Investigation into the microbiological causes of epizootics of Pacific oyster larvae (*Crassostrea gigas*) in commercial production

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Declaration

This thesis contains no material that has been accepted for the award of any other degree or diploma in any tertiary institution. To the best of my knowledge this thesis does not contain material written or published by another person, except where due reference is made.

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Abbreviations

DSS – days since spawning	MVDISP – multivariate dispersion index
% CL – percentage of larvae crossing midway line in cuvette	n – number of samples / replicates
% SL – percentage of larvae swimming in cuvette (above bottom)	NSW – natural seawater
CFU – colony forming units	PCR – polymerase chain reaction
CTW – clean tank water	ppb – parts per billion
DF – degrees of freedom	ppm – parts per million
DOC – dissolved organic carbon	ppt – parts per thousand
DPIPWE - Dept Primary Industries, Parks, Water and Environment (Tasmanian government)	Pr - probablility
EC50 – dose required to achieve a nominal effect in 50% of the population	SAS GLM – general linear model of statistical program SAS
F – F-value (statistics)	SSW – artificial sterilised seawater
FSW – filtered seawater	Stdev – standard deviation
HSP – heat shock proteins	STE – sodium-tris EDTA buffer
IC system – intensive culture, flow-through system	TCBS - thiosulfate citrate bile salts sucrose agar
LD50 – dose required to kill 50% of the population	THC – total heterotrophic counts
LSD – least significant difference	TRFLP – terminal restriction fragment length polymorphism
MA – marine agar	TSW – time since water change
MC – microbial community	TVC – total viable culturable bacteria
MFDP – marine farm development plans	WC – water column

Abstract

An investigation was undertaken into the cause of Pacific oyster (*Crassostrea gigas*) larvae epizootics occurring in a commercial hatchery located at Bicheno, Tasmania. An extensive monitoring survey was conducted at the hatchery to characterise the microbiological environment in the immediate vicinity of the larvae associated with different production outcomes. The surveys were performed over a 12-month period and included eight different production runs. Seven of the eight production runs terminated in disease incidence with larvae exhibiting disease symptoms consistent with bacterial infection previously described as bacillary necrosis, caused primarily by pathogenic *Vibrio* spp. Using production data records, physiochemical data, dissolved nutrient analysis, bacterial cultivation, TRFLP fingerprinting and 16S rRNA gene clone library analysis, two separate investigations were undertaken. In the first investigation microbial communities in each compartment of the larvae tank (water column, larvae, biofilm) as well as inputs into the tank (algae feed, seawater, and eggs) were characterised in both an intensive flow-though system and low intensity batch system, in order to understand the microbial ecological context in which disease occurred. Temporal variability of microbial communities was measured as an indicator of system stability. It was shown that microbial communities of the larvae and water column varied primarily with larvae age and sampling period and that the most likely cause of variability with sampling period was variability in the seawater. Altered culture conditions changed the microbial communities of the water column but larvae communities were shown to be largely resistant to change experienced in the water column. Larvae microbial communities were closely related to the indigenous microbial communities of the egg. Thus formation of the indigenous microbial community during spawning and fertilisation may be a control point for management of the microbial composition of the larvae and potentially for managing disease incidence. The presence of predominant non-typical marine species of the genera, *Sphingomonas* and *Ramlibacte*, in eggs and larvae samples, indicated a non-marine source of contamination occurring during spawning and fertilisation. The second investigation characterised the microbial environment associated with the emergence of disease

symptoms, and the underlying cause of disease. There was no predominant characteristic microbial community in the larvae or water column associated with disease and no recognised bacterial pathogens were detected using culture-independent methods of assessment. *Vibrio* population numbers peaked with the emergence of disease symptoms but remained only a minor component of the total population as indicated by prevalence in 16S rRNA gene clone libraries and next generation sequencing. Larvae aggregative behaviour near the tank bottom prior to the development of definitive disease symptoms indicated a non-microbiological primary cause of disease, or a microbiological etiology that occurred below the detection limits of 16S rRNA gene-based analyses used. Following the monitoring study an investigation was undertaken into the effect of environmental stressors on the susceptibility of larvae to bacterial challenge. Larvae were exposed to different levels of copper for 24 and 48 h before being challenged with three different bacterial species. It was shown that sub-lethal levels of Cu decreased larvae activity and increased larvae susceptibility to bacterial attack under some conditions. Relative to sub-lethal levels larvae exposed to the lethality threshold of 25 ppb Cu had higher activity levels and higher survival rates in subsequent bacterial challenge, which may indicate induction of the so called heat-shock response. Larvae behaviour was modified at Cu levels as low as 2.5 ppb, which indicates that behaviour could be used as a sensitive biomarker of Cu stress and potentially other forms of chemical stress. The behavioural response to different concentrations of Cu was non-linear and differed with duration of exposure, indicating that behavioural assessments should be made across a range of concentrations and also across a 24 – 48 h time period.

Introduction

Mass mortality events in oyster hatcheries are a major constraint in all the significant oyster growing regions of the world. In Australia the problem is no less significant with losses estimated at $500,000 annually with seasonal undersupply approaching 50%. To address the problem the Australian Seafood Cooperative Research Centre, the Tasmanian Institute of Agriculture in the University of Tasmania (UTAS), the Tasmanian-based hatchery, Shellfish Culture Ltd, and the UTAS PhD supervisory team supported this PhD research project, which was undertaken by Chris Chapman at the Tasmanian Institute of Agriculture (TIA). The industry partner in this work, Shellfish Culture Ltd, currently supplies 60% of the Australian oyster market for oyster spat, and have two hatcheries, one based in Pipeclay lagoon (south east coast, Tasmania) and the other on the Gulch in Bicheno (east coast, Tasmania), where monitoring work for this project was conducted (Figure 1).

Disease problems in hatcheries involving bacterial infections are not a new problem. In fact the development of the industry in Australia since the 1970's has been a history of success and failures in overcoming disease problems as systems have developed and increased in capacity, with each new development causing added complication. In numerous studies pathogenic *Vibrio* spp. have been implicated in hatchery epizootics (DiSalvo et al 1978; Elston et al 2008) and the pathogenic mechanism at least partly elucidated (Hasegawa et al 2009). However, since these bacteria are often present in association with healthy larvae, it is not clear how pathogenicity is initiated (Elston et al 2008). Previous research into hatchery epizootics has been largely limited to studying only the culturable bacterial populations associated with larvae culture, which has been shown by Ferguson et al (1984) to represent only 0.01-12.5% of the viable bacterial population from the marine environment, although is likely to be higher in the eutrophic environment of the larvae tank (Lebaron et al 2000). As such, implication of bacterial pathogens has been achieved with little or no understanding of the non-culturable microbial community in which the pathogen has arisen. This knowledge gap is addressed

in this study, which has had the advantage of relatively new tools in molecular genetics, which have allowed whole bacterial communities to be characterised and described.

The severity of pathogen infection is recognized to be context dependent. This was perhaps first formalized in the disease triangle (McNew 1960), a conceptual framework that acknowledges infection outcomes as the product of three factors: inherent susceptibility of the host, the inoculum potential of the pathogen, and the environmental conditions that both experience during infection. With this in mind three research hypotheses were developed, in Table 1, with the ultimate purpose of reducing the incidence of failed production runs caused by disease. Hypothesis A was addressed in Chapter 2 and Chapter 3 to identify the underlying cause(s) of disease in a commercial oyster hatchery, through a 12-month monitoring study in which microbial communities were characterised in a commercial context using culture-independent methods of assessment, including 16S rRNA gene clone libraries and Terminal Restriction Fragment Length Polymorphism (TRFLP), to include the whole microbial community. In Chapter 2 the frame of view on disease incidence was widened to describe the microbial ecology of the larvae tank and inputs into the tank; the broader microbial context in which disease occurs. In Chapter 3, incidence of disease was examined in detail, with inclusion of characterisations of microbial communities in the larvae tank, larvae health data and physiochemical data of culture water; and was supported by larvae-bacteria challenge trials. Hypothesis B, addressed in Chapter 4, involved a shift in focus in McNew's disease triangle, from the microbial community (pathogen) to the larvae (host) and the potential impact of chemical stress (environment) on susceptibility to disease incidence. Copper stress was investigated since a potential source was located immediately adjacent to the hatchery in the form of a boat slip (Figure 1).

Table 1 – Hypotheses addressed in thesis and research objectives

Hypothesis	**Research objectives**
A. That disease of oyster larvae is characterised by the presence or absence of particular predominant bacterial groups in the larvae tank	1. Characterise the microbial ecology and temporal variability in the larvae tank with respect to tank inputs (Chapter 2) 2. Characterise the microbial community and environment associated with emergence of disease symptoms and identify microbial cause(s) of disease (Chapter 3)
B. That exposure to sub-lethal levels of toxins in culture water may affect larvae susceptibility to bacterial disease	3. Determine whether exposure to sub-lethal levels of copper affects larvae susceptibility to bacterial challenge or alters larvae swimming behaviour (Chapter 4) 4. Develop tools for testing hatchery water for sub-lethal levels of chemical toxins (Chapter 4)

Figure 1 – Google earth image of hatchery site including items of interest. A: Boat slip. B: Jetty. C: Shallow water source. D: Shellfish Culture oyster hatchery. E: Abalone farm. F: Boat ramp. G: Deep water source

1 Chapter 1: Literature review

1.1 Introduction

Prior to the 1980's production of oyster spat in Australia was achieved by collecting newly spawned oyster spat on concrete covered sticks place in bays and estuaries (Evans 2000). The sticks were then sold to oyster farmers, in bundles of around 40, to be nailed separately on racks where the spat could mature for sale. In the late 1970's production failures and increasing demand necessitated a new production system. Thus the first pilot hatcheries were established, which demonstrated that production of spat through larviculture could be achieved. Commercialisation of production occurred in Tasmania through formation of a company, modelled on fisherman's co-operatives, named Shellfish Culture Ltd, which established a hatchery at Bicheno, where it still is today. The science of spat production was still in its infancy and so significant research and development was required to overcome production challenges. Throughout this period disease problems were the primary production limitation and today remains as significant a problem as ever, as intensification of production has added new layers of complexity to the disease problem. Hatcheries around Australia and in other regions of the world all report similar problems, albeit with regional differences, such that disease is considered the main constraint in most oyster hatcheries (Elston & Leibovitz 1980; Elston et al 2008).

This literature review details the current understanding of the disease problem as is experienced in the commercial context. The framework of the literature review is based on McNew's disease triangle comprising host, pathogen and environment. For the host, the larvae, relevant topics include normal development, the larvae immune response and common environmental stressors in the hatchery. Recognised pathogens are identified and pathogenic mechanisms detailed. The microbial ecology of known bacterial pathogens is described in their ecological and hatchery contexts including a description of selective pressures affecting microbial communities in the larvae tank and microbial niches typically encountered. The literature review is divided into four main parts as follows:

- Normal development of oyster larvae
- Causes of larvae mortality
- Larvae immune response
- Microbiological aspects of oyster hatcheries

1.2 Normal development of Oyster larvae

The duration of the Pacific oyster (*C. gigas*) larval period is largely dependent on temperature but is typically in the range of 14 to 17 d under culture conditions (Elston 1999). Larvae undergo a number of well defined developmental stages in transition from gametes to their juvenile form known as "spat". The following description of these stages has been adapted from (Loosanoff & Davis 1963) and is summarised in Figure 2.

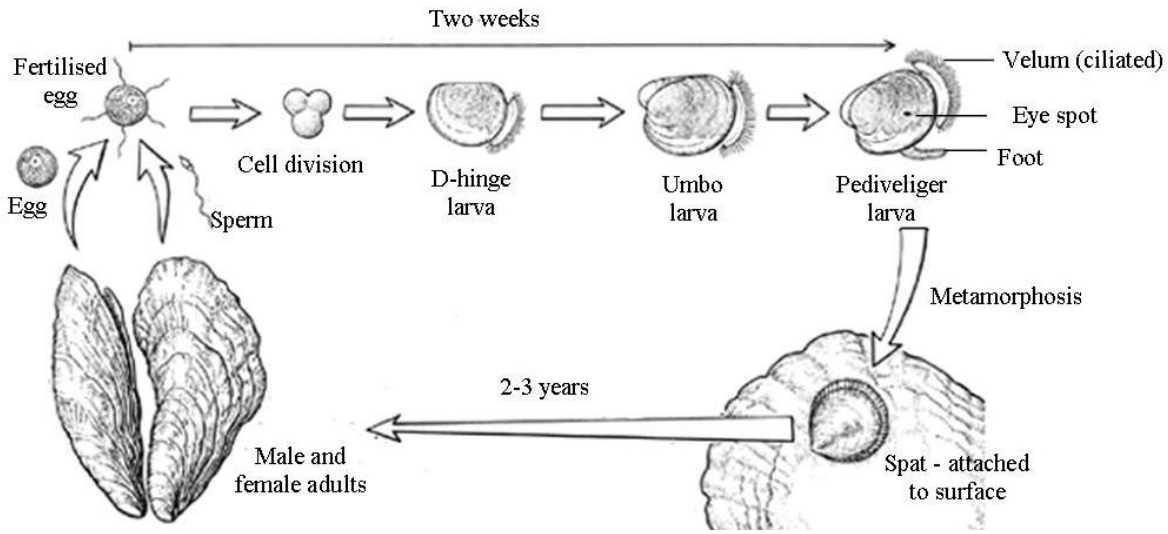


Figure 2 – Life cycle of oyster showing larval stages

Following spawning of gametes into the aqueous environment fertilisation occurs and the egg fertilisation membrane is raised from the egg surface and a polar body is formed on the outside edge; these developments are viewed microscopically by hatchery technicians to assess fertilisation success (Helm & Bourne 2004). Cell division occurs within 30 min

and functional organs are formed while still encased within the membrane including the digestive system, apical plate, kidneys and genitals and the prototrochal girdle (velum precursor). The egg membrane bursts and releases a free swimming trochophore larva of approximately 60 µm in diameter and protected by a thin horny cuticle. Upon release from the egg membrane the larva begins to drink almost immediately for osmoregulation.

Within only 24 h the trochophore larva develops into a veliger larva through enlargement of the prototrochal girdle to form the velum and secretion of two hinged calcareous valves. The velum is a ciliated ring used in swimming and directing food particles towards the mouth. This stage is known as the prodissoconch I stage or more commonly the straight hinge or D-hinge stage. Larvae at only one-day old are capable of ingesting small algal cells such as diatoms. Subsequently, the larva develops into the prodissoconch II stage, or umbo larvae, as the straight hinge becomes rounded. Near the end of the larval period as shell diameter approaches 300-350 µm the larva, or pediveliger, develops an eye spot, a prominent foot containing the byssal gland and gill rudiments. At this stage the larva is settlement competent and exhibits bottom directed behaviour including crawling along the substratum using its protracted foot. Settlement behaviour is often exhibited a day or two before larvae are metamorphosis competent. Once the metamorphosis competent larva finds a suitable substratum metamorphosis is initiated and the larva attaches to the substratum, resorbs its velum and assumes a sessile life style as a juvenile commonly referred to as oyster spat.

1.3 Causes of larvae mortality

1.3.1.1 Overview

In the marine environment oyster larvae growth and survival can be affected by numerous factors including phytoplankton abundance and type (Helm & Bourne 2004), physiochemical properties including salinity, temperature and pH (Calabrese & Davis 1970), toxins from algal blooms (Stoecker et al 2008), anoxia and anthropogenic sources (Calabrese & Davis 1970; Parry & Pipe 2004), predators such as filter feeding

invertebrates, fishes and polychaete larvae (Johnson & Brink 1998), viruses (Hine et al 1992), and bacteria (Tubiash & Otto 1986). The hatchery environment is substantially different from the marine environment and as such mortality usually relates to different causes. Many of the stresses of the marine environment are removed or controlled in culture where conditions are optimised for larvae growth and survival. Optimal levels and tolerance ranges have been determined for phytoplankton quality and quantity, salinity, temperature, pH and oxygen levels (Calabrese & Davis 1970; Helm & Bourne 2004). As such significant mortality events relating directly to these factors are less frequent and can be identified from hatchery monitoring records. Predators of oyster larvae are excluded from hatchery seawater by filtration, excluding biota greater than 1-10 µm in size. However, larvae experience additional stresses during intensive cultivation compared to the marine environment, including density related stresses, high bacterial concentrations, tank water changes, and build up of toxic metabolites in larvae tanks (Helm & Bourne 2004).

As semi-closed systems hatcheries are exposed to external variables that are difficult to control. Factors such as toxins associated with algal blooms or anthropogenic contamination are hard to detect because of their high potency to oyster larvae and temporal nature – once mortality is observed the toxicant may no longer be present in the water. Removal of toxins cannot be achieved through filtration although other water treatments such as foam fractionation may be successful (Helm & Bourne 2004). Such problems tend to be localised or hatchery specific and require site specific solutions. Toxins could potentially increase the susceptibility of oyster larvae to disease (addressed in section 0).

Infectious diseases are a particularly noxious problem potentially affecting all hatcheries and often resulting in epizootics where the entire culture is lost. In Australia hatchery epizootics are an ever-present problem and currently contribute towards an estimated 50% seasonal undersupply of spat to oyster farms (Scott Parkinson pers. comm.). Hatchery epizootics have been related to both bacteria and viruses although there appears to be a more substantial number of publications implicating bacteria as the etiologic agent.

Overall, disease caused by bacteria is considered the most serious disease of hatchery-reared larval oysters (Elston & Leibovitz 1980; Elston et al 2008; Hada et al 1984).

1.3.1.2 Viral pathogens causing hatchery epizootics

Viral pathogens were first implicated in Pacific oyster larvae hatchery epizootics in 1991, simultaneously in France and New Zealand. In a New Zealand hatchery infected larval Pacific oysters experienced mortality rates of 60-100% at 7-11 d post fertilization (Hine et al 1992) and in a French hatchery abnormal mortality and morbidity was reported (Nicholas et al 1992). In both cases a herpes-like virus was implicated (Ostreid herpesvirus-1 or OsHV-1). Since that time OsHV-1 has caused chronic mortality in some European hatcheries particularly along the Atlantic coast of France. The only other known virus associated with significant mortality of oyster larvae is Oyster Velar Disease (OVVD), which was reported on the west coast of North America (Elston & Wilkinson 1985). Virus diseases have not been reported in larval or adult Pacific oysters in Tasmanian waters.

1.3.1.3 Bacterial disease causing hatchery epizootics

Bacterial disease has affected hatcheries wherever they have operated (Elston 1999) and numerous reports have identified bacterial species as proven pathogens causing hatchery epizootics (Table 2). Moreover, bacterial disease is usually the main factor limiting survival of larval bivalves in intensive culture systems (Elston & Leibovitz 1980).

The widespread prevalence and pervasiveness of bacterial disease in hatcheries may relate to the conditions of larviculture. Optimal conditions for growth and development of bivalve larvae in hatcheries enhance the growth and multiplication of bacteria and the accumulation of their metabolites (Brown & Tettelbach 1988). This was recognised early in the development of hatchery methodologies by Walne (1956) who showed that bacterial populations in larval cultures may be 100 times greater than those in the sea. Hansen and Olafsen (1999) suggested that the increase in bacterial populations in larval rearing tanks may be 1,000-fold. Evidently, since the earliest studies in which the feasibility of

culturing bivalve species was demonstrated (by Loosanoff and Davis (1963) and Davis and Ukeles (1961)) development of production methodologies have been shaped by disease pressure every step of the way.

Research by Tubiash et al (1965) to identify the etiologic agent of epizootics showed that diseased larvae exhibited a characteristic set of symptoms including deciliation and loss of velar epithelial cells, growth of bacteria along the internal shell and mantle, abnormal swimming behaviour and rapid mortality. The authors termed the disease "bacillary necrosis" and attributed it to species of the genus *Vibrio*. Numerous hatchery studies of epizootics have since been undertaken and particular bacterial species implicated as summarised in Table 2.

In the vast majority of cases reported epizootics have been attributed to bacterial species of the genus *Vibrio* (Table 2), hence bacillary necrosis has been alternatively named "Vibriosis". Paillard et al (2004) stated that whilst other species have been shown to cause disease the most severe mortalities are caused by members of the genera *Vibrio*, *Pseudomonas* and *Aeromonas*.

Table 2 – Implication of bacterial pathogens in bivalve hatchery epizootics

Hatchery location	Pathogenic species implicated	Bivalve species	Study reference
Bicheno, Tasmania	*Vibrio* spp. and *Alteromonas* spp.	*C. gigas*	(Garland et al 1983)
Bicheno, Tasmania	Mixed bacterial population	*C. gigas*	(Garland et al 1986)
Japan	*V. splendidus*	*C. gigas*	(Sugumar et al 1998)
Long Island, USA	*V. anguillarum*	*C. virginica*	(Brown 1981)
Coast of Galacia (NW Spain)	*V. neptunis*	*Ostrea edulis*	(Prado et al 2005)
California coast	*V. anguillarum*	*C. gigas*	(DiSalvo et al 1978)
USA	*Vibrio* spp.	*C. virginica*	(Brown & Losee 1978)
France	*V. splendidus*	*Pecten maximus*	(Nicolas et al 1996)
Chile	*V. anguillarum*	*Argopacten purpuratus*	(Riquelme et al 1995)
Coast of Galacia (NW Spain)	*V. tubiashii*	*Ostrea edulis*	(Lodeiros et al 1987)
USA	*V. tubiashii*	*C. gigas*	(Elston et al 2008)

Pathogenesis in the American oyster (*C. virginica)* caused by a range *Vibrio* spp. was described in detail by Elston et al (1980) who categorised three different types of pathogenesis (I, II and III). Types I and III diseased larvae became sedentary in the early stages of infection while type II larvae remained active. In type I infections bacteria attached to the shell and grew along the mantle preferentially, and invaded the visceral cavity whereas in type III bacteria invaded through the digestive system and caused progressive and extensive visceral atrophy. In type II infections the initial bacterial attack

was focussed on the velum, which showed a variety of velar damage. Retractor muscles detached and vela remained extended before larvae tissues were invaded late in the disease process. Type I affected all larvae stages while type II affected early veliger and type III affected late veliger. With this and other studies, tissues of bivalve larvae with bacillary necrosis have been well described with histological immunofluorescent and ultrastructure techniques (Elston & Leibovitz 1980; Elston et al 1981; Leibovitz 1978).

The mechanism of *Vibrio* pathogenicity has been partly elucidated in research conducted with supernatants of pathogenic *Vibrio* spp. Brown and Losee (1978) showed that cell free supernatant from *V. anguillarum* caused mortality in *C. virginica* larvae through production of an exotoxin. Brown and Rolan (1984) presented evidence that *Vibrio* produced an exotoxin that was a secondary metabolite and proteinaceous in nature. Kothary et al (2001) and Delston et al (2003) showed that *V. tubiashii* produced both a metalloprotease and a cytolysin. More recent work by Hasegawa (2009) showed culture supernatants of a variety of *Vibrio* spp. were highly toxic to oyster larvae and that the production of metalloprotease was required for this effect. Hence the importance of exotoxins and metalloprotease in particular, in *Vibrio*-pathogenicity has been demonstrated; although Elston et al (2008) reported two pathogenic *Vibrio* spp. that did not produce metalloprotease or cytolysin and suggested that there may be additional unknown pathogencity factors (Elston et al 2008).

Pathogenic mechanisms of *Vibrio* spp. involving secretion of exoproteins have been shown to be coordinated by quorum factors in a range of pathogenic species including *V. anguillarum*, *V. vulnificus*, and *V. harveyi* (Milton 2006). (Hasegawa et al 2008) suggested that *V. tubiashii* may take advantage of a quorum-sensing system similar to that of *V. vulnificus* in which the metalloprotease gene is activated at high cell population densities. Although, induction of the disease process does not appear to be dependent upon cell density alone and remains poorly understood (Elston et al 2008). According to Griffiths (2001) virulence determinants of pathogenic bacteria may be "regulated" by environmental conditions or may only be expressed under specific conditions. A study by Brown (1981) indicated that toxin production by a pathogenic *Vibrio* sp. may be related to

nutritional requirements. The pathogen grew when provided with media containing inorganic salts, glucose and asparagine but required hypoxanthine and either glutamic acid, histidine or thiosulphate for toxin production. However, research into characterisation of the environment required for toxin production does not appear to have been advanced since this time.

Thus disease is not related simply to the presence or absence of a pathogenic bacterial species in sufficient abundance. In fact since seawater may function as a medium for both transport and growth of microorganisms, marine organisms share an ecosystem with bacteria responsible for their disease (Hansen & Olafsen 1999). Numerous hatchery studies have reported co-existence of pathogenic species and larvae host without disease incidence (Bourne et al 2004; Brown 1981; Elston et al 2008; Schulze et al 2006).

