold	G-	Rod	+/-	+	-	-
82	Marine	brown centre, white halo	+	-	+/-	-	very small dark brown			+/-	-	-	-
83	Starch	clear yellow centre	+	+/-	+/-	+	wet yellow	G-	Cocci	+/-	+	-	-

Isolate No.	Media of initial isolation	Colony description at initial isolation	Growth on media			Iodine starch test	Colony description on marine agar	Gram Stain	Cell description	Nutrient utilisation assays			
			Mar	Sta	Cell					T-iso	Sta	Cell	Pro
84	Starch	apricot red	+	+	+	+	yellow brown			+	+	+/-	-
85	Starch	white concentric rings	+	-	+/-	-	cream			+/-	+	-	-
86	Starch	yellow/clear	+	+/-	+/-	+	small clear yellow			+/-	-	-	-
87	Starch	white	+	+	+	-	cream			+	+	-	-
88	Starch	clear	+	-	-	-	clear cream			+/-	+	-	-
89	Marine	red brown, white edge	+	+	+	+	clear brown			+	-	-	-
90	Marine	clear	+	-	-	-	very small clear			+	+	-	-
91	Marine	white, cream centre	+	+/-	+/-	-	very small cream	G-	Rod	+	-	-	-
92	Marine	clear white	+	+/-	+/-	-	cream white			+	+	-	-
93	Starch	red concentric rings	+	+	+	+	golden brown			+	-	+	-
94	Starch	yellow	+	+	+/-	+	clear brown/yellow			+	-	-	-
95	Starch	creamy yellow	+	+/-	+/-	-							
96	Starch	cream concentric rings	+	+	+	+	yellow cream			+	+	-	-
97	Starch	clear concentric rings	+	+/-	-	+	clear cream	G-	Rod	+/-	+	-	-
98	Starch	white concentric rings	+	+/-	+	+	cream			+	+	-	-
99	Starch	white	+	+	+	-	cream			+/-	+/-	+	-
100	Cellulose	golden brown	-	-	-	+	bright orange/yellow			+/-	-	-	-
101	Cellulose	white	+	+/-	+/-	-							
102	Cellulose	cream clear edge	+	+/-	+/-	+	small clear	G-		+	+	-	-
103	Cellulose	clear white	+	-	-	+	small clear	G-	Rod	+/-	+	-	-
104	Cellulose	reddish white	+	+	+	+	clear cream		Cocci	+/-	+	-	-

Table A4.3 Results of the reactions on the api 20E identification strip after 24 hours incubation. Where reactions were not clear +/- has been recorded. Tests are: Onpg = Beta-galactosidase, Adh = Arginine dihydrolase, Ldc = Lysine decarboxylase, Odc = Ornithine decarboxylase, Cit = Simmons citrate, H_2S = H_2S production, Ure = Urease, Tda = Tryptophane deaminase, VP = Voges Proskauer, Gel = Gelatin hydrolysis, Glu = Glucose, Man = Mannitol, Ind = Indole, Sor = Sorbitol, Rha = Rhamnose, Sac = Sucrose, Mel = Melibiose, Amy = Amygdaline, Ara = Arabinose, NO_2 = Nitrite production, N_2 = Nitrogen production.

ID	API tests																			
	Onpg	**Adh**	**Ldc**	**Odc**	**Cit**	**H_2S**	**Ure**	**Tda**	**VP**	**Gel**	**Glu**	**Man**	**Ind**	**Sor**	**Rha**	**Sac**	**Mel**	**Amy**	**Ara**	**NO_2**	**N_2**
1	+/-	-	-	-	-	-	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	+/-	-	-	-	-	-	-	+/-	-	-	-	+	-	-	-	+	-	-	-	+	
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	+/-	-	-	-	-	-	-	+/-	-	-	+	+	-	-	+	+	-	-	-	+	
16	-	-	-	-	-	-	-	+/-	-	+	-	+	-	-	-	+	-	-	-	+	
19	-	-	-	-	-	+	-	+/-	-	-	-	-	-	-	-	-	-	-	-	+	
20	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	+	-	+	-	+	
22	-	-	-	-	-	+	-	+/-	-	-	-	-	-	-	-	-	-	-	-	+	
23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	+/-	-	-	-	-	-	-	+	-	+	+	+	-	-	-	+	-	+	-	+	
26	+/-	-	-	-	-	-	-	+/-	-	+/-	-	+	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+/-	-	-
30	-	-	-	-	-	-	-	+/-	-	-	-	-	-	-	-	-	-	-	+/-	-	-
31	-	-	-	-	-	-	-	+/-	-	-	+	+	-	-	-	+	-	-	-	+	
36	-	-	-	-	-	+	-	+/-	-	-	-	-	-	-	-	-	-	-	-	+/-	
37	-	-	-	-	-	+	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	-
38	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
39	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
41	-	-	-	-	-	-	-	+/-	-	-	+	+	-	-	-	+	-	-	-	+	
45	-	-	-	-	-	-	-	+/-	-	-	+	+	-	-	-	+	-	+/-	-	+	
50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
58	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	+	+/-	+	+/-	+	
60	-	-	-	-	-	-	-	+/-	-	-	+	+	-	-	+/-	+	+/-	+/-	-	+/-	-

ID	API tests																			
	Onpg	**Adh**	**Ldc**	**Odc**	**Cit**	**H_2S**	**Ure**	**Tda**	**VP**	**Gel**	**Glu**	**Man**	**Ind**	**Sor**	**Rha**	**Sac**	**Mel**	**Amy**	**Ara**	**NO_2**	**N_2**
61	-	-	-	-	-	-	-	+/-	-	-	+	+	-	-	-	+	-	-	-	+	
64	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	+	-	+/-	-	-	+
65	+/-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	-	-
70	+/-	-	-	-	-	-	-	+/-	-	-	-	+	-	-	-	+	-	-	-	-	-
72	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	-	-
75	+/-	-	-	-	-	-	-	+/-	-	-	-	+	-	-	-	+	-	-	-	-	-
76	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	+	-	+/-	-	-	+
81	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
91	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
97	+/-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-
102	+/-	-	-	-	+	-	-	+/-	-	-	-	-	-	-	-	-	-	-	-	+	-
103	+/-	-	-	-	-	-	-	+/-	-	-	-	+	-	-	-	-	-	-	-	-	-

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