1.3.1.4 Microbial ecology of *Vibrio* spp.

The *Vibrio* genus is comprised of more than 50 species that are found free-living in marine coastal waters, sediments, and estuaries, and are particularly common in association with marine organisms in relationships that may be described as commensal, symbiotic or pathogenic (Thompson et al 2004). This diversity of habitat reflects a highly adaptive capacity to environmental variability. *Vibrio* spp. are able to survive for long periods during starvation by sequential changes in cell physiology and gradual changes in morphology (Morita 1993; Östling et al 1993). Moreover, some species develop the so-called viable but nonculturable (VBNC) state in response to certain stress conditions (Biosca et al 1996), which is an adaptative strategy of microorganisms against stress from which cells may be able to recover once optimal conditions are restored (Huq & Colwell 1996). In association with animals *Vibrio* spp. are exposed to an additional set of ecological challenges, such as evasion of the immune system, iron limitation, and oxidative stress (Milton 2006). This immense variability of *Vibrio* spp. to cope with broad variations in the ecology is reflected in the diversity of their genomes. Among marine bacteria, *Vibrio* spp. are prolific producers of antimicrobials as well as the most resistant (Milton 2006). Interspecies and intraspecies competitivness of *Vibrio* spp. is aided by

complex intercellular signalling involving quorum factors that enable *Vibrio* spp. to coordinate their activities and environment (Manefield et al 2004).

Vibrio spp. play a role in nutrient regeneration in the marine ecosystems by taking up dissolved organic matter, producing essential polyunsaturated fatty acids in the food web, and degrading chitin (Milton 2006). *Vibrio* spp. are well adapted to respond to nutrient pulses in the environment (Rehnstam-Holm et al 2010). Increases in the concentration of *Vibrio* spp. have been observed in association with algal blooms (Rehnstam-Holm et al 2010), upwelling of nutrients (Elston et al 2008) and carbohydrate-containing waste water (Larsen 1985). In aquacultural systems *Vibrio* spp. are ideally suited to conditions including high densities of animals, nutrients and bacterial densities.

1.4 Larvae immune response

The host defence of adult bivalves has been described by Pipe and Coles (1995). In summary the bivalve immune function is largely affected by circulating phagocytic coelomocytes (haemocytes) and also involves a range of non-immunoglobulin serum proteins which are probably secreted by the coelomocytes. Three types of coelomocytes exist and these are identified as hyalincytes, granulocytes and serus cells. Preceding both phagocytosis and encapsulation there is usually a measurable increase in the number of circulating coelomocytes, which probably results from migration from the tissues. Phagocytosis is accompanied by a repertoire of killing mechanisms including the release of degradative enzymes and the generation of reactive oxygen intermediates with accompanying antioxidant enzymes. Other soluble components released by the coelomocytes as part of their defence strategies include agglutinins, lysins and various other antimicrobial factors.

Immune reactions of larval bivalves are much less understood (Dyrynda et al 1995) although it appears that larvae have non-specific defence barriers to bacterial invasion as well as specific immune functions. Non-specific immune defence includes external and mucosal barriers and some adaptive components that are transferred from the mother, such as agglutinins, precipitins, lysins and immunoglobulins on the egg membrane surface

(Mulero et al 2007). External and mucosal barriers are the first line of defence since infection usually requires intimate association with larvae surfaces (Gómez-León et al 2008; Olafsen 2001).

Evidence of a specific immune response in *C. virginica* larvae was reported by Elston and Leibovitz (1980) while investigating the pathogenicity of *Vibrio* spp. against oyster larvae. The authors observed motile phagocytes containing bacterial fragments in the visceral cavity, and recorded removal of redundant phagocytes through the velum. Phagocytosis was observed in larvae of all ages. Some free-living cells in the visceral cavity increased their amount of smooth and rough endoplasmic reticulum in response to bacteria, which may have been associated with increased production of secretory products. In another study Elston (1980) described two different types of free-living coelomocytes, one was described as phagocytic and the other contained significant amounts of smooth endoplasmic reticulum. Both cell types were observed in the visceral cavity and the nascent circulatory system.

Formation of abscesses in the mantle was observed by Elston et al (2008) in larvae suffering from low level *Vibrio* infections, which indicated an immune response of containment of the bacterial pathogen. Although such containment can prevent near-term mortality, bacterial abscesses are likely to reduce growth and overall health, and result in ultimate failure of production (Elston et al 2008).

In a study of the immune system of larvae of mussels (*Mytilus edulis*) Dyrynda et al (1995) demonstrated a number of immune defence functions including production of degradative enzymes phenol oxidase and arylsulphatase, phagocytosis of *E. coli* cells and generation of reactive oxygen metabolites. Immunity attributes were compared to those of adults in Table 3 (Dyrynda et al 1995), which indicates that some elements of the immune system in adult bivalves also appear in the trochophore and veliger larvae.

Table 3 – Summary of defence mechanisms recorded in mussels (*Mytilus edulis)*

Activity	Adults	Larvae	Reference
Degradative enzymes	+	+	(Pipe 1990)
Phagocytosis	+	+	(Noel et al 1993)
Cytotoxic reactions	+	ND[A]	(Leippe & Renwrantz 1988)
Reactive oxygen metabolites generation	+	+	(Pipe 1992)
Nitric oxide generation	+	ND	(Ottaviani et al 1990)
Lymphokine-like activity	+	ND	(Hughes Jr et al 1990)
Antimicrobial factors	+	ND	(Nottage & Birkbeck 1990)
Agglutinins	+	ND	(Renwrantz & Stahmer 1983)

(A) ND – not determined

The adult bivalve immune system does not appear to operate autonomously in regulation and action as previously thought, but rather may interact with the nervous and endocrine systems (Koller 1990). These links may increase the complexity of the immune response and potentially make it more sensitive to environmental stressors (Parry & Pipe 2004). Once again it is not clear how the different systems interact in larvae.

With respect to bacillary necrosis larvae appear to be more sensitive at particular life stages. Whilst all life stages can be affected by disease, larvae susceptibility appears to differ dependent upon life stage. Garland et al (1983) reported disease peaks in larvae 7-10 d old and in older larvae just before metamorphosis. There also appears to be genetic variation in susceptibility. Gomez-Leon et al (2008) tested larvae from three different lines of the American oyster (*C. virginica*) against a pathogenic *Vibrio* spp. and found that susceptibility varied significantly between lines.

1.5 Microbiological aspects of oyster hatcheries

1.5.1.1 Selective pressures affecting microbial communities in bivalve hatcheries

Oyster hatcheries are typically semi-closed systems in which production is land-based and water is exchanged between the hatchery and the marine environment. As a semi-closed system, differences may exist between microbial communities associated with larvae rearing and that of the sea from which the water is sourced. Several authors have reported such differences in the microbial communities of intensive aquacultural systems (Bourne et al 2004; Payne et al 2006; Verschuere et al 1997). Differences arise because of selective pressures operating within the hatchery, which in particular include water treatment, sanitation, and eutrophication.

Semi-closed systems enable a degree of control over water quality through treatment of incoming seawater including treatments such as filtration, UV exposure, pasteurisation, foam fractionation and chemicals such as chlorine, EDTA and antibiotics (Helm & Bourne 2004). The choice of water treatment often varies within a single hatchery depending upon the intended purpose. Hatcheries will often use different water treatment for broodstock, larviculture and algae production with usually increasing treatment effort in that order. From a microbiological perspective the degree of change experienced with water treatment varies substantially. Some treatments such as filtration to 0.2 µm or UV exposure can produce near sterile water while others provide a selective pressure that favours some species over others.

Filtration is often used for larviculture water and is often performed at 1-10 µm. Such treatment removes detritus and larger predators such as protozoa, which is advantageous to both larvae and bacteria. Without filtration larvae may be adversely affected by predators and parasites such as protozoans. With respect to the microbial community filtration to 1 µm is at best a selective pressure against the largest of bacteria and particle-associated bacteria. Hobbie et al (1977) found that 87% of bacteria from a fresh water

lake, which were noted to be a similar size to marine bacteria, passed through a 1.0 µm Nucleopore filter and 93% passed through a 3.0 µm filter. Ninety-nine percent of bacteria were retained by a 0.2 µm filter. Ferguson and Rublee (1975) found similar results with most marine bacteria being cocci of 0.5 µm or less in diameter. Removal of particle-associated bacteria through filtration may not substantially change the microbial composition of the water since free-living bacteria predominate in the marine environment (Bidle & Fletcher 1995).

Heat pasteurisation is often used in hatcheries for algae production and is conducted at 80^{o}C for 60 min (Helm & Bourne 2004). The treatment is effective in killing bacteria but is selective for spore-forming bacteria such as those of the genus *Bacillus*, which are able to survive such treatment by formation of heat-resistant spores (Novak et al 2005).

Sanitation in hatcheries is considered a necessary measure for reduction in disease incidence (Tubiash & Otto 1986) and biofilms of the larvae tank are highly disturbed through cleaning, sanitation and desiccation between cultures. Although such practice is likely to substantially remove biofilms, residual structures remain, which may reduce diversity and stability (Verschuere et al 1997).

Eutrophication within the hatchery environment can also affect microbial composition (Bourne et al 2004; Payne et al 2006; Verschuere et al 1997). There is much evidence to suggest that eutrophication in intensive culture systems favours development of bacterial communities dominated by fast-growing, r-selected species[1]. Lebaron et al (2000) showed that when a sample of seawater was incubated in a tank with added organic matter, the diversity of the bacterial populations dropped and the percentage of culturable bacteria increased markedly. McIntosh et al (2008) suggested that the high organic load associated with intensive production of live feed cultures (such as micro-algae) selectively induces an increased proportion of fast-growing opportunistic bacteria. Similarly, Vershuere et al

[1] R-selected species are prone to high reproduction at low cost per an individual offspring, while K-selected species expend high cost in reproduction for a low number of more difficult to produce offspring.

(1997) showed that composition of bacterial species in *Artemia* tanks favoured r-strategists as incubation time increased and with reference to the untreated seawater, which was dominated by K-strategists[1].

The reduction in diversity observed by Lebaron et al (2000) with addition of organic matter to a tank may indicate a general trend in intensive aquaculture systems. Nogales et al (2010) observed that while diversity usually increases with eutrophication in the marine environment, the situation is often reversed in intensive aquaculture systems. The authors hypothesized that when the eutrophic conditions of culture are more constant in amount, composition and periodicity of additions (as in aquacultural systems) this might lead to the development of microbial communities highly specialised in processing this particular type of organic load, which may be dominated by a few very efficient micro-organisms and therefore less diverse.

1.5.1.2 Microbial niches in hatcheries

Distinct microbial niches exist in hatcheries, that are subject to a different set of environmental conditions and selective pressures, which fosters development of distinct microbial communities. In this section the microbial niches of algae, biofilms and the larvae tank are briefly described.

Algae

Unicellular micro-algae cultures are used as live feed in bivalve hatcheries for all larval growth stages. Although some non-living and artificial food products have been developed, live algae remain the principal food source in most hatcheries. A range of species is used to meet larvae nutritional requirements. Micro-algae types include diatom and flagellate species. Commonly used commercial diatom species include *Chaetoceros calcitrans, Chaetoceros gracillis, Thalossiosira pseudonana, Skeletonema* and *Phaedactylum tricornutum*. Flagellate species include *Tetraselmis suecica, Duniella tertiolecta, Isochrysis galbana* and *Pavlova lutherii* (Helm & Bourne 2004).

Algae are produced in a variety of culture systems usually at high densities under highly eutrophic conditions. Algal cultures often have continuous nutrient input and algae harvest that may be maintained in a stationary phase for up to three months before a decline phase begins (Helm & Bourne 2004). Algae production systems have received much attention from microbiologists due to the high density of bacteria reached in these cultures. In a survey of eight European bivalve hatcheries Nicolas et al (2004) found that the average concentration of bacteria in algal cultures ranged from 1.3×10^5 to 5.3×10^8 ml^{-1} (direct counts) while culturable bacteria varied from 10 to 60% of total bacteria. The culturable fraction of the microbial community associated with algal cultures, as identified by partial sequences of 16S rRNA, was of low diversity. Greater than 80% were composed of two or three strains, and in 9/19 algal cultures one strain comprised >50% of the bacteria. Algal cultures were dominated by certain bacterial species which belong to distantly separated groups, in particular members of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) and the *Roseobacter* clade (Nicolas et al 2004). Similarly predominance by CFB and *Alphaproteobacteria* in mico-algae cultures was also observed by Nicolas et al (2004).

Although some algal species are inoculated with specific bacterial species, development of the microbial community in commercial algal cultures is usually left to chance and so may be highly variable in time and among culture bags. Nevertheless different algal species tend to harbour distinct bacterial populations (Grossart et al 2005; Nicolas et al 2004), which suggests a particular interaction between bacteria and microalgae.

Research by Lewis et al (1988) demonstrated that high bacterial density in microalgae cultures (greater than 6.3 log CFU/ml) was associated with high disease incidence. Measures to reduce bacterial numbers were successful in improving the culture success rate. The high density of microbial communities in algal cultures ensures that the microbial community from algal feed has a substantial impact on the microbial communities within the larvae-rearing tank. In a survey of bacterial communities associated with the early stages of Great Scallop, Sandaa et al (2003), concluded that a significant portion of bacteria in rearing tanks was contributed from algal cultures used as

feed. There is also substantial evidence to support the hypothesis that bacteria present in live feed can play a significant role in the development of the gut microbial community of marine fish larvae (Griffiths et al 2001; Hansen & Olafsen 1999).

Biofilm

Accumulation of organic matter at the biofilm interface can sustain a large and metabolically diverse microbial population (Karunasagar & Otta 1996) that can be 1,000 to 10,000 times higher than the surrounding water (Bruhn et al 2007). Furthermore surface-associated microbial community composition differs from free-living microbial composition (DeLong et al 1993). Hence biofilms provide an ecological niche within hatcheries in which microbial community function and dynamics may differ substantially from that of the water column and this may have implications for larvae production. Biofilms in hatcheries may play a greater role in hatcheries than in the marine environment due to the high surface to volume ratio in pipes and small tanks.

The production of secondary metabolites such as anti-microbial compounds, exotoxins and quorum factors appears to be greater in biofilms. Porsby et al (2008) demonstrated that *Roseobacter* spp. produced secondary metabolites and anti-microbial compounds when conditions were suitable for biofilm formation. Long et al (2001) demonstrated that particle-associated *Roseobacter* spp. were 13 times more likely to produce anti-microbial compounds compared to free-living cells and concluded that antagonistic behaviour within microbial communities is more common in particle-associated bacteria. Similarly Yan et al (2002) demonstrated that the production of antibiotic substances by two-seaweed associated *Bacillus* spp. was dependent upon biofilm formation by the bacteria. Such antagonistic behaviours may prevent pathogenic bacteria from proliferating and researchers in the area of probiotics have identified specific inhibitory activity of bacterial species against pathogenic *Vibrio* spp. (Porsby et al 2008; Prado et al 2009; Verschuere et al 2000a).

Greater metabolic diversity of biofilms facilitates a greater role in nutrient cycling, which is important for maintenance of water quality in many intensive aquacultural systems

(Jorquera et al 2001). In fact this faculty of biofilms is used in bioreactors and biofilters to improve water quality in aquacultural and industrial applications (Seca et al 2011).

Biofilms may also provide a means by which bacteria can survive between water changes within the larvae tank (Estes et al 2004) as biofilms are usually incompletely eradicated even when disinfectants are used (Verschuere et al 2000b). Protection of bacteria from adverse environmental conditions, including desiccation, has been described as one of the ecological advantages of biofilms (Davey & O'Toole 2000).

Larvae and culture water

Evidence of bacterial colonisation of eggs while still in the gonadal tissue was presented by Riquelme et al (1994) who reported concentrations of 0.24 CFU per ovum cells in suspensions of the scallop (*Argopecten purpuratus)* that were spawned under sterile conditions. Larvae surfaces are extensively colonised within hours of fertilisation following rupture of the fertilisation membrane. Larvae begin to imbibe water for osmoregulation immediately and in doing so also ingest bacteria which colonise the digestive tract before feeding commences. The influence of bacteria brought by live food organisms on the intestinal microbial community is particularly dramatic during first feeding (Munro et al 1993). Hence an indigenous microbial community becomes established, which is influenced by the microbial community of the egg and the rearing water, and may persist throughout much of the larval period (Olafsen 2001).

Bacterial colonisation of the intestinal tract and internal surfaces of the larvae may require specialist adaptations. Attachment to fish mucosal surfaces requires specific mechanisms such as cell surface hydrophobicity or expression of adhesion receptors (Hansen & Olafsen 1999). Typical intestinal bacteria of mature oysters include species such as *Pseudomonas* and *Vibrio* spp. but it is thought that the intestinal tract of bivalve larvae is largely influenced by the surrounding water due to continuous intake of seawater and the absence of a gastric barrier (Gatesoupe 1999). As such, the intestinal microbial composition may change rapidly with the intrusion of bacteria coming from water and

food (Gatesoupe 1999). The influence of microbial communities associated with live feed, on gastrointestinal microbial communities in fish larvae is evident (McIntosh et al 2008).

The larvae culture water is a highly dynamic environment with elevated temperatures, high inputs of organic material and regular disturbance through water changes conducted every 1-3 d and biofilm disturbance through cleaning and sanitation. Microbial communities of seawater, usually minimally altered through treatment, are combined with high bacterial densities of algae, with larvae and residual biofilm-associated bacteria. The eutrophic conditions of the larvae tank provide a highly selective environment for r-strategist species as discussed in section 1.5.1.1. In a culture of *Artemia* Verschuere et al (1997) observed a general increase in the proportion of *Vibrio* and *Pseudomonas* spp. in the culture water with incubation time. Growth in the tank was initially rapid with the total number of heterotrophic bacteria reaching a peak in 36 h followed by a subsequent decrease of 2 log CFU/ml.

1.5.1.3 Sources of bacteria within hatcheries

Studies on mollusc hatcheries have confirmed the existence of three main pathways in which bacteria enter culture systems: from the water supply, broodstock and microalgae feed (Elston 1984). Broodstock have been implicated as a source of pathogenic bacteria in other studies of bivalves (Elston 1989; Sainz-Hernanndez & Maeda-Martinez 2005) and Sainz-Hernandez notes that it is probably impossible to prevent transfer of bacteria from broodstock to larvae. As noted in section 1.5.1.2, algae have bacterial communities of high bacterial density and low diversity and also have been associated with disease (Elston et al 2008; Lewis et al 1988; Sainz-Hernanndez & Maeda-Martinez 2005). Seawater, when minimally treated with filtration to 1-10 µm is relatively unchanged from the marine environment and thus provides a diverse range of bacteria to the hatchery most of which are adapted to the oligotrophic conditions of the marine environment and may not be competitive in the eutrophic conditions of the hatchery (section 1.5.1.1). Other minor sources of bacteria exist within the hatchery including air supply, tap water, equipment,

and human skin. Sainz-Hernandez et al (2005) found that *Vibrio* spp. in tap water used for washing and rinsing were blamed for contamination of microalgae cultures.

1.5.1.4 Microbial relationships in the hatchery environment

The bacterial species that live in association with cultured bivalve larvae are not passive entities in the growth and survival of larvae nor simply pathogenic or innocuous to larvae. But rather larvae live in a complex microbial ecosystem in which bacteria affect larvae directly through relationships that may be pathogenic, beneficial or innocuous (Schulze et al 2006), and indirectly through inter-bacterial species relationships, and microbial community metabolism that alters the physio-chemical environment in larvae rearing tanks (Verschuere et al 2000b). These relationships are complex and poorly understood (Schulze et al 2006).

Experiments in which larvae have been grown under axenic conditions illustrate that bacteria may be beneficial to larvae growth and survival. Gnotobiotic Pacific oyster larvae fed with axenic algae supplemented with a bacterial strain had enhanced growth (16% to 21%) and survival (21% to 22%) compared to controls without the added bacterial strain (Douillet & Langdon 1993). Garland et al (1986) found that development of Pacific oyster into D-larvae was abnormal when bacteria were removed from culture water through filtration to 0.2 μm.

Numerous benefits have been assigned to bacteria in larviculture of invertebrate larvae: nutritional contribution, competitive exclusion of pathogens, inhibition or interference of pathogen growth, stimulation of the immune system (Tinh et al 2008), and improvement of water quality by removal of toxic waste products (Jorquera et al 2001).

Nutritional contribution can be made through larvae digestion of bacteria or by bacterial assistance in digestion. It is thought that the gastrointestinal microbial community plays an important role in the nutrition of host organisms (Tinh et al 2008) and that bacteria may play a role in furnishing cell substances or micronutrients such as essential fatty acids, vitamins, minerals or even enzymes (Hansen & Olafsen 1999; Seguineau et al 1996).

McHenry and Birbeck (1986) note that bacteria can constitute a substantial proportion of carbon and nitrogen requirements in bivalve diets.

Competitive exclusion of pathogens can occur through microbial competition for attachment sites and scarce resources. Attachment of the bacterial pathogen to the mucosal surface or gastrointestinal tract is considered the first step of bacterial infection (Bengmark 1998). Therefore competition for attachment sites may serve as the first barrier against invading pathogenic bacteria (Vine et al 2004) and colonisation of attachment sites by non-pathogenic species may prevent infection. Bacterial competition for nutritional resources has also been shown to protect cultured aquacultural species. Rica-Mora et al (1998) inoculated a diatom culture (*Skeletonema costatum*) with a bacterial strain that prevented establishment of an introduced *Vibrio alginolyticus.* The authors assumed that the bacterial strain was able to outcompete *V. alginolyticus* due to its ability to metabolise the exudates of the diatom.

Inhibition of pathogen growth by other species of bacteria has been demonstrated in a number of *in vitro* experiments. Prado et al (2009) tested 523 bacterial strains for their ability to inhibit growth of three pathogenic *Vibrio* spp. including *V. anguillarum* and *V. neptunius* on solid media. Four similar strains belonging to the genus *Phaeobacter* were effective at inhibiting the *Vibrio* spp. in solid media tests and in tests in seawater. Bruhn et al (2007) demonstrated production of antimicrobial compounds by a *Silicibacter* spp. isolated from a dinoflagellate culture (*Pfiesteria piscicida*) that were active against a range of non-*Roseobacter* clade marine bacteria. The authors proposed that production of anti-microbial compounds as well as biofilm formation gave *Roseobacter* clade species a selective advantage, enabling them to dominate marine algae microbial communities. Gibson et al (1998) isolated a strain of *Aeromonas media* that protected Pacific oyster larvae when challenged against *V. tubiashii*.

Interference with quorum-sensing mechanisms is another example of bacteria-to-bacteria antagonism. Quorum-sensing molecules have been found to be involved in the regulation of virulence factors in many pathogenic bacteria (Federle & Bassler 2003), and Tihn et al

(2008) proposed that quorum-sensing in pathogenic species could be disturbed by other bacterial species.

Stimulation of the immune system of fish by probiotic bacteria has been reported (Verschuere et al 2000b); however, there does not appear to be any equivalent research for bivalves. Nevertheless it remains a possibility that certain bacterial species may cause an enhanced immunological response or otherwise generate some synergism with local immunological responses (Hansen & Olafsen 1999).

Improvements in water quality through elimination of toxic metabolites in aquacultural systems have been attributed to bacteria. For example build-up of ammonia associated with catabolism of protein is a common problem in intensive aquacultural systems that can be prevented through the activity of nitrifying bacteria that incorporate ammonia into bacterial biomass (Ebeling et al 2006).

2 Microbial ecology in a commercial oyster larvae tank

2.1 Abstract

A microbiological study of a commercial oyster hatchery in Bicheno, Tasmania, was undertaken to enhance understanding of hatchery epizootics attributed to microbiological causes. Both culture-dependent and culture-independent tools were used, including TRFLP and 16S rRNA gene clone libraries. A total of eight production runs were monitored over a 12-month period, seven of which terminated in epizootics. The microbial communities in each compartment of the larvae tank (water column, larvae, biofilm) as well as inputs into the tank (algae feed, seawater, and eggs) were characterised and the relationships among these compartments were established. Microbial communities of all compartments were dominated by members of class *Alphaproteobacteria*, primarily members of the *Roseobacter* clade. This study demonstrated the existence of three distinctly different microbial niches within the larvae tank (larvae, biofilm and water column) and significant seasonal variation in both larvae-associated and water column microbial communities. Microbial communities of the water column were influenced by larviculture management while microbial communities associated with the larvae were resistant to such changes and remained closely related to the indigenous microbial community of the fertilised egg. Thus the microbial community of the larvae is influenced by microbial communities introduced at spawning and fertilisation.

Keywords: Oyster larvae, hatchery, disease, microbial community, *Vibrio.*

Abbreviations: DSS – days since spawning, WC – water column, CTW – clean tank water.

2.2 Introduction

Mass mortality events in oyster hatcheries are a major constraint in all the significant oyster growing regions of the world. In Australia the problem is no less significant with losses estimated at $500,000 annually with seasonal under-supply approaching 50%. These epizootics are usually attributed to bacterial infections. In numerous studies pathogenic *Vibrio* spp. have been implicated in oyster hatchery epizootics (DiSalvo et al 1978; Elston et al 2008) and the pathogenic mechanism at least partly elucidated (Hasegawa et al 2009). However, since these bacteria are often present in association with healthy larvae, it is not clear how pathogenicity is initiated (Elston et al 2008). Research into hatchery epizootics has been largely limited to studying only the culturable bacterial populations associated with larvae culture, even though other research has shown the culturable population in marine waters to represent only 0.01-12.5% of the total viable population (Ferguson et al (1984).

In general pathological terms, the severity of pathogen infection is recognized to be context-dependent. This idea was first formalized in the disease triangle (McNew 1960), a conceptual framework that acknowledges infection outcomes as the product of three factors: inherent susceptibility of the host, the inoculum potential of the pathogen, and the environmental conditions that both experience during infection. Previous studies, due to being limited to studying only the culturable bacterial population, have not taken full account of the disease triangle through neglecting the microbial environment in which disease has occurred. Implication of bacterial pathogens has been achieved with little or no understanding of the non-culturable microbial community in which the pathogen has arisen.

In this study a monitoring survey of an oyster hatchery in Bicheno, Tasmania was undertaken to address this knowledge gap using microbiological tools that enable whole communities to be characterised, including 16S rRNA gene clone libraries and terminal restriction fragment length polymorphism (TRFLP) analysis. The research objective was

to enhance understanding of the microbial ecology of the larvae tank and inputs into the tank and thus develop a deeper concept on the reasons for disease incidence in oyster hatcheries. Here microbial communities of the larvae tank and inputs into the tank are described, relationships among these niches characterised, and temporal variability within larvae and culture water communities are reported with respect to tank inputs. A total of eight production runs were monitored over a 12-month period, seven of which terminated in disease incidence.

2.3 Methodology

2.3.1 Monitoring program design

A total of eight commercial production runs were monitored from spawning through to metamorphosis or an epizootic event. Monitoring was focussed on compartments of the larvae tank (larvae, water column (WC) and biofilm) and inputs into the tank (algae, seawater (clean tank water - CTW) and eggs), referred to collectively as "sample types". Monitoring involved daily data and sample collection including cultivation of samples for bacterial enumeration, physiochemical data on the larvae tank WC, larvae production and health records, and samples for TRFLP analysis, and 16S rRNA gene clone libraries. Monitoring was conducted across three different sampling periods from 16/01/2009 to 01/02/2010: Summer 2009 (16/01/2009 to 12/02/2009), Spring 2009 (07/09/2009 to 03/10/2009), Summer 2010 (27/11/2009 to 18/01/2010).

Microbial communities were assessed according to four independent variables: sample type, larvae age (days since spawning (DSS)), sampling period, and tank type. Tank type was included in analysis because two different production systems were used in the hatchery including a flow-through and a batch production system and these were conducted in different tank types. Tank type could not be controlled because commercial operations took precedence over experimental design.

2.3.2 Hatchery production systems

The flow-through system was conducted in tanks of approximately 1,000 L ("IC tanks") in which the culture water was replaced by a continuous flow of 1-10 µm filtered seawater pre-heated to 26^{o}C (CTW) at a rate of 0.6-3.0 L/min and IC tanks were changed every 24 h. The batch system consisted of tanks of 11,500 L capacity ("Batch tanks") that were filled with CTW approximately 16 h prior to the addition of larvae, and were used for a period of 48 h without any renewal of culture water before tanks were changed. The culture water, following addition of larvae and algae, was referred to as the WC. Larvae were held at higher densities in the IC tank system (3-6 to 30-40 larvae/ml for 10 d old larvae in Batch and IC tanks, respectively). Larvae began life in the Batch tanks and either completed the full larval cycle there or were transferred to the IC tanks at any age beyond four days old.

Larvae were spawned either by strip spawning or by "natural" spawning using controlled temperatures. Eggs and sperm were combined in a 20 L vessel in CTW and allowed to fertilise for approximately 1 h at 25^{o}C before being placed into Batch tanks at densities of 30-45 larvae per ml. Larvae were fed a mixture of live diatom and flagellate algae species grown on site. Three algal species were used as larvae feed in mixes that changed throughout the production cycle to suit changing dietary requirements. *Chaetoceros calcitrans* was batch-cultured under axenic conditions in 18 L autoclaved carboys. *Isochrysis galbana* and *Pavlova lutheri* were grown in a flow-through 500 L bag system in xenic conditions with filtered (1-10 µm) and pasteurised seawater (heated to 80°C for 1 h). All algae species were grown at 22°C with nutrient media and aerated with CO_2 enriched air (approximately 1% CO_2). Feed rates were based on assessments of larvae size, residual feed in the WC, health and with use of feed charts. During larvae tank water changes larvae were collected on 60 µm screens as tanks were emptied, separated into size classes and culled at this point as required before being placed into new tanks. Used tanks were cleaned, chlorinated and were not re-used for at least 12 h before being filled again. The production cycle took at least two weeks and metamorphosis of eyed pediveligers was induced with epinephrine. Spat were then transferred to a "nursery".

2.3.3 Analytical procedures

2.3.3.1 Determination of microbial abundance

Cultivation of bacteria from larvae, WC, algae and CTW samples from all eight production runs was undertaken and data analysed with respect to independent variables: DSS, sampling period, and tank type. The biofilm was not evaluated because it was largely removed through cleaning and sanitation prior to use, which results in difficulty in sampling residual biofilm structures consistently. In a separate analysis cultivation data was used to calculate the total number of bacteria, and the total number of *Vibrio* spp., entering Batch tanks through the CTW, larvae and algal. The number of bacteria contributed from each source was calculated by multiplying the total viable culturable (TVC) count (CFU per ml or per larvae) by the total volume (algae, CTW) or number (larvae) added to the tank over 48 h between water changes. Similar calculations were also undertaken for *Vibrio* counts. Statistical analyses were undertaken using SAS software (version 9.2) general linear model (GLM) and regression analysis (REG). F-values and probability values were calculated using type III sum of squares (otherwise referred to as partial sum of squares).

Liquid samples from the larvae tank, including CTW and WC, were sampled directly from the top 300 ml of the WC using sub-surface sampling techniques. WC samples were filtered through an alcohol-disinfected 60 µm screen to remove larvae. Algae were sampled from a composite harvest line from 10-20 upright 500 L bags and were not filtered. Heterotrophic culturable bacteria in WC, CTW, and algae were enumerated by triplicate serial dilution in artificial sterile seawater (SSW) (35 g/L Red Sea Salt, Red Sea Fish Pharm, Eilat, Israel) and cultivation on marine agar (MA) (5 g peptone, 5 g yeast extract, 35 g Red Sea Salt and 15 g agar per L) and thiosulfate citrate bile salts sucrose (TCBS) agar (Oxoid Pty Ltd, Basingstoke, Hampshire, UK). Colony counts on MA were defined as TVC and those on TCBS were defined as presumptive *Vibrio* spp. (referred to as simply *Vibrio*). The ratio of these two counts, expressed as percentage, was also

reported and referred to as *Vibrio*/TVC. Plates were incubated at 25°C for 24 h (TCBS) or 48 h (MA) before enumeration. Detection limits were 10 CFU/ml.

Larvae samples were collected from the top 300 ml of the WC using 75 mm lengths of PVC pipe with a 60 µm mesh screen glued on one end using Loctite 401 (Henkel Australia Pty Ltd, Kilsyth, Australia). Larvae samples were washed with 3 × 1 L of sterilised seawater on alcohol disinfected 60 µm screens. A 1.0 ml aliquot of the larval suspension was transferred into a sterile glass grinding tube (Corningware) and larvae were counted in 3 × 0.1 ml subsamples before the remainder was homogenised until particulates were no larger than 5 µm at their greatest diameter (measured by light microscopy). The homogenate was serially diluted in SSW and cultivated on MA and TCBS in triplicate as above. The non-homogenised larvae were then stored for DNA extraction (see below).

2.3.3.2 DNA sample collection, extraction and purification

Extraction of microbial DNA from larvae and egg samples was undertaken according to the following protocol modified from Griffiths et al (2000). Larvae samples were collected as described above while fertilised egg samples were collected in a similar fashion using 20 µm screens from a bucket in which fertilisation occurred. Three separate replicates were collected for each sample. A volume of approximately 3-4 mm^3 of larvae was transferred into 2 ml screw-capped microcentrifuge tubes containing 0.1 g each of 0.1 mm and 1.0 mm zirconia/silica beads. A volume of 0.5 ml of hexadecyltrimethylammonium bromide (CTAB, pH 8) extraction buffer was added and vortexed briefly. A volume of 0.5 ml phenol:chloroform:isoamylalcohol (25:24:1) was added and tubes were bead-beaten at 20 oscillations /s for 1 min. Following bead beating, tubes were centrifuged at 16,000 × *g* for 5 min at 4^{o}C. The aqueous top layer was removed to a new tube and an equal volume of chloroform: isoamylalcohol (24:1) was added. Tubes were mixed well before centrifugation at 16,000 × *g* for 5 min at room temperature. The aqueous layer was removed to a new 1.5 ml microcentrifuge tube. Two volumes of polyethylene glycol (PEG, 30%) plus NaCl (1.6 M) precipitate solution was added and incubated at room temperature for 2 h. The precipitate was centrifuged at

18,000 × *g* for 10 min at 4°C. The pellet was washed in ice cold 70% ethanol, dried and resuspended in 10 mM Tris buffer (pH 8).

Extraction of microbial DNA from WC and CTW followed a modified version of a protocol used by Fuhrman et al (1988). Water samples of 500 ml were filtered through 0.2 µm filter paper and filter paper was placed in a sterile 15 ml centrifuge tube and stored at -18°C. Frozen filters were thawed, cut into strips with a sterile razor blade and resuspended in 1.5 ml of STE buffer (100 mM NaCl, 10 mM Tris HCl; 1 mM disodium EDTA, pH 8) in a sterile 15 ml centrifuge tube and vortexed. To lyse cells, sodium dodecyl sulphate (SDS, final concentration 1% wt/vol) was added and the 15 ml tubes were held in boiling water at 100°C for 2 min and then cooled on ice. The lysate was extracted twice with equal volumes of phenol: chloroform: isoamylalcohol (24:1) with centrifugation at 10,000 × *g* for 15 min at 4°C, and once with chloroform: isoamylalcohol (24:1) with centrifugation at 10,000 × *g* for 15 min at room temperature. DNA was precipitated by adding two volumes of ice cold absolute ethanol and 0.2 volumes of 3 M sodium acetate (pH 8.0). The sample was centrifuged in a 15 ml centrifuge tube at 12,000 × *g* for 30 min at 4°C. The pellet was washed in ice cold 70% ethanol, dried and resuspended in 10 mM Tris buffer (pH 8).

Larvae tank biofilm samples were collected using 100 mm sterile cotton swabs (Copan Italia S.p.A, Brescia, Italy) following emptying of tanks during water change. Swabs were wiped along the insides of the larvae tank until the whole cotton bud was discoloured from biofilm. The sample was then placed in a 15 ml centrifuge tube and frozen at -18°C until further processing. For DNA extraction the same methodology as the WC and CTW was used starting from addition of 1.5 ml STE buffer.

Extraction of microbial DNA from algae samples followed a modified protocol from Burke et al (2009) as described in Powell et al (2012). Algae samples of 50 ml were collected in the hatchery and frozen at -18°C until further processing. Briefly, 0.5 ml of 3 M rapid enzyme cleaner (3 M Australia) and 1.0 ml of 0.5 M EDTA were added to a 50 ml algal sample. Samples were incubated at room temperature for 2 h with gentle agitation before centrifugation at 1,000 × *g* for 30 min. The supernatant was removed and DNA

extracted using the UltraClean 15 kit (MoBio laboratories Inc, Carlsbad, USA) following the manufacturer's protocol.

2.3.3.3 TRFLP analysis

Two different TRFLP analyses were undertaken. The first analysis included egg, larvae and WC samples from all eight production runs to compare microbial communities with respect to independent variables: sample type, DSS, sampling period, and tank type. DSS was categorised into three fixed time points (2-5, 5-10, and > 10 DSS) from each production run. The second TRFLP analysis was undertaken for comparison of microbial communities of all sample types independently of sampling period and DSS, which involved collection of all sample types, except for eggs, on the same days: 09/09/2009, 15/09/2009, 20/09/2009, 24/09/2009, 20/01/2010 and 26/01/2010. Egg samples were collected on the day of spawning and fertilisation.

Amplification of the 16S rRNA gene was undertaken with PCR using labelled primers. A blocking primer developed by Powell et al (2012) was used to prevent amplification of algae DNA (907R_block). PCR reaction components included Immomix Mastermix (Bioline) with 1 ng BSA, 0.1 µM of primers 10F (GAGTTTGATCCTGGCTCAG) and 907R (CCGTCAATTCCTTTGAGTTT) and 2 µM of 907R_block (TGAGTTTCACCCTTGCGAGCG_C3 spacer). Primers were labelled on the 5' end with either WellRED dye D3 or D4 (SigmaProligo). PCR was carried out in a thermal cycler with a program consisting of a 10 min initial denaturation step at 95°C, followed by 32 cycles of 1 min at 94°C; 1 min at 55°C and 1 min at 72°C, with a final step of 10 min at 72°C.

For each sample replicate two separate PCR reactions were performed and PCR products pooled before digestion with three different enzymes: *Hae*III, *Hinf*I and *Msp*I (New England BioLabs). Digestion reaction components included 1 µl of enzyme buffer, 3 µl of combined PCR reaction, 1 µl of water and 5 U of enzyme and the digest was carried out at 37^{o}C for 3 h. The resulting digest was diluted × 10 to 100 µl and 5 µl of each diluted digest was cleaned by ethanol precipitation within a 96-well plate. The cleaned digests

were resuspended in 30 µl of CEQ sample loading solution (Beckman Coulter) with 0.25 µl of GenomeLab size standard 600 (Beckman Coulter). The fragments were separated on a Beckman Coulter CEQ Genetic Analysis system.

Initial data processing was carried out using CEQ software (Beckman Coulter). The raw data consisted of a chromatograph of absorbance intensity versus peak length for each digest reaction. For each sample replicate there were three digest reactions (one from each enzyme (*Hae*III, *Hinf*I and *Msp*I), from which a total of six chromatographs were generated (3 enzymes × D3 and D4 labels). A matrix was generated of peak area and fragment length for each enzyme-label combination excluding peaks with a height less than 500 absorbance units. The percentage peak area of the sample replicate was calculated for each fragment and fragments that made up less than 1% of the total peak area for a sample were given a value of zero. Analysis was also undertaken using only presence-absence data to investigate differences between sample types. Except where indicated otherwise, all TRFLP analyses reported were undertaken using abundance data. Data from the six enzyme-label combinations were combined into a single matrix of percentage peak area and fragment length for all sample replicates.

Statistical analysis was carried out using computer software Primer6 (Clarke & Gorley 2006) with the additional add-on package PERMANOVA+ (Anderson et al 2008). A number of different statistical analyses were carried out on a similarity matrix including non-metric multidimensional scaling ordination (MDS), Multivariate Dispersion indices (MVDISP), and PERMANOVA analysis (permutational multivariate analysis of variance).

To visualize patterns in bacterial community structure based on the TRFLP data, MDS plots were constructed using the Bray-Curtis similarity matrix (Clarke 1993). For each MDS plot a stress value was given for the two-dimensional fit. The lower the stress the better the data were represented, with values of 0.2 indicating a potentially useful representation that needs to be interpreted with caution (Clarke & Warwick 1994). The statistical significance of differences within TRFLP data sets was analysed using PERMANOVA. All tests were performed using type III sums of squares (as any missing data points caused the data to be unbalanced) and 9999 permutations with unrestricted

permutations of the raw data. Multivariate dispersion indices (MVDISP) were used to measure the variation within different sample types as done in work by Chong et al (2009) in comparing bacterial community structure of different soil types. Standard diversity measures were calculated in Primer 6 including Shannon-Weiner (H'), Margalef's (d) , and Pielou's evenness (J') (Clarke & Warwick 1994). These diversity indices were calculated using each TRF as representative of a single species, although different species can share the same TRF but nevertheless provides an estimate of species diversity. Diversity indices were exported from Primer and statistical analysis was undertaken using SAS GLM as described previously.

2.3.3.4 Clone libraries

A total of 18 clone libraries were undertaken to support TRFLP analysis in comparison of all sample types. Comparisons among clone libraries using Principal Component Analysis (PCA) and phylotype analysis (further details below) were made from samples collected from a particular larvae tank on a single day (15/09/2009). Summary details of clone libraries are given in Appendix 1.

The 16S rRNA gene was amplified using the same protocol described for TRFLP except the 10F and 907R primers were not labelled. PCR product was cleaned and cloned using the TOPO-TA cloning kit (Invitrogen, California) and the standard kit protocol was employed. 16S rRNA gene inserts were amplified with M13 primers (F: GTAAAACGACGGCCAG and R: CAGGAAACAGCTATGAC). M13 PCR reactions were precipitated overnight in absolute ethanol before being centrifuged to form a DNA pellet, which were cleaned in 70% ethanol. Dry pellets were sent to Macrogen Inc (Seoul, South Korea) for sequencing.

Sequences were identified to class level using the BLAST algorithm to search GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi; 01/01/2012) for similar matches. A phylogenetic tree was constructed for the major classes identified as represented in Appendix 3 to 9. A phylogenetic distance matrix was generated from each tree that was imported into DOTUR software, which was used to group sequences into operational taxonomic units or

phylotypes at 97% similarity (Appendix 2). A representative was chosen from each phylotype and the Pintail tool (http://www.bioinformatics-toolkit.org/Web-Pintail/; 01/01/2012) was used to check for chimeric sequences. Representative sequences from each phylotype were compared to 16S rRNA gene sequences of type strains from the Genbank database (http://www.ncbi.nlm.nih.gov/genbank/; 01/01/2012) using DOTUR. Where identified phylotypes are referred to in the Results and Discussion sections, the species name is followed by the percentage similarity to the species type strain in brackets and subscripted e.g. *Staphylococcus sciuri*$_{(97\%)}$. Representative sequences from all phylotypes were submitted to Genbank: JX486558 - JX486676.

Percentage coverage was calculated using Good's formula (Good 1953). Species diversity was calculated using Chao-1 estimator (Simberloff 1978), http://www2.biology.ualberta.ca/jbrzusto/rarefact.php, 01/01/2012. Phylogenetic diversity was calculated using Phylocom software (Webb et al 2008), http://www.phylodiversity.net/phylocom/; 01/01/2012, which was used to provide a calculation of Faith's phylogentic diversity index based on a tree distance matrix.

Comparisons of clone libraries were performed using the Unifrac analysis (http://128.138.212.43/unifrac; 01/01/2012), which uses phylogenetic distance-based information for comparisons (Lozupone & Knight 2005). The Unifrac distance metric was used to undertake PCA to compare the similarity of a number of clone libraries simultaneously. This was undertaken using both abundance data and presence-absence data. Pair-wise comparisons were made between some clone libraries using the Unifrac significance test (Bonferroni correction). In addition to PCA analysis clone libraries were compared by determination of the number of common and unique phylotypes among libraries.

2.3.3.5 Physiochemical data

In conjunction with cultivation of bacteria from liquid samples (algae, CTW and WC), measurements were taken for temperature, and pH. For WC samples dissolved oxygen was measured using an OxyGuard® portable meter (Technolab Marketing Pty Ltd,

Mornington, Tasmania). Physiochemical data were examined with respect to independent variables using SAS GLM as described above.

2.4 Results

2.4.1 Bacterial communities of the larvae tank

A total of 18 clone libraries were generated for the different sample types including algae (3), CTW (1), eggs (1), biofilm (3), WC (2) and larvae (5). A total of 119 phylotypes were identified among a total of 1390 clones obtained from all clone libraries combined (Appendix 2). Species composition data are summarised by class for the different sample types in Figure 3. Bacterial community composition of all sample types was dominated by members of class *Alphaproteobacteria* (algae = 60.4%, biofilm = 62.1%, CTW = 64.4%, eggs = 52.3%, WC = 66.2% and larvae = 58.3%). *Flavobacteria* was the second most predominant bacterial class associated with the algae, biofilm, and CTW (30.0, 19.3, and 17.8%, respectively). *Betaproteobacteria* was the second most predominant bacterial class in eggs (43.7%) and were also well represented in the biofilm and larvae (8.6 and 10.0%, respectively). *Gammaproteobacteria* was the second most predominant class in larvae and WC with 13.4 and 15.4%, respectively.

The most commonly encountered bacterial species in all sample types were typical marine heterotrophs. *Ramlibacter* and *Sphingomonas* sp., which were the two most prevalent species in the egg clone library and were also predominant in larvae clone libraries, are not typical marine species. Both of these species appear to have had a close association with the egg and larvae samples since they were otherwise absent from the system. Members of the *Roseobacter* clade were particularly common in all sample types while SAR 11 species were notably absent.

2.4.2 Relationships among microbial niches of the larvae tank

2.4.2.1 TRFLP analysis

Comparisons among the microbial communities of the different compartments of the larvae tank (larvae, WC and biofilm) as well as tank inputs (eggs, CTW and algae) were made using TRFLP data with some additional evidence from clone libraries and culture-based methods. TRFLP analysis indicated that microbial communities associated with different sample types were significantly different to each other (Figure 4 (B), $Pr < 0.0001$). Pair-wise PERMANOVA tests, using both abundance data and presence-absence data, indicated that all sample types were significantly different ($Pr < 0.0002$) except for larvae and eggs ($Pr > 0.05$). Abundance and presence-absence pairwise comparisons were similar, which indicates that differences between different sample types existed in both species composition and relative abundance of common species.

TRFLP analysis of microbial communities associated with larvae, WC and egg samples in relation to DSS time points supports the previous observation that bacterial communities associated with different sample types were different (Figure 4 (A), $Pr < 0.0001$). Pairwise PERMANOVA comparisons, using both abundance and presence-absence data, indicated that the difference between larvae and eggs was statistically significant, in contrast to comparisons in which DSS time points were not considered.

The Shannon-Weiner index (H') indicated that bacterial species diversity was significantly different for different sample types (H': $Pr < 0.0001$). Species diversity was highest in the WC (3.82) followed by the larvae (3.54) and then eggs (3.21). Indices for species richness and evenness indicated that the difference in species diversity was due to differences in species richness rather than species evenness (species richness index (d): $Pr < 0.0001$; species evenness index (J'): $Pr = 0.6273$)).

2.4.2.2 Clone library analysis

Unifrac PCA comparison of clone libraries derived from all different sample types studied was based on samples obtained during the production run starting 07/09/2009 and ending

with larval mass mortality on 16/09/09 (clone library summaries in Appendix 1). The data indicates that WC, algae and biofilm clone libraries were most similar (as evidenced from clustering) when only presence-absence data were analysed but less similar when abundance data were included (Figure 5). Conversely CTW was distinctly different from WC, algae, and biofilm when only presence-absence data were used but similar when abundance data were included. In support of TRFLP analysis when DSS was not specifically considered (Figure 4 (B)), larvae and eggs plotted closely in the PCA plots (Figure 5), using both abundance and presence-absence data. Larvae and eggs plotted differently to other sample types regardless of whether abundance data were used or not.

Comparison of the number of unique and similar phylotypes among clone libraries sampled on two successive days (15/09/09 and 16/09/09) supported Unifrac PCA analysis. CTW had the largest number of unique phylotypes (22 out of a total of 29 phylotypes). The WC may be considered a subset of the other sample types since only 4 out of 21 phylotypes observed were unique and it had the highest number of shared phylotypes with each sample type (Table 4). Of the 6 phylotypes CTW shared with the WC, three were absent from the algae. Only 3 out of 14 phylotypes of the algae were unique with a large number of algae phylotypes persisting in the WC (10 out of 14) and to a lesser extent in the biofilm (6 out of 14) and larvae (4 out of 14). Of the 10 phylotypes algae shared with the WC, seven were absent from the CTW (Table 4).

Comparison of the number of unique and similar phylotypes in the clone libraries of eggs and larvae indicated that approximately 50% (9 out of 21 and 9 out of 18 for seven DSS and nine DSS larvae, respectively) of phylotypes found in larvae clone libraries were also encountered in egg samples. Only a small number of phylotypes encountered in eggs were absent from larvae samples (2 out of 11 in both comparisons).

2.4.2.3 Microbial abundance data

Regression analysis among microbial abundance data from production run monitoring indicated that there was a significant correlation between the *Vibrio* population in the larvae and the WC (larvae *Vibrio* and WC *Vibrio*: $Pr < 0.0001$). *Vibrio* abundance in the

larvae was independent of bacterial abundance in the WC (larvae *Vibrio* and WC TVC: Pr < 0.52).

The relative contribution of bacteria to the Batch tank WC from the algae, CTW and larvae is represented in Figure 6. Algae were the greatest source of TVC bacteria (86.8%) into the larvae tank (Batch system), followed by CTW (9.7%) and then larvae (3.5%) (Pr < 0.0001). A higher number of *Vibrio* CFU were contributed from the CTW (72.2%) than from the larvae (27.8%) and *Vibrio* were not detected in the algae (Pr < 0.0001). The combined totals of TVC and *Vibrio* counts of larvae, CTW and algae inputs accounted for 16.3% and 12.8%, respectively, of the total WC count after 48 h with the remainder assumed to be from residual tank biofilm or replication within the larvae tank over the 48 h period.

2.4.3 Tank type and associated microbial communities

Microbial communities of the WC differed significantly between the two tank types (batch and IC). When time points for DSS were considered, TRFLP analysis revealed different microbial composition of the WC between the Batch and IC tanks (Pr < 0.0001) as illustrated in Figure 7. Batch tank WC samples had marginally higher TVC counts (Pr = 0.0495) while the IC tank WC samples had higher *Vibrio* counts (P = 0.0017) and *Vibrio/*TVC (10.0% and 0.6% for IC and batch tank, respectively; Pr < 0.0001). By contrast, larvae microbial communities were much less affected by tank type. TRFLP analysis indicated larvae had similar microbial composition in both tank types but had higher *Vibrio/*TVC percentages in the IC tank (P = 0.0276) (15.8% and 3.7% for IC and Batch tanks, respectively). Differences in both temperature and pH were observed between the two tank types. On average temperature was higher in IC tanks compared to Batch tanks (25.9^{o}C and 23.4^{o}C, respectively; P = 0.0001) and pH was lower in IC tanks compared to Batch tanks (7.95 and 8.06, respectively; P = 0002).

2.4.4 Temporal variability in microbial communities of larvae and WC

TRFLP analysis indicated that microbial composition of larvae samples was affected by both sampling period and DSS at a high level of significance ($Pr < 0.0001$ and $Pr = 0.0003$, respectively, Figure 8). Pair-wise PERMANOVA tests of DSS groupings showed older larvae, >10 DSS, were different from 2-5 DSS and 6-10 DSS larvae, and that the difference was greater between the oldest and youngest larvae. Microbial composition of WC samples was also affected by both sampling period and DSS ($Pr < 0.0001$ for both variables, Figure 9). Pair-wise PERMANOVA tests of DSS groupings showed that all DSS groups were different although the difference between 2-5 DSS and 5-10 DSS was more highly significant. *Vibrio* spp. were not enhanced as a proportion of the total culturable population with increasing age ($P = 0.6717$).

2.5 Discussion

2.5.1 Bacterial communities of the larvae tank

The water chemistry within a larval rearing environment is highly eutrophic compared to the marine environment, and consequently the microbial composition of the larvae tank may be expected to differ substantially from that of raw seawater (Bourne et al 2004). Such a difference was observed in the present study where CTW microbial communities were significantly different from larvae tank microbial communities. Eutrophication of seawater generally favours fast-growing, r-strategist species, which may explain the absence of SAR11 clade members and other strictly oligotrophic taxa in hatchery samples. The great majority of bacterial species encountered in all compartments of the larvae tank were typical marine heterotrophs, primarily of the class *Alphaproteobacteria*, which are widely distributed in marine waters and are the predominant bacterial group in coastal surface bacterioplankton communities (Bruhn et al 2007).

In other hatchery studies predominance by members of *Alphaproteobacteria* was also observed but, in contrast to the present study, members of *Gammaproteobacteria* were equally abundant. In a multispecies fish and shellfish hatchery on Vancouver island the

majority of bacteria isolated from larvae and other hatchery sources were instead members of *Gammaproteobacteria* (76% of phylotypes) with only 7% contribution by *Alphaproteobacteria* (Schulze et al 2006), although characterisations were limited to culturable bacteria. Nakase (2007) conducted a study in a Red Sea bream hatchery that showed predominance of *Alphaproteobacteria* and members of the phylum *Bacteroidetes* in culture water in association with successful larvae production whereas high levels of *Gammaproteobacteria* were associated with disease. In the present study, predominance by *Alphaproteobacteria* was not associated with successful production outcomes.

Members of the *Roseobacter* clade were particularly common in all parts of the hatchery. *Roseobacter*-related bacteria were also reported as a predominant group found in a Spanish turbot larval unit where a number of specific subtypes appeared as stable colonizers of the rearing unit (Hjelm et al 2004). In the marine environment the *Roseobacter* clade is known to dominate algae-rich environments (Buchan et al 2005), which may explain their predominance in the larvae tank. *Flavobacteria* are also known to dominate marine algae-rich environments (Nakase et al 2007) and were accordingly observed as relatively abundant in algae, CTW, WC and biofilm samples (18-30% of clones). Domination of algae-rich environments is favoured by a number of specific adaptations. In the *Roseobacter* clade these adaptations may include capacity for aggressive surface attachment involving formations identified as rosettes (Bruhn et al 2007), degradation of algal exudates such as dimethylsulfoniopropionate (DMSP) (Miller & Belas 2004), production of secondary metabolites including antibiotics, and quorum factors to facilitate coordinated activities within the population (Bruhn et al 2007). Such adaptations might be a pre-requisite for predominance in the algae-dominated, highly competitive, high surface/volume ratio environment of the larvae tank. Colonisation of oyster larvae may require a different set of specialised adaptations such as those for mucosal adhesion including cell surface hydrophobicity or expression of adhesion receptors (Hansen & Olafsen 1999), which may explain the relative absence of algae-associated bacteria from larvae microbial communities.

2.5.2 Relationships among microbial niches of the larvae tank

The major bacterial inputs into the larvae tank, including seawater, algae and eggs were not controlled and consequently may have caused variability within microbial communities of the larvae tank. Treatment of seawater for larviculture, involving filtration to 1-10 µm, which would not exclude bacteria since they are mostly less than 0.5 µm in diameter (Ferguson & Rublee 1975). While heating of seawater to larvae culture temperatures (approximately 26^{o}C) may have altered the microbial composition, the CTW is likely to have reflected the microbial composition of the raw seawater. With exception of the inoculation of some specific bacteria into algae-starter cultures, inoculation of algae cultures was left to chance. Seawater used as culture medium for algal cultures was pasteurised at 80^{o}C for 60 min, which reduced the microbial diversity such that this was not considered a major route for bacteria into the larvae. Broodstock treatment prior to spawning, involving scrubbing and physical removal of surface biofilms, while potentially reducing contamination risk, cannot prevent transfer of bacteria from the broodstock to the eggs since bacteria may be associated with the gonads (Lodeiros et al 1987).

Thus whilst the microbial communities of the seawater, algae and eggs, originating from outside the larvae tank, were uncontrolled and subject to independent influences, microbial communities of the larvae tank, including larvae, WC and biofilm, were subject to the variability caused by these inputs. Accordingly, clone library analysis indicated that microbial communities associated with the IC tank WC at nine DSS, had few unique phylotypes (4 out of 21) and may have been a subset of other sample types; although TRLP analysis indicated that WC was distinctly different from communities of other sample types. In fact, TRFLP analysis indicated that microbial communities associated with all sample types were distinctly different (section 2.4.2.1). The nature of the relationships among the different sample types is depicted conceptually in Figure 10. Because larvae were not fed xenic algae until at least two DSS, Figure 10 is divided into two parts (A and B), where A represents interactions among microbial niches on the day of spawning, and B represents interactions occurring after two DSS.

Evidence indicated that the larvae microbial community was influenced by the indigenous microbial community of the fertilised eggs. TRFLP analysis showed that while the microbial communities associated with the fertilised eggs and larvae were different, the difference was less substantial than the difference among other sample types (section 2.4.2.1). Clone library comparisons of samples from the production run starting 07/09/2009 showed that the majority of phylotypes in fertilised egg samples were also present in larvae samples from seven and nine DSS (9 out of 11), which indicates that the indigenous microbial community of the fertilised eggs was persistent.

Further evidence that the larvae microbial community was persistent was found in a comparison between the two tank types. TRFLP analysis revealed different microbial composition of the WC between the Batch and IC tanks ($Pr < 0.0001$; section 2.4.3). Batch tank WC samples had marginally significant higher TVC counts ($Pr = 0.0495$) while the IC tank WC samples had higher *Vibrio* counts ($P = 0.0017$) and *Vibrio*/TVC ($Pr < 0.0001$). By contrast, larvae microbial communities were much less affected by tank type. Larvae had similar microbial composition in both tank types, as indicated by TRFLP, but had higher *Vibrio*/TVC percentages in the IC tank ($P = 0.0276$). These results indicate that larvae microbial communities were somewhat resistant to change occurring in the WC caused by altered culture conditions, as experienced in the different tank types.

In other studies, Olafsen (2001), similarly showed that bacteria associated with oyster eggs established an indigenous microbial community that persisted throughout the larval period. According to Olafsen (2001) the indigenous microbial community is formed within hours of fertilisation following rupture of the fertilisation membrane as larvae begin to imbibe water for osmoregulation and in doing so also ingest bacteria which colonise the digestive tract and external surfaces before feeding commences. Thus an indigenous microbial community becomes established, which is influenced by the microbial community of the unfertilised egg and the rearing water (Figure 10A), and persists throughout much of the larval period (Olafsen 2001).

The predominance of egg and larvae microbial communities by *Ramlibacter* sp. and *Sphingomonas paucimobilis* is of interest because these species are not usually

encountered in marine environments and are more typically found in soil and fresh water environments. Their persistence in the unfavourably saline environment of the oyster larvae provides further evidence of the persistence of the indigenous microbial community and indicates a source of bacterial contamination not originating from seawater, algae or eggs. Sources of contamination occurring during spawning and fertilisation may have included fresh water used for washing, water splashing off the hatchery floor, human skin, and equipment used such as buckets and meshes. Olafsen (2001) affirms that the microbial population of the egg incubator may differ considerably from that of the sea. Broodstock may have also been a source of contamination (Elston 1989). Broodstock were kept in another hatchery in a semi-closed farm environment that may have harboured substantially different microbial communities from the typical marine environment. Verschuere et al (1997) stated that the indigenous microbial community of *Artemia* eggs is influenced by both deterministic and stochastic factors. They also postulated that the presence of indigenous bacterial species poorly adapted to the conditions of culture may be less persistent and therefore inherently less stable throughout the larval period.

Beyond two DSS, the microbial communities of the larvae tank may have been influenced by the variability in the microbial communities of the seawater and algae inputs (Figure 10B). Algae-associated bacteria mostly survived in the IC tank WC at nine DSS with 10 out of 14 phylotypes detected in an algae sample also detected in the WC (seven of which were absent from the CTW), which indicates that conditions in the IC tank suited these bacteria (section 2.4.2.2). In contrast the majority of phylotypes (22 out of 29) detected in the CTW were not detected in the WC, which indicates that the majority of CTW bacterial species were not competitive in the conditions of the IC tank. Unifrac PCA analysis, using presence-absence data showed that algae, WC and biofilm microbial communities were similar, which indicates a primary role of algae in determining the species composition of the WC and biofilm in the IC tank (Figure 5). Although when abundance data were used, CTW plotted close to algae and WC samples in the Unifrac PCA plot indicating that these samples shared similar predominant bacterial species and that CTW may have contributed towards their relative abundance in the IC tank WC.

In the Batch tank system, an evaluation of the total numbers of bacteria contributed to the WC over 48 h at 13 DSS showed that algae were the largest source of bacteria ($Pr < 0.0001$; section 2.4.2.3). Algae contributed 14.2% of the bacteria in the larvae tank, compared to 1.6% from the CTW and 0.6% from the larvae. The remaining 83.7% resulted from remnant biofilm structures or replication within the larvae tank. This result indicates a primary role of algae in influencing Batch tank WC microbial communities, as was also observed in the IC tank. Sandaa et al (2003) reached a similar conclusion in a study of a Great scallop hatchery in which a significant portion of bacteria in larvae tanks was contributed from algal cultures. However, the influence of algae is dependent upon the volume of algae-feed added to the larvae tank, which was greatly reduced at the beginning of the production run, hence the influence of CTW on larvae tank microbial communities may have been greater at this time.

Algae microbial communities may have affected larvae communities either indirectly, via the WC, or directly, through concentration of algae and associated bacteria in the larvae intestinal tract. Yet, there was no significant evidence that larvae were substantially affected by either route. Unifrac PCA analysis indicated that the bacterial communities associated with the larvae and algae were not similar using either presence-absence or abundance data (Figure 5) and only four out of 14 algae-associated phylotypes were also found in larvae. These results indicate that algae-associated bacteria mostly did not persist in association with the larvae or were numerically overwhelmed by bacteria consumed through drinking or bacteria already resident in the larvae. Similarly in a study by McIntosh et al (2008) it was observed that few bacterial phylotypes associated with feed-rotifers were also found in cod larvae. Blanch et al (2009) in a study of aquarium fish intestinal microbial communities found that the microbial population in the water had a greater impact on the intestinal community compared to the microbial composition of the feed.

Aside from influences on larvae tank microbial communities from external inputs (seawater, algae and eggs), relationships among microbial communities of the larvae tank must have also existed. While the influence of tank biofilms on the microbial community variability in the larvae tank was not investigated, analysis of the number of shared

phylotypes in samples from production run starting 07/09/2009 (section 2.4.2.2) showed that the biofilm and WC shared a high proportion of phylotypes (8 out of 15 biofilm phylotypes). This indicates a possible interdependent relationship between the biofilm and WC communities. Research by Verschuere et al (1997) demonstrated that biofilm and cleaning practices employed between water changes could influence the WC. They showed that even after cleaning and chlorination, walls of four different *Artemia* culture tanks still contained bacteria in minute fissures and cracks and the surviving bacterial composition was very different in each tank. The initial inoculum affected the microbial community that developed in the culture water and the four tanks remained distinctly different for the duration of the culture (six days, without water change). The work of Verschuere and colleagues demonstrates the potential influence that cleaning of biofilm may have had on variability in WC microbial communities.

The existence of a relationship between the microbial communities of the larvae and WC was also indicated by the correlation between larvae *Vibrio* counts and WC *Vibrio* counts ($Pr < 0.0001$; section 2.4.2.3) and is supported by other research. According to Gatesoupe (1999), the microbial community of the larvae is largely influenced by the surrounding water due to continuous drinking of seawater and the absence of a gastric barrier. Whilst, in contrast to Gatesoupe's finding, larvae microbial communities were shown to be somewhat resistant to change occurring in the WC, there appears to be little evidence for the reverse relationship in which larvae microbial communities exerted significant influence on WC communities. Bacteria associated with larvae were out-numbered in the Batch tank by bacteria originating from both CTW (by 2.7-fold) and algae (by 25-fold), and the specialised adaptations required for colonisation of larvae surfaces may not be advantageous in the WC. Hence, WC and larvae microbial communities may share an unequal relationship where the larvae community is influenced by the WC community (Figure 10).

2.5.3 Temporal variability in microbial communities of larvae and WC

2.5.3.1 Sampling period variability

Sampling period affected the microbial composition of the larvae and the WC equally (Larvae: Pr < 0.0001. WC: Pr < 0.0001; section 2.4.4) and in both larvae and WC the sampling period, Summer 2009, was the most different. This indicates that sampling period variability in both larvae and WC could have been due to a single common influence. Such an influence may be related to larvae tank inputs (seawater, algae and eggs) and the conceptual model developed in the preceding section (Figure 10). Accordingly it seems unlikely that microbial communities of the eggs could influence larvae and WC communities equally since egg communities have considerable influence on communities of the larvae but not the WC (Figure 10A). It also seems unlikely that change experienced in microbial communities of the algae could influence larvae and WC communities equally since algae communities have a large influence on WC communities but little influence on larvae communities (Figure 10B). However, microbial communities of the seawater could influence communities of the larvae and WC equally during formation of the larvae indigenous microbial community (Figure 10A). Seawater microbial community composition is known to vary temporally with predictable seasonal patterns (Nogales et al 2010), which is consistent with the temporal nature of sampling period variability. Thus according to the conceptual model of Figure 10 it seems plausible that seasonal variability in microbial communities of seawater could be the primary cause of sampling period variability in communities of the larvae and WC.

2.5.3.2 DSS variability

DSS was a significant factor explaining variability in the larvae and WC microbial communities and was equally significant in both the larvae and WC (Larvae: Pr = 0.0003. WC: Pr < 0.0001; section 2.4.4). This result contrasts with a study by Sandaa et al (2003) in which the microbial community composition of scallop larvae *(Pecten maximus)* remained stable throughout the larval period, although the study used DGGE analysis,

which is less sensitive than TRFLP so may not detect changes occurring in less dominant parts of the community. As for sampling period, microbial community variability in larvae and WC communities may be related to tank inputs (seawater, algae and eggs) and the conceptual model developed in Figure 10. Of the three major inputs only microbial communities of the seawater may exert equal influence on both larvae and WC communities. However, microbial communities of seawater alone could not account for the variability in larvae and WC communities with DSS since seawater would exert greater influence on communities of WC than larvae in later stages of larval development because of the persistence of larvae communities (Figure 10B). Instead variability in microbial communities of the WC with DSS may have been related to changing feed demands of larvae as they develop. Changes in the quantity of feed and algae-species composition over the larval period may have been accompanied by changes in the associated microbial community. The larvae microbial community may have been subjected to age dependent influences such as microbial succession that may not have affected the WC. Thus DSS variability in larvae and WC could be due to different factors.

2.6 Conclusion

This study demonstrates the existence of three distinctly different microbial niches within the larvae tank (larvae, biofilm and WC) and significant seasonal variation in both larvae-associated and WC microbial communities. It further shows that while microbial communities of the WC can be influenced by conditions of culture, larvae-associated microbial communities are somewhat resistant to such changes and more closely related to the indigenous microbial community of the egg. Thus the microbial community of the larvae is influenced by microbial communities introduced at spawning and fertilisation.

Table 4 – Unique and common phylotypes among samples from production run 070909

Sample type	Clone library ID	No. of unique phylotypes[a]	Total No. of phylotypes	Percentage unique phylotypes	No. of phylotypes shared with:				
					WC	Larvae	Biofilm	Algae	CTW
WC	W_160909	4	21	19%	x	6	8	10	6
Larvae	L_160909	10	18	56%	6	x	5	4	3
Biofilm	B_160909	6	15	40%	8	5	x	6	2
Algae	A_150909	3	14	21%	10	4	6	x	3
CTW	C_150909	22	29	76%	6	3	2	3	x

a - No. of unique clones is in comparison to all other samples combined

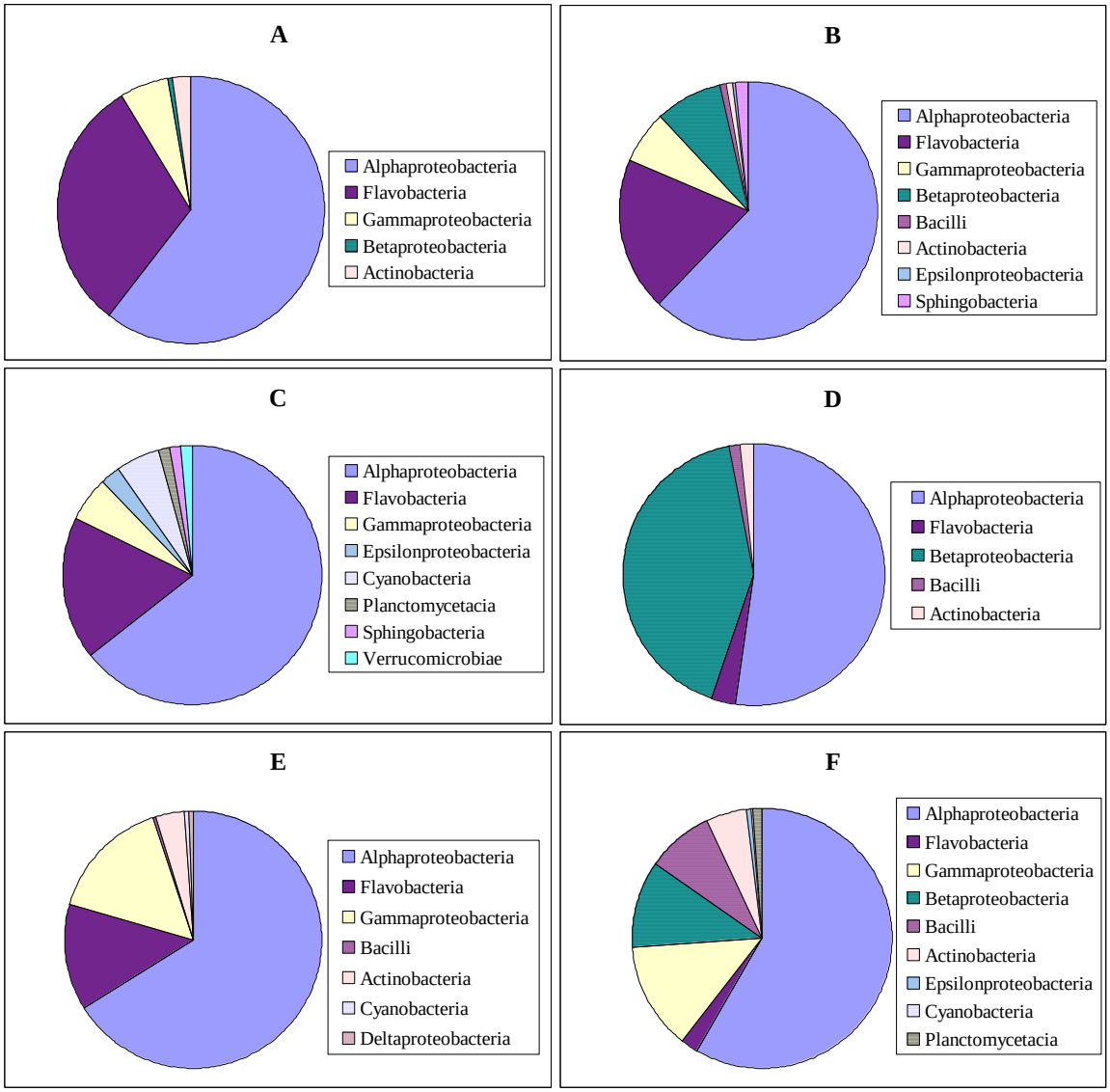


Figure 3 – Percentage composition of combined clone libraries for algae (A: 3 samples, 187 clones); biofilm (B: 3 samples, 243 clones); CTW (C: 1 sample, 74 clones); eggs (D: 1 sample, 65 clones); WC (E: 2 samples, 196 clones) and larvae (F: 8 samples, 350 clones)

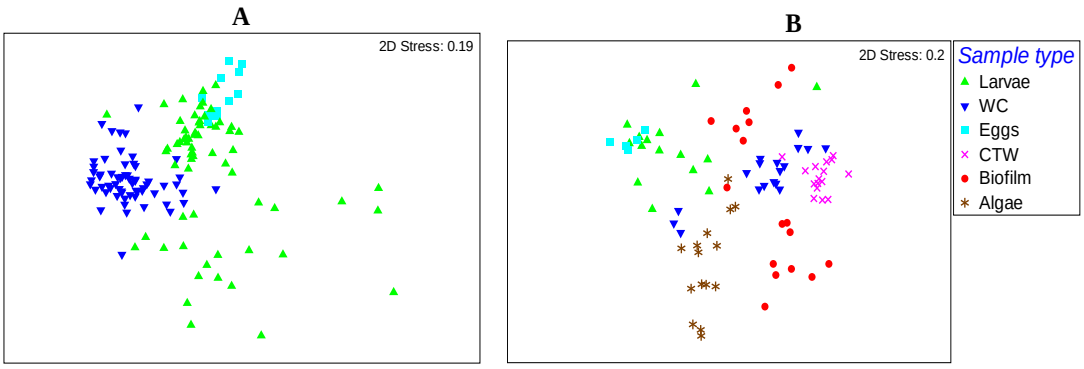


Figure 4 – MDS plots showing microbial composition similarity of sample types using two different sets of TRFLP data. A: Dataset includes egg, larvae and WC samples taken over three fixed time points (2-5, 5-10, and > 10 DSS) from each of eight production runs; Pr (sample type) < 0.0001. B: Dataset includes all sample types and is independent of sampling period and DSS; Pr (sample type) < 0.0001. In both MDS plots the closer points are to each other the more similar they are.

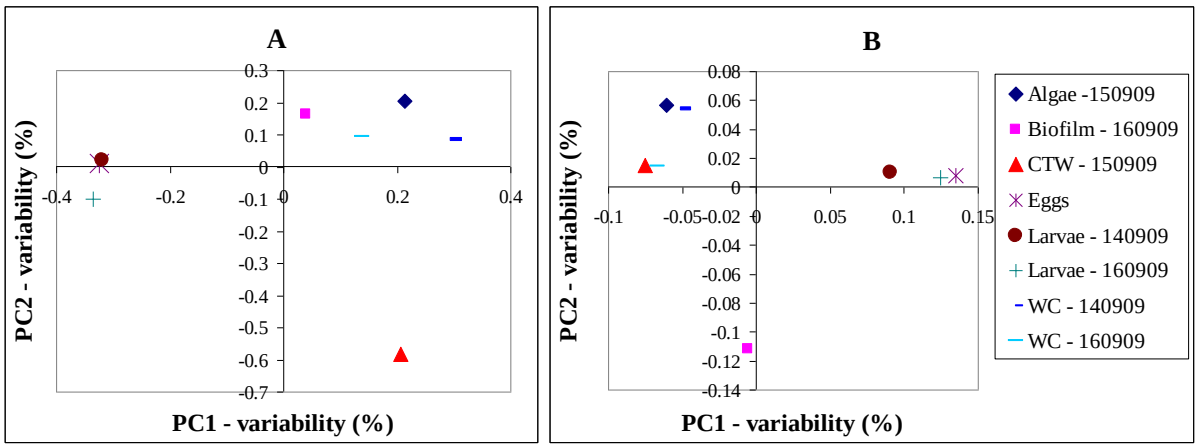


Figure 5 - PCA of clone libraries from different sample types in production run 070909. Clone libraries were analysed using Unifrac and either presence-absence data (A) or abundance data (B). The first two vectors explain 57.8% and 63.0% of the variability respectively.

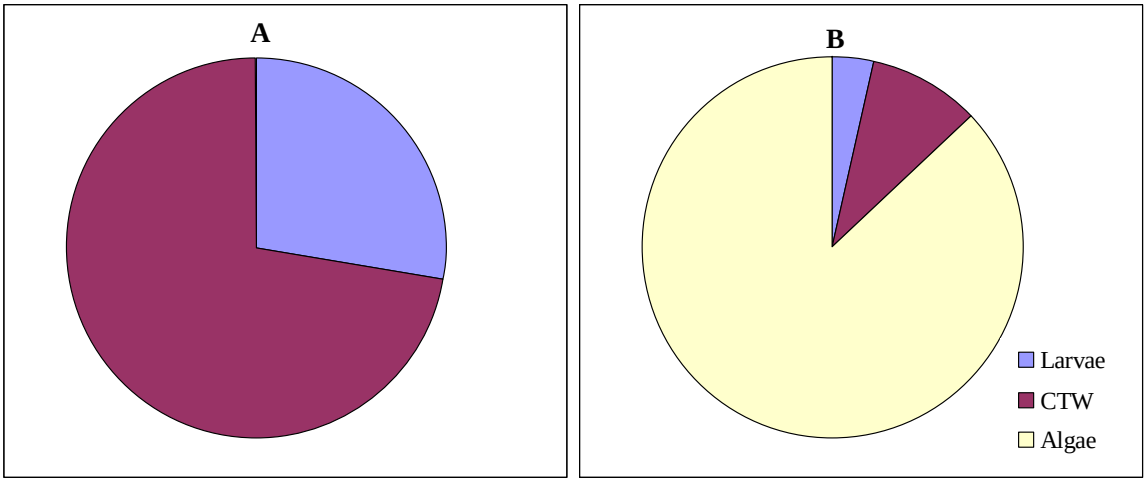


Figure 6 – Relative contribution of bacteria to the Batch tank WC from algae feed, CTW and larvae over a 48 h period. A: Vibrio counts; Pr (sample type) < 0.0001. B: TVC counts; Pr (sample type) < 0.0001.

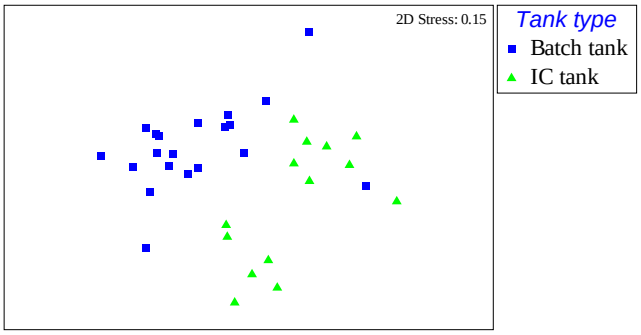


Figure 7 – MDS plot showing the similarity of the microbial communities in WC samples from different tank types based on TRFLP analysis. The closer points are to each other, the more similar they are. Pr (tank type) < 0.0001.

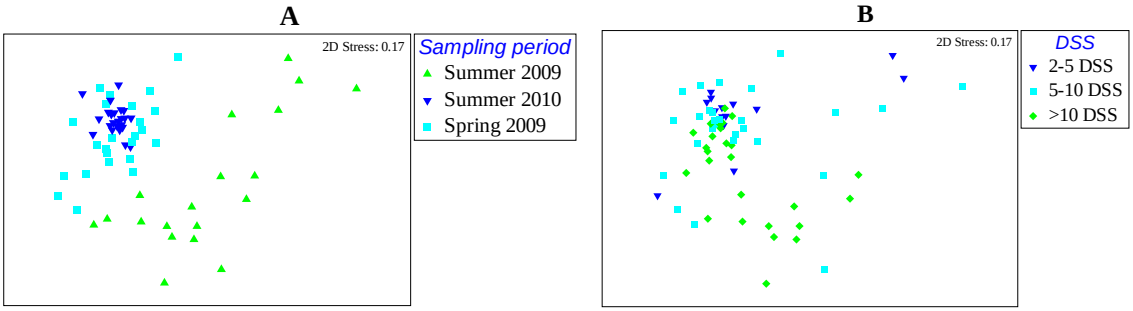


Figure 8 – MDS plots showing similarity of the microbial communities associated with larvae samples from different sampling periods (A: Pr (sampling period) < 0.0001) and DSS groupings (B: Pr (DSS) < 0.0001). In both MDS plots the closer points are to each other the more similar they are.

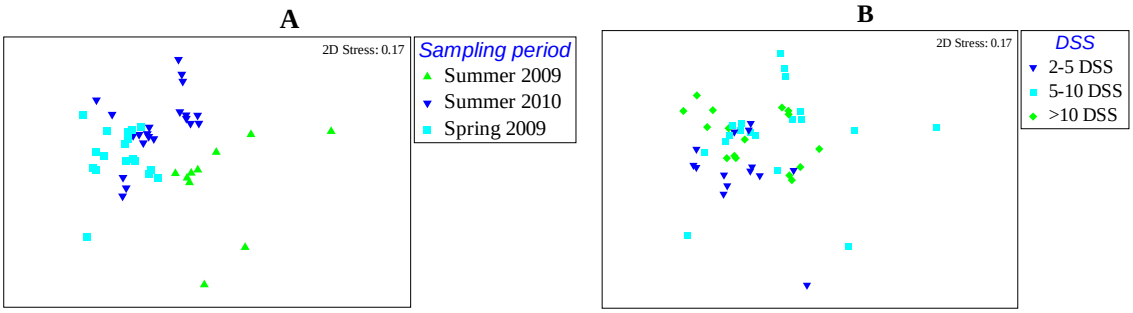


Figure 9 - MDS plot showing similarity of microbial communities of WC samples from different sampling periods (A: Pr (sampling period) < 0.0001) and DSS groupings (B: Pr (DSS) < 0.0001). In both MDS plots the closer points are to each other the more similar they are.

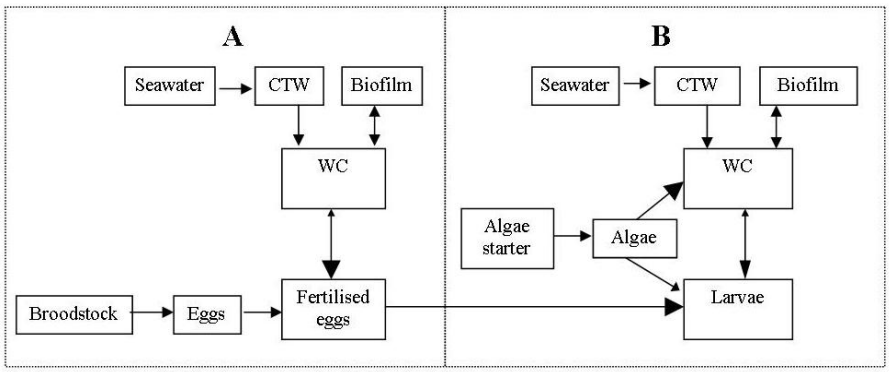


Figure 10 - Relationships among microbial niches of the larvae tank. A: At spawning, fertilization occurs in a fertilisation chamber before transfer to a tank after approximately one hour. B: After two DSS xenic algae are added to the feed mix. Arrows indicate the polarity of relationships among sample types.

3 Microbial communities associated with epizootics in a commercial oyster hatchery

3.1 Abstract

A microbiological study of a commercial oyster hatchery in Bicheno, Tasmania, was undertaken to compare microbial communities associated with apparently healthy and diseased larvae tanks. Both culture-dependent and culture-independent tools were used, including TRFLP, 16S rRNA gene clone libraries and next generation sequencing. A total of eight production runs were monitored over a 12-month period, seven of which terminated in epizootics. The study determined that epizootics may be attributed to bacterial disease but there was no predominant characteristic microbial community in the larvae or tank water associated with disease. *Vibrio* spp. comprised only a minor component of the total microbial community of the larvae tank (larvae, tank water, biofilm) in association with both apparently healthy and diseased larvae as indicated by culture-independent methods. However, using culture-dependent methods disease was associated with peaks in the relative abundance of *Vibrio* in larvae and WC, as high as 100% of the total culturable population, but was not consistently associated with peaks in total abundance of *Vibrio* in larvae or WC. Larvae aggregative behaviour was flagged as a potential contributing factor to disease incidence. Future studies should employ microbiological tools capable of characterising the *Vibrio* population and should include cultivation of both *Vibrio* and total heterotrophic bacteria for relative *Vibrio* abundance. This study is the first to characterise the microbial community associated with epizootics in a bivalve hatchery using culture-independent microbiological tools.

Keywords: Oyster larvae, hatchery, disease, epizootics, microbial community, *Vibrio.*

Abbreviations: DSS – days since spawning, WC – water column, CTW – clean tank water.

3.2 Introduction

Epizootic events occurring in commercial oyster hatcheries are a major production constraint and are attributed to both bacterial and viral infections. In Tasmania, which supplies at least 60% of oyster spat to Australian oyster farms, the viral agent, Ostreid Herpesvirus-1, appears to be absent. In 2011, as part of the National Ostreid Herpesvirus-1 survey conducted by the Animal Health Laboratories (Dept Primary Industries, Parks, Water and Environment (DPIPWE)), 1530 Tasmanian Pacific oysters from more than 15 farms covering every MFDP area in Tasmania, were tested and found negative by PCR for Ostreid Herpesvirus-1. Neither has histological evidence of virus infection been detected since DPIPWE began surveillance in 1995. As such epizootics occurring in Tasmanian oyster hatcheries are thought to be caused by bacterial infections.

Numerous studies have implicated pathogenic *Vibrio* spp. in hatchery epizootics including *V. tubiashii* (Elston et al 2008; Lodeiros et al 1987), *V. splendidus* (Sugumar et al 1998) and *V. anguillarum* (DiSalvo et al 1978). Another study implicated *Aeromonas* sp. and mixed bacterial species in incidence of disease (Garland et al 1983) but the vast majority of epizootics have been attributed to *Vibrio* spp. The pathogenic mechanism of pathogenic *Vibrio* spp. have been at least partly elucidated (Hasegawa et al 2009). Kothary et al (2001) and Delston et al (2003) showed that *V. tubiashii* produced both a metalloprotease and a cytolysin toxic to bivalve larvae. Milton (2006) demonstrated that such exoproteins were controlled through quorum sensing but it is not yet known how production is initiated. Other pathogenic *Vibrio* spp. have been shown to cause disease in oyster larvae without production of metalloprotease or cytolysin, which indicates that there may be other pathogenic mechanisms involved (Elston et al 2008).

Induction of disease in oyster larvae has been reproduced reliably by numerous authors under *in vitro* conditions in larvae-bacterial pathogen challenge trials where pathogens have been implicated in epizootics according to Koch's postulates. Yet, evidence from hatchery-based studies indicates that incidence of disease does not consistently relate to the presence of pathogenic species in high abundance (Elston et al 2008). Since challenge trials are normally conducted in the absence of other bacteria, as is required by Koch's

postulates, this indicates that induction of pathogenesis in the commercial hatchery might relate to some characteristic of the whole microbial community in association with larvae rather than only the presence and abundance of the pathogenic species. According to Paillard et al (2004) the requirement of Koch's postulates for isolation of pathogen and host is a limitation of the methodology because it takes no account of pathogens that cause disease only in the presence of other pathogens (co-infections or synergistic infections) or under environmental conditions favourable for the pathogen and not for the host, or only when the host is immunodepressed.

To date characterisations of microbial communities in bivalve hatcheries have been largely limited to the culturable community despite the availability of microbiological tools designed to study whole microbial communities for more than a decade. Hence a detailed case study has been undertaken here of epizootics occurring over a 12 month period in a commercial oyster hatchery located on the east coast of Tasmania, at Bicheno. The emphasis on whole microbial communities in experimental design is further warranted because of anecdotal evidence from the hatchery that disease is not always associated with peaks in *Vibrio* abundance in the larvae tank water. A total of eight production runs were monitored over a 12-month period, seven of which terminated in disease incidence. Cultivation-based and genetic tools including 16S rRNA gene clone libraries, terminal restriction fragment length polymorphism (TRFLP) and next generation sequencing (NGS) were used to characterise whole microbial communities of the larvae tank and compare communities associated with apparently diseased and healthy larvae. This investigation was undertaken as part of a larger investigation to characterise microbial communities in hatcheries, as the broader microbial context in which disease occurs (Chapter 2).

3.3 Methodology

3.3.1 Monitoring program design

A total of eight commercial production runs were monitored from spawning through to metamorphosis or an epizootic event. Monitoring was focussed on compartments of the larvae tank (larvae, WC and biofilm), referred to collectively as "sample types". Monitoring involved daily data and sample collection including cultivation of samples for bacterial enumeration, larvae production and health records, and samples for TRFLP analysis, 16S rRNA gene clone libraries, and next generation sequencing (NGS). Monitoring was conducted across three different sampling periods from 16/01/2009 to 01/02/2010: Summer 2009 (16/01/2009 to 12/02/2009), Spring 2009 (07/09/2009 to 03/10/2009), Summer 2010 (27/11/2009 to 18/01/2010).

Microbial communities were assessed statistically according to disease status: a classification of larvae health into "apparently healthy", "diseased" or "moribund" (detailed further below). Statistical analysis included four other independent variables in order to separate the effects of disease status in a multi-variate environment: sample type, larvae age (days since spawning (DSS)), sampling period and "tank type". Tank type was included in analyses because two different production systems were used in the hatchery including a flow-through and a batch production system and these were conducted in different tank types according to the methodology detailed in Chapter 2. A greater level of detail was reported for three production runs (identified as "280109", "070909, and "170909") in order to characterise changes occurring in larvae tank microbial communities in the days leading up to epizootic events.

3.3.2 Analytical procedures

Cultivation of bacteria from larvae and WC samples from all eight production runs was undertaken according to the protocol detailed in section 2.3.3.1. Data were compared with respect to disease status using SAS software (version 9.2) general linear model (GLM). F-values and probability values were calculated using type III sum of squares (otherwise

referred to as partial sum of squares). Daily microbial abundance data for production runs 280109, 070909 and 170909 was reported for analysis of microbial abundance changes in the days leading up to disease incidence.

For DNA-based analyses DNA extraction and purification was undertaken on larvae, WC and biofilm samples as detailed in section 2.3.3.2. Microbial communities of larvae and WC samples were compared with respect to disease status using TRFLP and PERMANOVA analysis according to the protocol given in section 2.3.3.3. Data from previously constructed clone libraries (GenBank accession numbers: JX486558 - JX486676) were analysed with respect to samples from diseased and apparently healthy larvae according to the protocol given in section 2.3.3.4.

3.3.2.1 Next generation sequencing (NGS)

NGS (454 pyrosequencing) was employed for a total of eight samples including larvae, WC and biofilm from both diseased and apparently healthy larvae tanks for the purpose of determining the relative abundance of *Vibrio* spp.. This work was undertaken following completion of clone library analysis to support the finding that *Vibrio* spp. comprised only a minor component of the microbial community of all sample types. A different primer set was used in PCR in order to eliminate potential PCR-bias relating to possible primer-template mismatches.

Extracted DNA was sent to MR DNA (Molecular Research LP) (503 Clovis Rd, Shallowater, TX 79363, USA) and all analysis was undertaken there according to their standard protocols, which are paraphrased below. Amplification of the 16S rRNA gene was undertaken with PCR using primers 104F (GGCGVACGGGTGAGTAA) and 530R (CCGCNGCNGCTGGCAC). Reads data derived from the sequencing process was processed using a proprietary analysis pipeline (www.mrdnalab.com). Within the pipeline barcodes and primers were removed from sequences and then poor quality sequences were removed including: short sequences (< 200bp), sequences with ambiguous base calls, and sequences with homopolymer runs exceeding 6 bp. The remaining sequences were then denoised and chimeras removed. Operational taxonomic units

(OTUs were defined at 3% divergence (97% similarity) after removal of singleton sequences. OTUs were taxonomically classified using BLASTn against a curated GreenGenes database (DeSantis et al 2006) and compiled into each taxonomic level into both "counts" and "percentage" files. Counts files contain the actual number of sequences while the percent files contain the relative percentage of sequences within each sample that map to the designated taxonomic classification.

3.3.2.2 Larvae health and production records

Larvae health was measured daily by hatchery staff using a range of assessments performed under a light microscope, including five variables: activity levels, velum condition, gut contents; lipid content, and faeces compactness. Reduction in activity, velum damage and poor faeces consistency are characteristic symptoms of bacterial infection (Tubiash et al 1965) and are therefore important health measures. Inspection of gut contents indicates whether larvae have been ingesting algae feed normally. Lipid reserves are an important indicator of fitness that is especially important for successful metamorphosis (Helm & Bourne 2004). All variables were rated on a scale of one to five where one represented very poor health and five represented very good health. Values were combined and averaged to make a composite health score that was used in data analyses. Production notes were also recorded daily and included daily larvae attrition percentages and observations of disease symptoms. Diseased larvae were those with a health score of two or less whereas apparently healthy larvae had a health score above two. Moribund larvae were apparently diseased larvae that had ceased swimming in the WC and formed dense aggregations on the tank bottom. Larvae aggregating towards the tank bottom were described as having "dropped out" of the water column.

3.4 Results

3.4.1 Observation of disease symptoms

Production run 280109 larvae were cultured in the Batch tanks until seven DSS when the larvae were moved to IC tanks. The larvae were healthy and growing well prior to 14 DSS, although minor velum damage was noted on 11, 12 and 13 DSS. From the emergence of first disease symptoms on 14 DSS health deteriorated rapidly and terminated in mass mortality at 16 DSS as reflected in the health score (Figure 11A). On the morning of 14 DSS a decline in larvae velum condition and minor mortality (less than 5% total population) was noted. The population was culled to remove larvae of smaller size. Throughout 15 DSS larvae began releasing large amounts of dark mucus-like faeces into the WC. On 16 DSS black spotting appeared on the inside of the tank at the water line, high mortality was observed (greater than 30%) and the culture was abandoned. Diseased larvae had badly damaged velum, high activity levels and erratic swimming behaviour. Moribund individuals had necrotic internal regions.

Production run 070909 larvae were initially cultured in the Batch tanks. On the morning of three DSS, larvae were found in dense aggregations near the bottom of the tank that were visible from the surface. Under microscopic examination larvae appeared active, healthy, and had no disease symptoms. Following water change, larvae dropped out and aggregated again but were dispersed using a plunger. By the afternoon larvae were behaving normally again. At five DSS larvae aggregative behaviour was observed again with no disease symptoms and high activity levels. Larvae recovered upon agitation with a plunger. At 6 DSS larvae were moved to the IC tanks and by eight DSS larvae were in excellent health and growing well. At nine DSS larvae health score dropped (Figure 11B) as symptoms of disease emerged: stomachs light in content and non-compact faeces with mucus entrail, reduced activity and progressively worse throughout the day. At 10 DSS high mortality was observed (greater than 50%) and surviving larvae had damaged or detached velum, erratic swimming behaviour and moribund larvae had necrotic internal regions.

Production run 170909 was conducted entirely in the Batch tanks. At four DSS larvae dropped out of the WC in all tanks and formed dense aggregations near the tank bottom. Microscopic examination activity levels remained high and no disease symptoms were observed. Recovery followed water change and feeding. Larvae dropped out again at six DSS but recovered following addition of algae feed. Again there were no other symptoms indicating disease. At 10 DSS, larvae health declined (Figure 11C) with a noted reduction in activity levels, minor velum abnormalities and reduced gut contents; although faeces remained compact and only minimal mortality occurred. On the following day larvae health had improved and larvae were feeding as normal. At 16 DSS larvae health deteriorated again with decreased gut content, non-compact faeces, poor development and approximately 10% mortality although velum and activity levels were relatively normal. At 17 DSS larvae health deteriorated further with reduced activity and velum damage. Metamorphosis was chemically induced with epinephrine despite disease problems. At 18 and 19 DSS larvae metamorphosis was weak and disease symptoms continued with heavy attrition every day until the culture was abandoned.

3.4.2 Microbial communities and disease incidence

Comparison of larvae microbial communities from eight production runs using TRFLP PERMANOVA indicated no significant difference between diseased and apparently healthy larvae (Pr = 0.0633, Figure 12A). Analysis of WC microbial communities indicated a significant interaction between disease status and sampling period (P = 0.0033, see Figure 12B), indicating that there may have been a difference between communities of WC associated with diseased and apparently healthy larvae but the difference was dependent upon the sampling period. Thus there was no significant evidence of a predominant characteristic community in the larvae or WC associated with disease incidence across production runs.

Clone library composition of moribund larvae from three production runs is summarised to class level in Figure 13. Moribund larvae of production run 280109 were dominated by members of the *Bacilli* and *Gammaproteobacteria*. A total of 15 phylotypes were

identified and the most predominant phylotype was *Staphylococcus sciuri*$_{(97\%)}$ with 42.5% of all clones identified, followed by *Marinovum algicola*$_{(97\%)}$ at 21.9%, *Arcobacter nitrofigilis*$_{(93\%)}$ at 6.8%, and *Pseudoalteromonas marina*$_{(97\%)}$ at 6.8%. Moribund larvae of production run 070909 were dominated by *Alphaproteobacteria* and also had a high proportion of *Flavobacteria*. A total of 15 phylotypes were identified and the most predominant included *Nautella italica*$_{(97\%)}$ at 61.4%, an unidentified seawater clone GQ274082$_{(98\%)}$ at 19.3% and *Marinovum algicola*$_{(97\%)}$ at 9.1%. Moribund larvae of production run 170909 were dominated by *Alphaproteobacteria* and *Betaproteobacteria*. A total of 14 phylotypes were identified and the most predominant species included *Ramlibacter tataouinensis*$_{(93\%)}$ at 34.2%, *Shimia marina*$_{(97\%)}$ at 24.6%, *Sphingomonas paucimobilis*$_{(97\%)}$ at 17.5%, and *Arcobacter nitrofigilis*$_{(93\%)}$ at 8.8%. Among other clone libraries taken for these three production runs, including biofilm and WC taken at the same time as moribund larvae samples, no bacterial species known to be overtly pathogenic to oysters were identified.

3.4.3 Vibrio abundance and disease incidence

Of a total of 18 clone libraries, with a combined total of 1390 clones, only three *Vibrio* clones were identified. NGS data confirmed that *Vibrio* spp. were a minor component of the total microbial community of larvae, biofilm and WC irrespective of disease status (Table 1). The highest percentage of *Vibrio* sequences detected using NGS was 2.81% in a moribund larvae sample (Table 5). The moribund larvae sample dated "010210" had a presumed high *Vibrio*/TVC (51.90%) but no *Vibrio* sequences were detected using NGS.

Microbial abundance data from eight production runs indicated a change in the *Vibrio* component of the population with disease status. Although neither TVC nor *Vibrio* counts were significantly different between diseased and apparently healthy larvae, the relative *Vibrio* abundance (*Vibrio*/TVC), was on average higher in diseased larvae (18.2%) than apparently healthy larvae (2.4%) (Pr = 0.0102). This trend was not observed in the WC.

Analysis of *Vibrio* abundance on a day-by-day basis throughout production runs 280109, 070909 and 170909 provided further insight into *Vibrio* population dynamics associated

with disease incidence. Prior to the development of clear disease symptoms in production runs 070909 and 170909 incidence of larvae drop-out was observed. This was not accompanied by a rise in *Vibrio* abundance although in both production runs *Vibrio* abundance in larvae and WC increased on the day following the second incidence of drop-out (Figure 11B and C) and continued to increase until disease incidence.

Emergence of clear disease symptoms was accompanied by a spike in the relative *Vibrio* abundance in the larvae and to a lesser degree in the WC in all three production runs. A similar spike in total *Vibrio* abundance was not observed consistently in either the larvae or WC. Larvae *Vibrio*/TVC spikes reached 100% for production run 280109, 35.4% for production run 070909 and 21.2% and 24.2% for production run 170909, which had two *Vibrio* spikes. However, *Vibrio*/TVC spikes did not coincide exactly with the emergence of disease symptoms in all cases. Whilst in production runs 280109 and 170909 disease incidence occurred with increasing *Vibrio* abundance, in production run 070909 disease incidence occurred the day following a *Vibrio* spike and mass mortality occurred another day later when *Vibrio* levels were comparatively low (<1%).

3.5 Discussion

The emergence of disease symptoms was often preceded by unusual larvae behaviour. In six out of the seven unsuccessful production runs monitored, some days prior to the emergence of disease symptoms, larvae dropped out near the bottom of the tank and forming dense aggregations or "swarms", which were visible from above the tank and in which activity levels remained high (henceforth referred to as "swarming"). Aggregative behaviour has been noted by a number of authors as an early disease symptom. DiSalvo et al (1978) noted that larvae progressively accumulated in spots on the bottoms of tanks in a commercial hatchery of European Flat Oyster (*Ostrea edulis*) and suggested that "spotting" may be due to bacterial toxins that inhibit larvae swimming. Prado et al (2005) noted similar aggregation in challenge tests with *Vibrio* spp. and described the phenomena as "an accumulation of larvae agglutinated in the bottom of the well". In a study of a commercial pacific oyster hatchery by Sugumar et al (1998), visible aggregation of larvae

on the tank bottom was noted as the first sign of disease. While these studies are in agreement that the first sign of disease may be aggregative behaviour, in the present study swarming does not appear to be directly related to incidence of disease because of the absence of other disease symptoms and the separation of swarming and disease symptoms by some days. Swarming may be a contributing factor to disease incidence due to the high densities achieved in swarms and their proximity to the tank bottom. High density has previously been shown to favour proliferation of opportunistic bacteria (McIntosh et al 2008; Sandaa et al 2003). Bottom-dwelling may also facilitate bacterial growth through accumulation of detritus in the tank including larvae faeces and sedimented feed algae (DiSalvo et al 1978). Moreover, swarming may be an indicator of larvae stress, which might increase larvae susceptibility to bacterial attack.

High activity levels observed in swarms indicates that swarming may be an active behaviour rather than passive sinking and aggregation due to tank flow dynamics. Swarming, as an active behaviour, does not appear to have been addressed in the literature despite being a well recognised phenomenon in zooplankton communities, where it is believed to be a typical response to increased predation risk (Banas et al 2004). It is thought that oyster larvae may have little influence over their position in the water column over the scale of metres due to currents in the marine environment (Finelli & Wethey 2003). However, larvae have been shown to possess several distinct swimming behaviours mediated by environmental cues (Finelli & Wethey 2003) and so it may be postulated that altered swimming behaviour may cause swarming in the relatively static conditions of the larvae tank.

Toxicants are known to alter larvae swimming behaviour. For example Thompson et al (1997) showed that mussel larvae (*Mytilus edulis*) swimming activity was temporally reduced and larvae sunk to the bottom of the experimental container when exposed to very small concentrations of chlorine (1 ppm NaOCl). After one hour activity levels began to increase and returned to normal after six hours although the larvae were still immersed in the chlorine medium. In response to mercury contamination, veligers of Pacific oyster were shown to swim nearer to the surface (His et al 2000). In general, behavioural

responses are seen at sub-lethal levels (Beiras & His 1994; His et al 1999; Prael et al 2001). Potential nearby sources of anthropogenic contamination included a boat slip, abalone factory, boat ramp, and forestry plantations. Natural toxins such as those associated with algal blooms cannot be discounted. Legacy issues of contaminated sediment might explain the hatchery manager's observation that windy conditions, which may increase sediment content in the water, correlate with poor production outcomes.

Discounting anthropogenic contamination, behavioural responses to natural chemicals in the marine environment are known to be profoundly important in mediating zooplankton behaviour (Zimmer & Butman 2000). For example the highly volatile chemical dimethylsulphide (DMS), which is a breakdown product of the algae osmoregulant dimethylsulfoniopropionate (DMSP) associated with algae blooms, is an important "info-chemical" in the plankton environment. It has been shown to elicit responses across a wide range of marine species including sea-birds (Nevitt and Haberman 2003), copepods (Steinke et al. 2006) and sea urchins (Lyons et al., 2006) and it maybe that DMS could theoretically cause swarming in zooplankton in response to increased predation risk.

Whilst larvae swarming may have been a contributing factor to disease incidence, the direct cause appears related to bacterial infection. Typical disease symptoms encountered included damaged or detached vela, abnormal swimming behaviour, reduced activity, poor faecal consistency, necrotic soft tissues and mass mortality, which are consistent with those caused by bacteria and described by Tubiash et al (1965) as "bacillary necrosis". A previous investigation carried out at the same hatchery demonstrated that epizootics experienced in 1981 were caused by bacteria (Garland et al 1983). Non-detection of viruses over 15 years of surveillance by DPIPWE, from 1995 to 2011, indicates that viruses are highly unlikely to have impacted upon this study.

TRFLP analysis of larvae and WC microbial communities indicated that there was no predominant characteristic community associated with diseased larvae across production runs (Figure 12). Non-detection of a characteristic microbial community in association with disease and non-detection of any recognised bacterial pathogens in diseased larvae,

moribund larvae, biofilm or WC samples indicates that a microbiological cause of disease may have occurred below the limits of detection.

Due to their notoriety as oyster larvae pathogens, *Vibrio* spp. may be suspected as the causal agent in hatchery epizootics although clone library analysis and NGS, using different primer sets, indicated that *Vibrio* spp. were only a minor component of the total microbial community of both healthy and diseased larvae. In contrast culture-based assessment indicated that *Vibrio* abundance was as high as 100% of the total heterotrophic bacterial count (*Vibrio*/TVC) in association with diseased larvae. Similar results were reported by Bourne et al (2004) in a study of microbial populations associated with larval Tropical rock lobster where *Vibrio* spp. were reportedly predominant in the larvae using culture-based analysis, but absent from molecular-based analysis. Culture counts over-estimate *Vibrio* abundance since the media used to selectively count *Vibrio* is not completely selective and may over-estimate *Vibrio* numbers (Pfeffer & Oliver 2003) whereas marine agar does not allow the growth of many marine bacteria hence underestimates total bacterial numbers.

Although *Vibrio* spp. were only a minor component of the microbial community, *Vibrio* may have been sufficiently abundant to cause disease. Larvae-bacteria challenge tests in other studies have shown that *Vibrio* spp. isolated from moribund larvae are able to cause mortality at very low concentrations. Elston et al (2008) showed that 100% larvae mortality was caused by a *V. tubiashii* strain at levels of 2 log CFU/ml in the culture medium after 67 h. Prado et al (2005) noted significant mortality in challenge tests after 24 h using 4 log CFU/ml of *Vibrio splendidus*. However, Elston et al (2008) reported that lower concentrations of bacteria were required to cause mortality in laboratory challenge tests compared to production facilities and proposed that the pathogenicity-disease curves generated in small scale laboratory experiments may not represent the same dose-disease relationships in large scale bivalve hatchery production. In hatchery assessments of total *Vibrio* abundance, Elston et al (2008) found a maximum *Vibrio* count of 4.52 log CFU/ml in the WC of oyster larvae hatcheries affected by disease in which *V. tubiashii* was implicated. Widman et al (2001) found that a total *Vibrio* count of 4 log CFU/ml in

hatchery water caused disease in scallop larvae, which as bivalve larvae are closely related. In the present study when disease symptoms first emerged *Vibrio* counts in the WC were 4.16, 4.85, and 3.21 log CFU/ml in production runs 280109, 070909 and 170909, respectively, which is a similar level to that encountered in other hatchery studies where *Vibrio* has been implicated as the primary etiologic agent.

The emergence of disease symptoms was consistently associated with a peak in relative *Vibrio* abundance (*Vibrio*/TVC) occurring either on the day of disease incidence or the day prior, while no such peak in total *Vibrio* abundance was observed. The larvae-associated *Vibrio*/TVC percentage peaked at 100%, 35.4%, and 24.2% in production runs 280109, 070909, and 170909, respectively (Figure 11). These "*Vibrio* spikes" indicate that *Vibrio* may have been the etiologic agent in hatchery epizootics.

It is interesting to note that in both production runs 070909 and 170909, neither larvae *Vibrio* counts nor *Vibrio*/TVC percentage increased at the first incidence of swarming but began to increase daily following the second swarming incident until a *Vibrio* spike occurred in association with a disease incident (Figure 11). This supports the idea that swarming may have been a contributing factor to disease incidence by providing conditions conducive to proliferation of a bacterial pathogen.

3.6 Conclusion

The study indicates that whilst *Vibrio* may be the primary etiologic agent in oyster hatchery epizootics attributed to bacillary necrosis, *Vibrio* comprise only a numerically minor component of the total bacterial population. Furthermore, monitoring of total *Vibrio* abundance in association with larvae and WC using cultivation-based methods should include TVC counts because only relative *Vibrio* abundance reliably correlated with disease incidence. Finally, this study flags the potential importance of induced larvae behaviour as a contributing factor to disease incidence.

Table 5 – Percentage of *Vibrio* sequences detected using NGS compared to cultivation (*Vibrio*/TVC)

Sample type	Larvae	Larvae	Larvae	Larvae	Biofilm	Biofilm	WC	WC
Sample date (dd/mm/yy)	150909	030209	010210	300909	160909	200110	011009	250909
Disease status[a]	D	A	D	A	D	A	A	A
Vibrio (%) - NGS	2.81	0.33	0.00	1.56	0.00	0.65	0.00	0.02
No. sequences - NGS	2257	5185	338	5285	2906	2497	530	4310
Vibrio/TVC (%) - cultivation	1.45	1.00	51.90	0.06	-	-	0.06	0.06

a – Disease status: D = diseased, A = apparently healthy

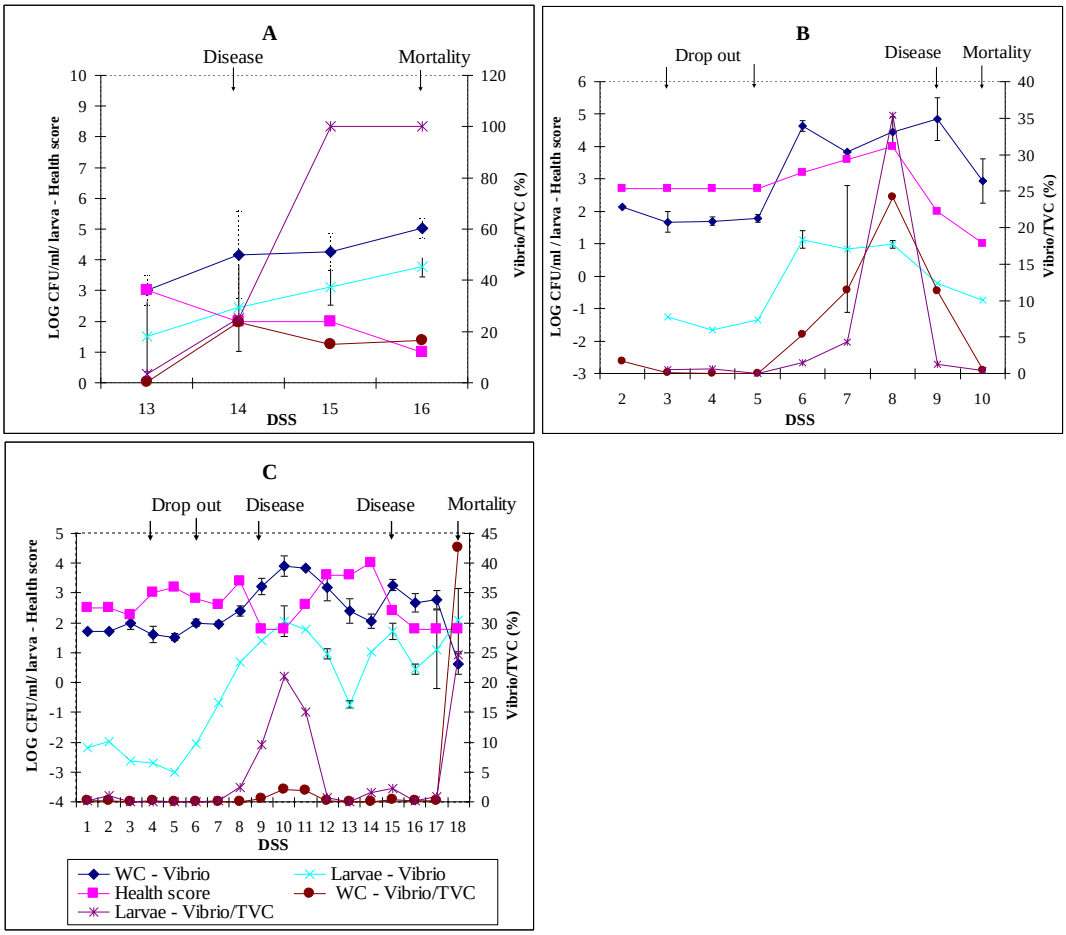


Figure 11 – *Vibrio* abundance in association with diseased production runs (A) Production run 280109 (B) Production run 070909 (C) Production run 170909

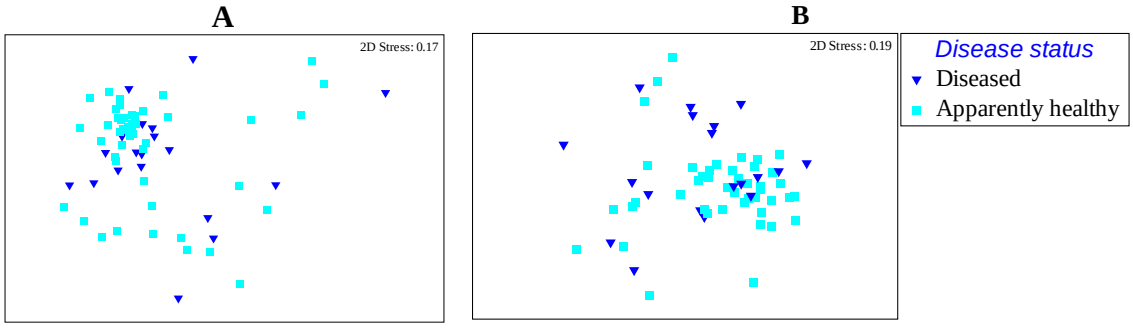


Figure 12 – MDS plot showing microbial composition similarity of diseased and apparently healthy larvae from seven runs (A) Larvae (Pr = 0.0633) (B) WC (Pr interaction: disease status and sampling period = 0.0033)

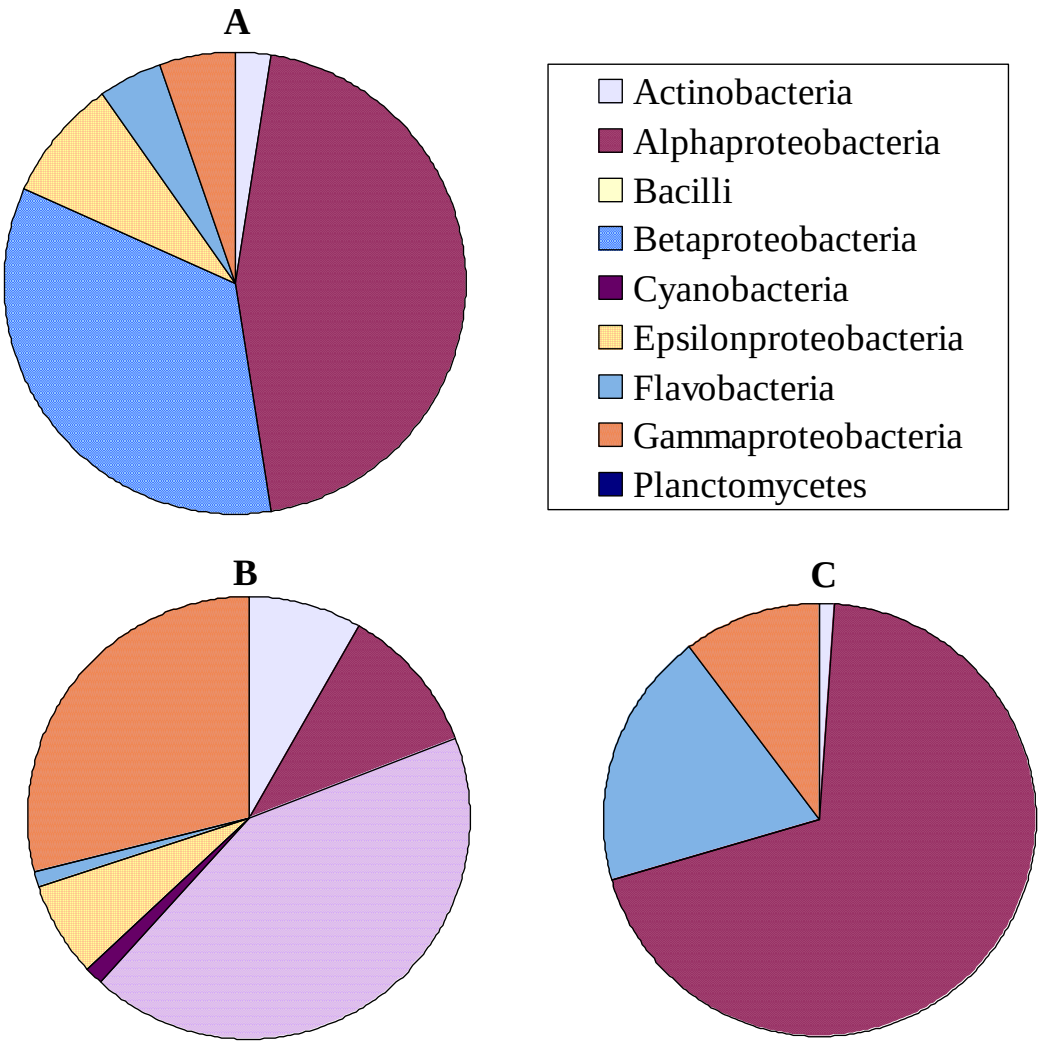


Figure 13 – Clone library data from moribund larvae from different production runs (A) Production run 170909 (sample D_031009) (B) Production run 280109 (sample D_100209) (C) Production run 070909 (sample D_150909)

4 Oyster larvae stress and susceptibility to bacterial disease

4.1 Abstract

An investigation was undertaken to test the hypothesis that oyster larvae susceptibility to bacterial disease may be affected by sub-lethal levels of stress. Larvae were exposed to Cu stress prior to bacterial challenge. It was shown that sub-lethal levels of Cu decreased larvae activity and increased larvae susceptibility to bacterial attack under some conditions. Relative to sub-lethal levels larvae exposed to the lethality threshold of 25 ppb Cu had higher activity levels and higher survival rates in subsequent bacterial challenge, which may indicate induction of the so called heat-shock response. Larvae behaviour was modified at Cu levels as low as 2.5 ppb, which indicates that behaviour could be used as a sensitive biomarker of Cu stress. The protocol developed might be of use in assessment of other types of chemical stress. The behavioural response to different concentrations of Cu was non-linear and differed with duration of exposure, indicating that behavioural assessments should be made across a range of concentrations and also across a 24 – 48 h time period.

Keywords: Oyster larvae, hatchery, disease, *Vibrio*, Cu, stress.

Abbreviations: % CL - percentage of larvae crossing midway line in cuvette, % SL - percentage of larvae swimming in cuvette

4.2 Introduction

4.2.1 Interaction between chemical stress and infectious disease

A number of researchers have suggested that a major cause of infectious disease in cultured larvae is opportunistic bacterial pathogens that normally coexist with the host but under sub-optimal and stressful conditions can take advantage of ecological changes to

cause mortalities (Bourne et al 2004; Morley 2010; Schulze et al 2006). The link between toxic environmental contaminants and incidence of non-infectious diseases in marine organisms is well supported (Pipe & Coles 1995; Sindermann 1990). However, there appears to be a scarcity of research to support the hypothesis that contaminant induced stress leads to enhanced susceptibility to infectious disease in oyster larvae. Moreover, the research that does exist appears to have been carried out with adult molluscs and not larvae.

Several examples of the interaction between stress and infectious disease in adult molluscs are given here that indicate stress may increase disease incidence but that the response is often complex with some examples showing stress may reduce infectious disease by stimulating adaptation that counters the pathogenic mechanism. Parry and Pipe (2004) note that exposure to environmental chemicals may result in complex immune responses due to reciprocal interactions between the immune, endocrine and nervous systems of bivalves and may include suppression or enhancement of the immune system.

Gagnaire (2007) conducted an experiment in which adults of the Pacific oyster were exposed to a mixture of eight different pesticides at environmentally relevant concentrations over a 7-day period before being exposed to bacterial challenge with *V. splendidus* related strains. The study showed that pesticide exposure decreased haemocyte phagocytosis and down-regulated genes involved in haemocyte function, particularly phagocytosis, and increased susceptibility to bacterial challenge. Although the author notes that the use of intra-muscle injection of *V. splendidus* bypassed the host's first line of defence and therefore the results should be interpreted with caution.

Parry and Pipe (2004) exposed adult mussels (*Mytilus edulis*) to Cu and bacterial challenge with *V. tubiashii* both sequentially and simultaneously. A number of immunological assays were carried out to obtain an overall view of immune function. Exposures to either Cu or bacterial challenge were shown to affect at least one aspect of haemocyte function although the relationship was complex due to interactions between the two factors and temperature. At low levels of Cu phagocytosis was stimulated but was inhibited at high levels of Cu. The study did not relate immunological function with

physiological end points such as disease symptoms nor was mortality reported. Hence the relevance of these results to infectious diseases is uncertain.

In another experiment, adult Taiwan abalone infected with *Vibrio parahaemolyticus* and exposed to either ammonia or nitrite had increased mortality compared to controls, and suppressed immune function, except for the activity of superoxide anion which increased (Cheng et al 2004a; b). The increased mortality in diseased abalone was attributed to the observed changes in immune function.

In the bivalve hatchery environment interaction between chemical stress and infectious disease may be caused indirectly through chemically-induced behavioural changes in larvae rather than by a direct impact on the larvae immune function. Valve closure and cessation of swimming caused by toxins is a recognised phenomenon in bivalve larvae (Wisely & Blick), which, in the larvae tank, results in bottom dwelling behaviour. Such behaviour is likely to create conditions conducive to proliferation of opportunistic bacterial pathogens within the larvae due to high densities of larvae and accumulation of tank bottom detritus including larvae faeces and sedimented feed algae (DiSalvo et al 1978). As such any chemical that may induce such changes in larvae behaviour may affect disease incidence.

4.2.2 Chemical stressors in the hatchery environment

Oyster larvae may encounter a range of stressors in the hatchery environment including: handling between water changes, poor diet, high larvae densities, inadequate aeration, physio-chemical properties of culture water, toxic metabolites, toxic compounds from outside and within the hatchery of both natural and anthropogenic origin. Toxins are of particular interest because they are often transient, difficult to measure, effective against bivalve larvae at concentrations as low as a few parts per billion, subject to influence from outside the hatchery, and increasing with urban influence on the coastal marine environment (Jones 2006).

Calabrese et al (1970) conducted a number of trials to determine the tolerance range of larvae of American oyster (*C. virginica*) to salinity, pH and temperature, and concluded

that larvae were "surprisingly tough" due to their ability to survive rather severe disturbances in these variables. Tolerance to a range of chemicals was also investigated and, by contrast, the authors concluded that larvae could be killed by "surprisingly low" concentrations of certain toxicants.

Typical contaminants of the urban-coastal areas include metallic, organometallic, and poly aromatic compounds (PAH); polychlorinated biphenyls (PCB); fertilizers and pesticides (Cravo et al 2009); many of which have been shown to be toxic to bivalve larvae. A number of studies have demonstrated that bivalve larvae are sensitive to minute concentrations of heavy metals (Beiras & His 1994; Geffard et al 2007; MacInnes & Calabrese 1979; Parry & Pipe 2004). In particular, Cu is highly toxic to bivalve larvae and is a common contaminant in the coastal marine environment. Calabrese et al (1977) found that Cu concentrations of 32.8 ppb resulted in 50% abnormal individuals following embryogenesis. Another study by Worbys et al (2002) indicated that embryogenesis may be sensitive to Cu levels as low as 0.6 ppb. Sources of Cu within the hatchery include brass fittings and pump bearings, both of which may "leak" Cu ions as corrosion takes place (Jones 2006). The main sources of Cu contamination in the marine environment are from industrial discharges and atmospheric deposition, particularly from foundries and metal processing operations. Fungicides, wood preservatives and boat antifouling paints can also contribute to high levels of Cu where high numbers of boats are moored or where boat works are undertaken.

Algal blooms are another potential source of toxins that have been shown to cause mortality in both adult and larval oysters. Natural mortalities of adult oysters in south Puget Sound have been correlated with blooms of *Ceratium* spp. (Westly et al 1989). While bacterial communities associated with blooms have been blamed for oyster mortalities (Connell et al 1997), algal toxins have also been shown to be toxic to oysters. Stoecker et al (2008) showed that high density cultures of the bloom-forming dinoflagellate *Karlodinium veneficum* prevented embryogenesis through toxin production when exposed to oyster embryos (*C. virginica*). The authors concluded that survival and maturation of embryos and larvae may have been reduced when oyster spawns coincided

with high bloom densities of *Karlodinium veneficum*. Some 40 or so species of algae produce toxins (Hallegraeff 1993) and some of these may be harmful to oyster larvae.

Many pollutants, including heavy metals, become concentrated in sediments and on suspended particles. Coastal sediments may acquire pollutant concentrations 5,000 times higher than those in the water column (Livett 1988) and re-suspension of sediments into the water column may increase the uptake of pollutants into aquacultural facilities. Sediments can be re-suspended by natural factors such as bioturbation, storms, wave and tide action, and by human activities such as dredging operations (Geffard et al 2007).

Contamination from within the hatchery may also cause stress to oyster larvae. Detergents and sanitizers used to clean larvae tanks and other hatchery surfaces may affect larvae if not sufficiently rinsed away. Embryogenesis of the pearl oyster *Pteria colymbus* and veliger larvae of the mussel, *Perna perna*, were affected by the presence of the common household detergent sodium dodecyl sulphate at concentrations of 0.8 ppm and 0.68 ppm, respectively (Jorge & Moreira 2005; Rumbold & Snedaker 1997). Thompson et al (1997) showed that concentrations of chlorine of 8 ppm (as sodium hypochlorite) caused mortality of all planktonic life stages of the mussel, *Mytilus edulis*, while concentrations of 1 ppm caused larvae to stop swimming and drop out of the water column for a brief period.

Stagnant water in pipelines and dead spaces in valves and fittings may also be a source of toxins that are damaging to larvae (Jones 2006). Stagnant water may become hypoxic, and anaerobic degradation of organic compounds may produce hydrogen sulphide (Wu 2002), which is toxic to bivalve larvae (Caldwell 1975). Stressed or dying algae and bacteria in stagnant water may release toxins that persist after the death of the producer organism (Jones 2006).

4.2.3 Measurement of chemically induced stress in oyster larvae

Because of their high sensitivity to a broad range of marine pollutants, bivalve larvae are frequently employed in marine pollution studies; in chemical toxicity tests and routine environmental monitoring. Wolke (1972) was one of the earliest researchers to standardize a “biomarker” approach using oyster embryos to test the toxicity of industrial

effluents. Various definitions of biomarker have been proposed but the broad definition proposed by Depledges and Fossi (1994) is used here: *a biological response to a chemical or chemicals that give a measure of exposure and sometimes also a "toxic" effect*. The use of biomarkers has great advantage over chemical analysis of water since only living systems are able to integrate the various complex effects of contaminants that are really bioavailable and additionally are less expensive and can be performed in 24 h (Chapman & Long 1983). Wolke (1972) proposed a bioassay using oyster embryos and utilising embryogenesis as the biomarker; upon exposure to potential chemical stressors or suspect environmental water samples, assessment was made of the percentage of the population that developed normally to D-stage larvae over 24 h or 48 h exposure. Embryos were used preferentially to larvae chiefly because factors such as conditions of culture, larvae condition, and algae feed did not need to be accounted for and also because embryogenesis was generally observed to be more sensitive than larvae survival across a broad range of toxicants (His et al 1999). As a general rule the lethality threshold increases with age from the most sensitive stage, embryo > veliger > pediveliger > adult (Wolke 1972).

Further development in marine pollution assessment beyond the embryogenesis test has occurred as researchers have sought to find more sensitive biomarkers. Some researchers have preferred to use growth of larvae as a more sensitive biomarker. Calabrese et al (1970) noted that reduction in growth was the first symptom observed upon exposure to contaminants at levels lower than those required to kill oyster larvae. Beiras and His (Beiras & His 1994) found that growth of Pacific oyster larvae was reduced upon exposure to 4 ppb of mercury (Hg), which compared to a decrease in successful embryogenesis at 8 ppb Hg. The authors noted inhibition of swimming at concentrations approximately 30 times lower than those causing lethal effects and suggested that behaviour may be a more sensitive indicator of stress; although swimming behaviour was not rigorously assessed in the study. Prael et al (2001) similarly suggested the use of swimming behaviour for assessment of sub-lethal effects of toxicants because of its sensitivity, ease of use and rapid results. The authors investigated the effects of leachate from wood treated with the biocide copper-chrome-arsenic (CCA) and observed larvae behaviour in 4.5 ml cuvettes.

Larvae, three and seven days old, were observed swimming two to three times faster in leachate than in plain seawater and moved up and down more in leachate.

Physiological biomarkers (such as embryogenesis, metamorphosis, and growth) and behavioural biomarkers signal adverse impact at the level of the whole organism and likely impact on an organism's survival. Yet departures from health are initiated at a biochemical level prior to the development of physiological and behavioural responses (Depledge & Fossi 1994; McCarthy & (eds.) 1990). Thus the measurement of larvae biochemical biomarkers has been undertaken and appears to be the most sensitive indicator of pollutant exposure (Stegeman et al 1992). In experiments with heavy metal exposure to Pacific oyster embryos and larvae, Geffard et al (2007) found the following order of sensitivity: biochemical marker-metallothionein > larvae growth > abnormal embryogenesis. Biochemical biomarkers indicate that exposure has taken place and additionally can be used to identify the nature of stress imposed upon the larvae and the type of pollutant in environmental samples (see Table 6) but contribute little to the prediction of the direct consequences for the organism or population in question (Depledge & Fossi 1994).

Table 6 – Biochemical markers used in oyster larvae

Biochemical marker	**Indicates:**	**Reference**
Glutathionione S-transferase (GST) activity	Organochlorine compounds and polychlorinated biphenyl (PCB)	Quiniou et al (2007) Damiens et al (2004)
Catalase (CAT) activity	General oxidative stress	Damiens et al (2006) Damiens et al (2004)
Thiobarbituric acid reactive substances (TBARS)	Lipid peroxidation – response to oxidative stress	Damiens et al (2006) Quiniou et al (2007)
Acetylcholinesterase (AChE) activity	Organophosphorous and carbamate compounds	Quiniou et al (2007) Damiens et al (2004)
Metallothionein (MT)	Exposure to heavy metals	Geffard et al (2007) Damiens et al (2006) Quiniou et al (2007)

In practice the choice of biomarker depends on a compromise between sensitivity and feasibility. Embryogenesis success is usually the method of choice because it can be conducted in 24 h or 48 h and does not require feeding, and is therefore easier to standardize. In contrast growth biomarkers, whilst potentially more sensitive, need to be conducted for a week or more and are difficult to standardise with results dependent upon many factors such as algae feeding and husbandry practices. Depledge and Fossi (1994) advocate using a suite of biomarkers (physiological, biochemical and behavioural) where biochemical biomarkers should reveal the type of detoxification mechanisms induced by an exposure while physiological and behavioural biomarkers will signal exposures resulting in adverse affects at the level of the whole organism. For application in the hatchery environment biomarkers need to be simply and rapidly applied. Determination of growth, embryogenesis and behaviour may meet these requirements and be useful tools for testing hatchery water with unknown potential toxicants in a commercial setting. Behaviour, in particular is the least developed of these biomarkers and may be a highly sensitive indicator of chemical stress and a promising area of research.

4.2.4 Research objectives

This chapter involves an investigation of the hypothesis that sub-lethal levels of Cu may affect larvae susceptibility to bacterial disease. Copper was chosen because it is a common contaminant of the coastal marine environment and because the hatchery is immediately adjacent to a boat slip where Cu-based anti-fouling paints are used regularly (Figure 1). The investigation includes assessment of larvae behaviour in recognition that any stress causing tank-bottom dwelling behaviour may increase disease incidence without a direct affect upon larvae immune function. Larvae behaviour is considered as a potential biomarker for application in the hatchery environment.

4.3 Methodology

4.3.1 Copper sulphate exposure followed by bacterial challenge

Larvae six DSS were collected from the Bicheno hatchery during a commercial production run, and transported in an ice-cooled esky (approximate temperature 5^{o}C to 10^{o}C) to laboratories at the university in Sandy Bay (3 h), where exposure to different concentrations of copper sulphate ($CuSO_4$) began 10 h after pick-up. Larvae were exposed to concentrations of $CuSO_4$ ranging from 10 ppb to 3,000 ppb for 24 h and 48 h. The control was exposed to the same treatment as $CuSO_4$ exposed larvae except no $CuSO_4$ was added. All 16 treatments were done in triplicate, which made a total of 48 replicates.

Copper sulphate was diluted in a small volume of sulphuric acid and diluted with autoclave sterilised artificial seawater (SSW) to 10,000 ppb for a stock solution. SSW contained de-ionised water combined with 35 g /L Red Sea Salt (Red Sea Fish Pharm, P.O. Box 4045, Eilat, Israel), which was then filtered to 0.45 µm to remove undissolved particulate matter, and autoclaved. Red Sea Salt, made from water from the Red sea, contained insignificant quantities of Cu. Full chemical analysis is available online from http://www.redseafish.com/ (01/01/2012). Treatments solutions of 200 ml were prepared in 250 ml Erlenmeyer beakers from stock solutions and diluted with SSW to required concentration. Adjustments were made for pH to a range of 8.1 to 8.2 using 5M sodium hydroxide.

Larvae were added to Erlenmeyer beakers at a density of 100 larvae per ml (a total of 20,000 larvae in 200 ml). Beakers were incubated in the dark at 25^{o}C for treatment duration. On conclusion of the $CuSO_4$ treatment larvae were removed and rinsed with 200 ml SSW on a 63 µm screen. Larvae were then placed into 12-well tissue culture plates in separate challenge tests with three different bacterial species (*Vibrio pomeroyii*, *Staphylococcus sciuri* and *Pseudomonas fluorescens)* at three different concentrations, plus a no-bacteria control. All challenge tests were done in triplicate using all Cu treatment replicates. Larvae were challenged in 12-well tissue culture plates (in 3 ml per 4 ml well), according to the methodology described by Estes et al (2004) at a density of

approximately 20 larvae per ml. Larvae mortality was assessed *in situ* in the 12-well plates at 24, 48 and 72 h using a dissection microscope. Larvae were counted as dead if no movement, including internal cilia movement, was observed. The total number of larvae in each well was determined at the end of the experiment.

Statistical analyses were conducted separately for each combination of $CuSO_4$ exposure time and bacterial species, which included a total of six analyses. All statistical analyses were conducted using SAS GLM (SAS software (version 9.2) general linear model (GLM)). Statistical analyses included modeling of main effects of $CuSO_4$ level and bacteria level as Class variables, as well as the interaction between these two factors. Statistically significant interaction between bacteria and $CuSO_4$ treatment levels was taken as an indication that $CuSO_4$ treatment may have increased disease susceptibility.

4.3.2 Copper sulphate exposure and larvae behaviour

In a separate trial run in conjunction with $CuSO_4$ exposure and bacterial challenge, larvae behaviour was assessed in 4.5 ml (dimensions: 12.5 x 12.5 x 45 mm) spectrophotometer cuvettes (Sigma-aldrich, St. Louis, USA) in response to exposure to the same concentrations of $CuSO_4$ used above for a five exposure periods from 0.5 h to 54 h. All treatment combinations were conducted in triplicate, which made a total of 120 replicates (8-$CuSO_4$ (including control) x 5-exposure periods x 3-replicates). Two measurements of behaviour were made: (1) the number of larvae swimming in the WC above the floor of the cuvette expressed as a percentage of the total population (% SL); and (2) the number of larvae crossing a line in the middle of the cuvette, whether ascending or descending, expressed as a percentage of the total population (% CL).

The protocol for assessment of larvae behaviour was adopted from Prael et al 2001. Larvae were obtained from the same batch as the previous trial. Larvae were suspended in $CuSO_4$ solutions in cuvettes at a density of 20-40/ ml and kept in a Styrofoam box in which the cuvettes were originally purchased, and stored in the dark at 25^{o}C. At each exposure time three replicates belonging to a single treatment were carefully removed and placed in position to film in-situ behavior for 2 min. Filming was conducted using a

Canon digital camera (PowerShot A720 IS) on movie function. Visual clarity was improved with use of a candescent beam of light projected from the side of the cuvettes. A still image of a movie is given in Appendix 10. Filming took approximately one hour for all replicates to be assessed. Hence the assessment times indicated above represent the time that filming commenced. All treatments were filmed beginning with the lowest $CuSO_4$ concentration and moving through to the highest concentration. After filming, the movies were downloaded to a PC computer and each replicate was assessed for the % SL and % CL. Full counts of larvae in each cuvette were made at the conclusion of the trial so that % SL and % CL could be calculated accurately. Statistical analyses were conducted using SAS GLM (SAS software (version 9.2) general linear model (GLM)) and included modeling of main effects of $CuSO_4$ level and exposure time, as well as the interaction between these two factors.

4.4 Results

4.4.1 Copper sulphate exposure followed by bacterial challenge

The effect of $CuSO_4$ exposure and bacterial challenge on mortality rates is represented in Figure 14 (A-F), where percentage mortality was plotted against $CuSO_4$ treatment levels. Statistical significance values given in the figure relate to the interaction between $CuSO_4$ and bacterial challenge, although the main effects of both variables were highly significant. In brief, $CuSO_4$ level and bacteria level positively correlated with mortality. In the no-bacteria control, there appeared to be a lethality threshold at approximately 60-100 ppb $CuSO_4$ in both 24 h and 48 h treatments. The rise in mortality levels with increasing $CuSO_4$ levels was more gradual in the 24 h treatment, reaching 26% mortality at the maximum dose of 3,000 ppb $CuSO_4$, while the 48 h treatment reached 80% mortality at the maximum $CuSO_4$ dose. Interaction between $CuSO_4$ treatment and bacterial challenge was significant for four of the six analyses: 48 h exposure followed by *V. pomeroyii* challenge (see Figure 14 (B), $Pr = 0.0018$); 24 h exposure followed by *P. fluorescens* challenge (see Figure 14 (E), $Pr < 0.0001$); 48 h exposure followed by *S. sciuri* challenge

(see Figure 14 (D), Pr = 0.0279); and 24 h treatment followed by *S. sciuri* challenge (see Figure 14 (C), Pr = 0.0279), although the latter two were of marginal significance.

The strongest interactions between $CuSO_4$ and bacterial challenge were seen in Figure 14 (B and E) and these had a similar characteristic response to $CuSO_4$ stress with at least one level of bacteria (all three levels in *Vibrio* and 5 log CFU/ml in *Pseudomonas*). The characteristic response curve under bacterial challenge showed a general increase in mortality with increasing $CuS0_4$ concentrations but a decrease in mortality at 100 ppb, while the no-bacteria control showed no increase in mortality until 60-100 ppb. In Figure 14 (E) the 5 log CFU/ml challenge followed the characteristic response described, whereas the 6 and 7 log CFU/ml challenge did not exhibit an increase in mortality at low $CuSO_4$ levels, as mortality was near 100% even with no $CuSO_4$ treatment, although there was a sharp decrease in mortality at 100 ppb.

Marginal interactions, seen in Figure 14 (C and D) reflected similar trends in a less consistent way. Similarly to the 6 and 7 log CFU/ml challenge of Figure 14 (E and C) had high mortality, greater than 90%, in 6 log and 7 log CFU/ml challenges in the $CuSO_4$ control and lower levels of $CuSO_4$; followed by a sharp decrease in mortality in the 7 log CFU/ml challenge at 100 ppb $CuSO_4$ and a sharp decrease in mortality at 30 ppb $CuSO_4$ in the 5 log CFU/ml challenge. Figure 14 (D) showed an increase in mortality from 10 ppb to 60 ppb followed by a very slight decrease in mortality at 100 ppb, in the 7 log CFU/ml challenge.

4.4.2 Copper sulphate exposure and larvae behaviour

Larvae behaviour in response to different $CuSO_4$ concentrations was plotted in Figure 15 (A and B for % SL and % CL, respectively). Statistical analysis indicated that larvae behaviour was significantly affected by $CuSO_4$ exposure (% SL: $Pr < 0.0001$; % CL: $Pr < 0.0001$) and the duration of exposure (% SL: $Pr < 0.0001$; % CL: $Pr < 0.0001$). The main trends were an increase in larvae behaviour with increasing exposure duration, even in the no-$CuSO_4$ control, and a decrease in mobility with increasing $CuSO_4$ levels. However, since the interaction between $CuSO_4$ level and exposure time was also significant (% SL:

Pr < 0.0001; % CL: Pr < 0.0001), it appears that the effect of $CuSO_4$ may have been dependent upon exposure time. The interaction between $CuSO_4$ and exposure time is evident in Figure 15 (A and B) by the different shaped curves of different time series. Apart from the assessments at 0.5 h in % SL, and 0.5 h and 13 h in % CL, the general shape of the response curve was a reduction in activity at 10 ppb $CuSO_4$ followed by a plateau or increase in activity from 10 ppb to 100 ppb followed by a further decline in activity at higher levels. The different time series appear to have been most divergent at 30 ppb to 100 ppb $CuSO_4$ where activity levels increased in 30 ppb to 100 ppb treatments with longer exposure times, in particular in the longest exposure of 54 h, which had much elevated larvae mobility at 60 to 100 ppb compared to other time series in both % SL and % CL. In addition the reduction in larvae mobility at 10 ppb $CuSO_4$, compared to the control, appears to have been more substantial with increasing exposure duration.

Sensitivity of larvae to 10 ppb $CuSO_4$ was further evidenced by inclusion of LSD analysis of larvae behaviour as reported in Table 8. Because of the interaction between $CuSO_4$ level and duration of exposure the LSD needs to be interpreted cautiously and so has been used here only to look at the difference between the control and 10 ppb $CuSO_4$. The % SL, averaged over all time series, was significantly higher in the no-$CuSO_4$ control than the 10 ppb (21.45% and 14.89%, respectively) while the % CL was not significantly different, although Figure 15 (B) indicates that the reduction in activity at 10 ppb was only experienced by larvae at longer exposure times.

4.5 Discussion

Oyster larvae susceptibility to disease increased upon exposure to Cu at sub-lethal levels, although in an inconsistent manner, at levels as low as 10 ppb $CuSO_4$ (2.5 ppb Cu). In those stress-bacteria combinations in which the interaction between $CuSO_4$ level and bacterial level was highly significant (Figure 14(B and E)), a characteristic response curve was evident: mortality increased from 0 to 60 ppb $CuSO_4$, and then decreased at the lethality threshold of the no-bacteria control (100 ppb), before increasing again at higher levels. In those stress-bacteria combinations in which the interaction was marginally

significant (Figure 14(C and D)), some evidence of increased mortality at sub-lethal $CuSO_4$ levels and decreased mortality at the lethality threshold was also evident, albeit inconsistently. Two stress-bacteria combinations (Figure 14(A and F)) showed no significant interaction between $CuSO_4$ stress and bacteria levels.

It is not clear why the interaction between Cu stress and bacterial challenge level was not consistent. The interaction was not unique to 24 h or 48 h exposures nor favored by particular bacterial species. Such inconsistency indicates that the effect of Cu in increasing disease susceptibility at sub-lethal levels may not have been substantial. However, disease is a physiological endpoint in a complex interplay of factors involving environment, larvae and pathogen, in which Cu induced stress is but one factor that may tip the balance towards a disease outcome of such magnitude that is not simply explained by additive effects of Cu; i.e. something akin to "the butterfly effect". In any case, the effect of Cu stress does not need to be reliably reproduced under all sets of laboratory conditions to establish that sub-lethal levels of Cu may increase larvae susceptibility to disease in the hatchery environment. A small increase in disease incidence may be amplified under hatchery conditions where a reduction in swimming activity caused by disease results in dense aggregations of larvae at the tank bottom, which has been shown to favor contagious spread of disease (DiSalvo et al 1978).

Reduction in mortality at the lethality threshold of 100 ppb $CuSO_4$ relative to lesser concentrations of $CuSO_4$ indicates that the effect of Cu was not simply additive. Such a response may be explained by what has been termed, somewhat misleadingly, as the "heat shock response"; which is a pro-survival strategy occurring at the cellular level in response to sub-lethal stress. The heat shock response involves an increase in the number of cell membrane bound proteins known as molecular chaperones or heat shock proteins (HSP), which assist in the folding, assembly and transport of nascent proteins (Gething & Sambrook 1992) and also function as cellular defences, aiding in the refolding and removal of proteins denatured by biotic and abiotic stress (Sanders 1993). These activities provide a transient protective effect against a variety of different stressors (Hochachka & Somero 2002). Following the discovery of the heat shock phenomena it has since been recognized that many stimuli, other than heat stress, can activate the heat shock response,

including oxidative stress and heavy metals (Fulda et al 2010). Miller et al (2003) demonstrated that Cu could induce HSP production in Sea anemone (*Anemonia viridis*). The induction of the heat shock response has been demonstrated in oyster larvae (Brown et al 2004; Ueda & Boettcher 2009) and although the induction of heat shock response by heavy metal exposure has not been investigated in oyster larvae, it appears a likely explanation of the observed reduction in larvae mortality occurring at 100 ppb $CuSO_4$ in the present study.

Assessment of larvae behaviour provided further evidence that larvae were sensitive to sub-lethal levels of Cu as low as 10 ppb $CuSO_4$, which was 10 times lower than the lethality threshold. The high sensitivity of larvae behaviour towards Cu has led other researchers to suggest that larvae behaviour could be used as a biomarker for assessment of sub-lethal effects of toxicants (Beiras & His 1994; His et al 1999; Prael et al 2001). A standardised larvae behaviour protocol could be useful in commercial hatcheries for testing of water quality under different treatment regimes. In feasibility terms monitoring of larvae behaviour need not be more complex than assessment of embryogenesis and is simple enough for application in a commercial hatchery. Behaviour can be assessed in a similar time frame of 24 h to 48 h and does not require feeding of larvae, and so could be easily standardized. Hence the value of swimming behaviour as a biomarker would depend upon demonstration that it is at least as sensitive as embryogenesis. Indications from the present study are that swimming behaviour may be comparable to embryogenesis in terms of sensitivity. Altered swimming behaviour was observed at 10 ppb $CuSO_4$ (2.5 ppb Cu), although lower levels were not tested, while other studies have indicated a range of Cu concentrations for embryogenesis as indicated in Table 7 adapted from His et al (1999). The average EC50[2] for Cu was 22 ppb, although values ranged from 2.72 through to 130 ppb. Thus it appears that swimming behaviour may be at least as sensitive as embryogenesis.

[2] EC50: Effective concentration - represents the concentration of a compound where 50% of the population exhibit a response

Table 7 – Concentrations of Cu causing abnormal embryogenesis in *C. gigas*

Exposure conditions (time, temperature, salinity, density, food, seawater)	EC50 (ppb Cu)	Reference and notes
27^{o}C	32-100	Okubo and Okubo (1962); $CuSO_4$
48 h, 20^{o}C, 33 psu, 28-38 ml^{-1}, 1 µm FSW[A] UV sterilized	10	Coglianese and Martin (1981); $CuNO_3$; abnormal larvae excluded
48 h, 25^{o}C, 34-35 psu, 5 µm FSW	5.3	Martin et al (1981); $CuSO_4$; abnormal larvae excluded
48 h, 26^{o}C, 28 psu, 30 ml^{-1}, 0.8 µm FSW	130[B]	His and Robert (1981); abnormal larvae excluded
24, 48 h, 24^{o}C, 32 PSU, 20 ml^{-1}, 0.2 FSW	13	His and Robert (1982), abnormal larvae excluded $CuSO_4$
48 h, 20^{o}C, 28-36 ml.$^{-1}$, 1 µm FSW uv sterilised	10	Coglianese (1982); $CuNO_3$; abnormal larvae excluded
24 h, 24^{o}C, 20, 25, 30 psu, 0.2 µm FSW	5-6.5	Robert and His (1985); abnormal larvae excluded
32 h, 20^{o}C, 35 psu, FSW (pore size not given)	2.72	Worboys et al (2002)

(A) FSW – Filter sterilised water. (B) Extrapolated data

Research into oyster larvae behaviour in response to heavy metals appears to be somewhat conflicting in terms of the effect that heavy metal stress has on activity levels. Beiras and His (1994) observed inhibition of swimming activity in veliger larvae at a nominal 8 ppb Hg. Conversely His and Seaman (1999) observed an increase in activity of veliger larvae when exposed to 16 ppb Hg. Similarly Prael et al (2001) observed an increase in activity in response to heavy metal (copper-chrome-arsenic) leachate. Beiras and His (1994) noted that larvae behaviour in experimental vessels changes with larvae age and therefore urged caution in interpretation of behaviour experiments. The differential behavioural responses to different concentrations of Cu at different time periods of assessment noted in the present study (Figure 15) may provide an explanation for the differences noted in previous

research. In any case, in relative terms to a control, an increase or decrease in activity in response to a chemical may indicate stress.

The present study contributes to previous research on the development of larvae behaviour as a stress biomarker by indicating that when testing a potential stressor or water of suspect quality (1) testing should be conducted over a range of concentrations given that larvae mobility may decrease at sub-lethal levels and increase near the toxicity threshold (when testing environmental water quality, a concentration range can be achieved through dilution); and (2) the time frame of assessment is important and tests should be conducted over 24 h to 48 h (longer timeframes may increase sensitivity as the response to Cu appeared to be greatest at the longest time period of 54 h). Further research and development is required before a behaviour protocol can be recommended. Research should include a range of different toxicants and larvae behaviour should be compared to currently used biomarkers such as larvae growth and embryogenesis.

Further development of biomarker assessment may provide a simpler and more effective assessment of larvae stress but the relevance of such biomarker assessments to susceptibility of larvae to bacterial disease is yet to be established. Research into the effect of stress on biomarkers and susceptibility of larvae to bacterial attack may identify biomarkers that can be used to indicate heightened susceptibility to bacterial disease for application in research and potentially in commercial hatcheries. Such biomarkers may include existing biomarkers such as larvae behaviour and growth, and biochemical biomarkers as identified in Table 6, but characterisation of the stress response should not be limited to these and in particular inclusion of immunological defence activity may be advantageous in identifying new biomarkers. Among physiological processes possibly disturbed by pollutants, the immune system is likely to be one of the more sensitive (Fournier et al 2000) and is likely to be most relevant to incidence of disease.

4.6 Conclusion

Exposure of oyster larvae to sub-lethal levels of Cu may increase larvae susceptibility to bacterial attack and such exposure may be indicated by behavioural changes. These

behavioural changes could be used as a biomarker for water quality testing. Assessments of behaviour should include a range of toxicant concentrations (or dilutions of suspect water) and be conducted over a 24-48 h period. At the Cu lethality threshold increased larvae activity and improved performance under bacterial challenge indicates induction of the heat shock response in oyster larvae, which has not previously been demonstrated using heavy metal exposure.

Table 8 – LSD grouping of larvae behaviour comparing the control and 1 log ppb $CuSO_4$ treatment

Percentage of larvae swimming (% SL)				**Percentage of larvae crossing the line (% CL)**			
$CuSO_4$ level	**Mean**	**N**	**LSD Grouping**	**$CuSO_4$ level**	**Mean**	**N**	**LSD Grouping**
0	21.45	15	A	0	19.9	15	A
10	14.89	15	B	10	16.31	15	A
LSD = 2.17. Pr < 0.0001				LSD = 3.83. Pr < 0.0001			

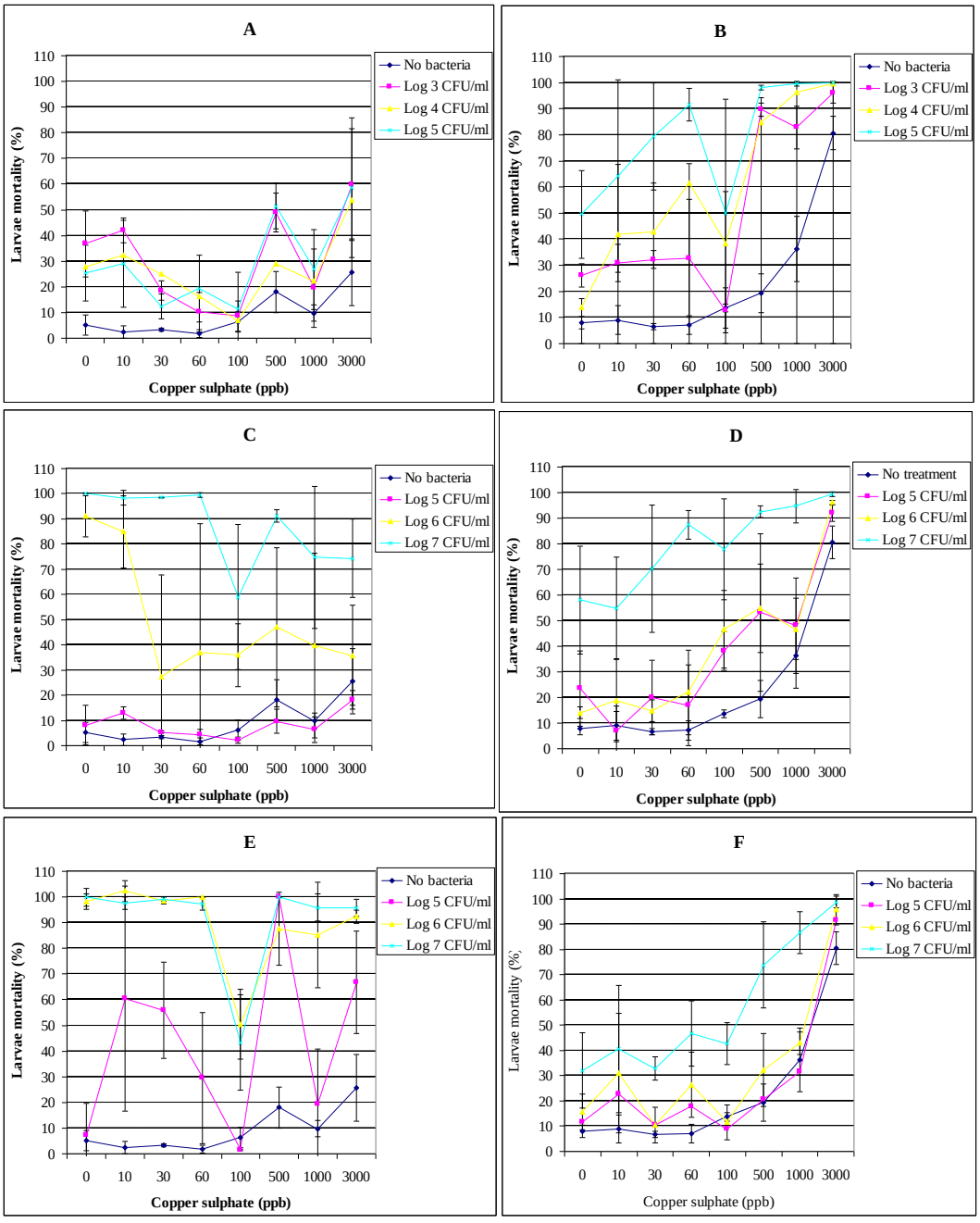


Figure 14 – Larvae mortality in response to 24 or 48 h $CuSO_4$ exposure followed by bacterial challenge. P = Interaction between $CuSO_4$ concentration and bacteria challenge: A: 24 h exposure and *V. pomeroyi* (Pr = 0.5238). B: 48 h and *V. pomeroyi* (Pr = 0.0018). C: 24 h and *S. sciuri* (Pr = 0.0296). D: 48 h and *S. sciuri* (Pr = 0.0279). E: 24 h and *P. fluorescens* (Pr < 0.0001). F: 48 h and *P. fluorescens* (Pr = 0.117).

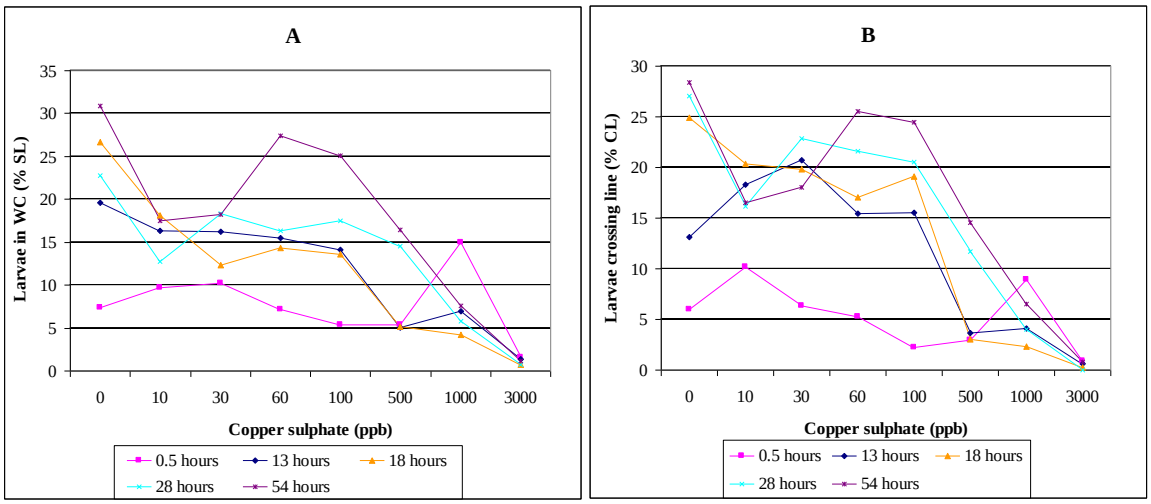


Figure 15 – Larvae swimming behavioural response to different periods of $CuSO_4$ exposure. A: % SL (Pr (interaction $CuSO_4$ concentration and exposure time) < 0.0001). B: % CL (Pr < 0.0001)

5 Synopsis

The investigation undertaken in this study into the occurrence of epizootics of oyster larvae at the Bicheno hatchery began with the hypothesis that disease incidence is characterised by the presence or absence of particular predominant bacterial groups in the larvae tank (hypothesis A; Table 1). To address this hypothesis, a monitoring study was undertaken using microbiological tools to study whole microbial communities (16S rRNA gene clone libraries and TRFLP analysis) in the larvae tank and tank inputs under commercial conditions. A total of eight production runs were monitored, seven of which terminated in epizootics. It was not originally planned to monitor as many as eight runs but additional monitoring was undertaken in hope of gaining more samples from successful runs for comparison with unsuccessful runs. Whilst this was not achieved the inclusion of additional runs provided a large data-set enabling a greater understanding of the microbial ecology of the larvae tank and statistical rigour in analysis of independent variables, which was especially important given the unbalanced data-set resulting from the uncontrolled nature of this observational study.

In Chapter 3 it was shown that there was no predominant characteristic microbial community in the larvae or tank water associated with disease symptoms characteristic of bacterial disease and hence hypothesis A was negated. Furthermore no species with any pathogenic record were identified in any compartments of the larvae tank. Through daily monitoring of microbial abundance in three production runs it was shown that *Vibrio* spp. experienced peaks in relative abundance in the larvae and WC in association with emergence of disease symptoms. *Vibrio* spp. reached as high as 100% of the total culturable population, but was not consistently associated with peaks in total *Vibrio* abundance in larvae or WC. This result explains anecdotal evidence from the hatchery that disease was not consistently associated with *Vibrio* abundance in the WC and hence it is recommended that hatchery staff monitor TVC counts in addition to *Vibrio* abundance for relative abundance.

Despite being relatively abundant in the culturable fraction of the microbial population *Vibrio* spp. were not detected in clone libraries of diseased and moribund larvae and other compartments of the larvae tank. It was acknowledged that non-detection of *Vibrio* spp. may have resulted from primer-template mismatch in PCR reactions. The primer-set used was not selected to target *Vibrio* spp. because the focus was on the whole microbial community as justified in the introduction of Chapter 3. To address this potential bias analysis of larvae tank samples including diseased land moribund arvae was undertaken using a different primer set and NGS, which confirmed that *Vibrio* were only a minor component of the microbial community. Although representing only a minor component of the total population *Vibrio* abundance in association with disease incidence was approximately 4 log CFU/ml in the WC, which has been shown by other researchers to be sufficient to cause bacillary necrosis. Thus *Vibrio* spp. may be suspected of having caused disease in monitored epizootics. It was recommended that future studies employ microbiological tools able to characterise minor components of the microbial population including *Vibrio* spp. in particular.

Larvae aggregative behaviour or "swarming" was the first disease symptom observed in 80% of disease incidents and occurred in the absence of any other disease symptoms or peaks in *Vibrio* abundance, although *Vibrio* abundance in larvae and WC increased following swarming incidents. It was argued that swarming could create conditions of locally high larvae-densities in association with tank-bottom detritus that may favour pathogen success. Swarming may have been due to a non-microbiological primary cause of disease, or a microbiological etiology that occurred below the detection limits of TRFLP and 16S rRNA gene clone libraries.

In Chapter 2, it was shown that all compartments of the larvae tank (larvae, biofilm and water column) were distinctly different microbial niches but all similarly dominated by members of class *Alphaproteobacteria*, primarily members of the *Roseobacter* clade. This study demonstrated the existence of three distinctly different microbial niches within the larvae tank and significant seasonal variation in both larvae-associated and WC microbial communities. The larvae indigenous microbial community, formed within hours of fertilisation, persisted throughout much of the larval period. The presence of predominant

non-typical marine species, *Sphingomonas paucimobilis*$_{(97\%)}$ and *Ramlibacter tataouinensis*$_{(93\%)}$, in eggs and larvae samples indicated a non-marine source of contamination occurring during or prior to fertilisation. Thus formation of the indigenous microbial community during spawning and fertilisation may be a control point for management of the microbial composition of the larvae.

As hypothesis A was negated, and larvae aggregative behaviour indicated the possibility of a non-microbiological primary cause of disease, it was hypothesized that disease incidence may have been caused by exposure to some toxin that predisposed larvae to bacterial attack either directly by compromising immune function or indirectly by altering larvae behaviour and causing swarming (hypothesis B; Table 1). Copper was examined in Chapter 4 as a potential toxin because nearby sources of copper were identified (boatslip in Figure 1) and because copper can persist in sediment. A key objective was development of an experimental protocol that could be used to assess other potential toxins or suspect hatchery water. It was shown that exposure to sub-lethal levels of copper could increase susceptibility of larvae to bacterial attack under certain conditions. It was further shown that sub-lethal copper levels induced behavioural changes in larvae, which might account for swarming behaviour. Thus hypothesis B was supported.

Behavioural changes occurring upon exposure to sub-lethal levels of copper as low as 2.5 ppb indicated that larvae behaviour might be a highly sensitive biomarker of copper contamination and potentially other toxins. Ease of application and sensitivity may make the behaviour assessment protocol useful for water quality monitoring in hatchery and research applications. The results indicated that behavioural assessments should be made using a broad range of concentrations across a 24 to 48 h time period.

Based on the findings of this thesis, the following recommendations are made for both hatchery management and further research.

Key findings for hatchery management:

1. Care should be taken during spawning and fertilisation to reduce the possibility of contamination by non-marine bacterial species. Sources may include fresh water, hatchery floor (through splashing), implements, buckets, tanks and broodstock.

The indigenous microbial community formed on eggs and larvae at this time is resistant to change and might relate to incidence of disease.

2. Daily monitoring of *Vibrio* levels expressed as a percentage of the total culturable population (*Vibrio*/TVC) may be more useful than *Vibrio* levels alone. Absence of a *Vibrio*/TVC growth spike during incidence of disease might indicate a non-microbiological cause.
3. Aggregative behaviour in the absence of any other disease symptoms or *Vibrio*/TVC peaks might be caused by a non-microbiological cause such as a toxin in the culture water.
4. Intensive culture conditions (IC tank) may favour *Vibrio* spp.; although it is uncertain how this may impact upon incidence of disease.

Recommendations for research:

5. Larvae behaviour may be a sensitive and effective biomarker for assessment of sub-lethal levels of toxins and water quality testing. Behaviour assessment should include a range of toxin concentrations and be evaluated over 24 to 48 h. Further research should include a range of different toxicants and larvae behaviour should be compared to currently used biomarkers such as larvae growth and embryogenesis so that a standard protocol can be recommended.
6. Future studies on bacterial communities should include microbiological tools capable of high resolution so that populations such as *Vibrio* spp. can be detected.

6 Appendix

Appendix 1 – Clone library details including coverage and species diversity

Production run	Sample source	Sample name and date (_dd/mm/yr)	No. of clones	No. of phylotypes	Coverage (%)	Chao-1 estimator (StDev)	Phylogenetic diversity: Faith's index
170909	Larvae	L_260909	90	17	0.91	27.7 (+/-7.9)	64
	Larvae	D_031009	114	14	0.96	18.2 (+/-4)	72
	Biofilm	B_031009	58	15	0.84	35.3 (+/-14)	70
070909	Egg	E_070909	65	11	0.91	29 (+/-15.2)	74
	Algae	A_150909	64	14	0.95	15.5 (+/-1.9)	61
	CTW	C_150909	74	29	0.78	93 (+/-33)	103
	Biofilm	B_160909	92	15	0.93	33 (+/-15.2)	74
	Larvae	D_150909	88	10	0.94	16.3 (+/-5.9)	54
	Larvae	L_140909	83	22	0.88	32 (+/-6.7)	91
	Larvae	L_160909	75	18	0.92	27 (+/-7.7)	90
	WC	W_140909	94	20	0.88	80.5 (+/-37.7)	80
	WC	W_160909	102	21	0.90	71 (+/-32.6)	87
280109	Larvae	L_030209	62	10	0.89	22.3 (+/-9.6)	47
	Larvae	L_050209	40	4	0.98	4.5 (+/-1.1)	24
	Biofilm	B_100209	93	34	0.81	74.5 (+/-19.8)	117
	Larvae	D_100209	73	15	0.90	23.2 (+/-6.5)	71
-	Algae	A_101209	56	17	0.88	21.1 (+/-3.4)	78
	Algae	A_200110	67	11	0.94	15 (+/-4.3)	66

Appendix 2 – Phylotypes identified in the hatchery

ID	Species (97%) or Genbank nearest match (Accession No.)	Genbank Accession No.	Genus (93%)	Family (90%)	Order	Class	Phylum
A120	*Microbacterium oxydans*	JX486658	*Microbacterium*	*Microbacteriaceae*	*Actinomycetales*	*Actinobacteria*	*Actinobacteria*
A152	*Propionibacterium acnes*	JX486659	*Propionibacterium*	*Propionibacteriaceae*	*Actinomycetales*	*Actinobacteria*	*Actinobacteria*
A162		JX486649	*Salinicola*	*Micrococcineae*	*Actinomycetales*	*Actinobacteria*	*Actinobacteria*
A58	*Demequina aestuarii*	JX486656	*Demequina*	*Cellulomonadaceae*	*Actinomycetales*	*Actinobacteria*	*Actinobacteria*
A9	*Salinibacterium amurskyense*	JX486655	*Salinibacterium*	*Micrococcineae*	*Actinomycetales*	*Actinobacteria*	*Actinobacteria*
SA5		JX486652	*Salinicola*	*Micrococcineae*	*Actinomycetales*	*Actinobacteria*	*Actinobacteria*
A114	Seawater clone JF488663	JX486657				*Actinobacteria*	*Actinobacteria*
A159	Soil clone JN409139	JX486660				*Actinobacteria*	*Actinobacteria*
A102		JX486624		*Cryomorphaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A104		JX486625	*Winogradskyella*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A14	*Polaribacter dokdonensis*	JX486608	*Polaribacter*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A141	*Marixanthomonas ophiurae*	JX486628	*Marixanthomonas*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A151	*Gramella echinicola*	JX486630	*Gramella*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A155	*Chryseobacterium gleum*	JX486631	*Chryseobacterium*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A17	*Croceibacter atlanticus*	JX486609	*Croceibacter*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A179		JX486634	*Mesoflavibacter*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A27	*Dokdonia donghaensis*	JX486611	*Dokdonia*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A31		JX486570	*Arenibacter*	*Flavobacteriacea*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A34		JX486612	*Tenacibaculum*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A46	*Muricauda ruestringensis*	JX486614	*Muricauda*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A47	*Kordia algicida*	JX486615	*Kordia*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A6		JX486607	*Brumimicrobium*	*Cryomorphaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A64		JX486617	*Mesoflavibacter*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A71		JX486619		*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*

ID	Species (97%) or Genbank nearest match (Accession No.)	Genbank Accession No.	Genus (93%)	Family (90%)	Order	Class	Phylum
A82		JX486621	*Tenacibaculum*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A88	*Algibacter mikhailovii*	JX486622	*Algibacter*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A107	Seawater clone FN433392	JX486626				*Flavobacteria*	*Bacteroidetes*
A118	Seawater clone GQ274082	JX486627				*Flavobacteria*	*Bacteroidetes*
A15	Seawater clone JF827568	JX486635				*Flavobacteria*	*Bacteroidetes*
A166	Uncultured Bacteroidetes AY580686	JX486636				*Flavobacteria*	*Bacteroidetes*
A170	Seawater clone FR648180	JX486632				*Flavobacteria*	*Bacteroidetes*
A172	Soil clone JN122782	JX486633				*Flavobacteria*	*Bacteroidetes*
A22	Seawater clone JN639288	JX486610				*Flavobacteria*	*Bacteroidetes*
A43	Seawater clone GC385400	JX486613				*Flavobacteria*	*Bacteroidetes*
A55	Uncultured Bacteroidetes AY225660	JX486616				*Flavobacteria*	*Bacteroidetes*
A65	Seawater clone FJ202704	JX486618				*Flavobacteria*	*Bacteroidetes*
A72	Seawater clone GQ325429	JX486620				*Flavobacteria*	*Bacteroidetes*
A96	Seawater clone HQ163249	JX486623				*Flavobacteria*	*Bacteroidetes*
SB14	Seawater clone JF683452	JX486629				*Flavobacteria*	*Bacteroidetes*
A50	*Cytophaga marinoflava*	JX486637	*Cytophaga*	*Cytophagaceae*	*Sphingobacteriales*	*Sphingobacteria*	*Bacteroidetes*
A183	Seawater clone HQ166790	JX486675				*Bacteroidetes*	*Bacteroidetes*
A89	Seawater clone HM437477	JX486670				*Bacteroidetes*	*Bacteroidetes*
A200		JX486676	*Prochlorococcus*	*Synechococcaceae*	*Synechococcales*	*Cyanobacteria*	*Cyanobacteria*
A109	*Listeria monocytogenes*	JX486665	*Listeria*	*Listeriaceae*	*Bacillales*	*Bacilli*	*Firmicutes*
A149	*S. sciuri*	JX486661	*Staphylococcus*	*Staphylococcaceae*	*Bacillales*	*Bacilli*	*Firmicutes*
A29	*Brochothrix campestris*	JX486667	*Brochothrix*	*Listeriaceae*	*Bacillales*	*Bacilli*	*Firmicutes*
A123	*Lactobacillus divergens*	JX486666	*Lactobacillus*	*Lactobacillaceae*	*Lactobacillales*	*Bacilli*	*Firmicutes*
A129	*Lactococcus piscium*	JX486662	*Lactococcus*	*Streptococcaceae*	*Lactobacillales*	*Bacilli*	*Firmicutes*
A135	*Vagococcus fluvialis*	JX486663	*Vagococcus*	*Enterococcaceae*	*Lactobacillales*	*Bacilli*	*Firmicutes*

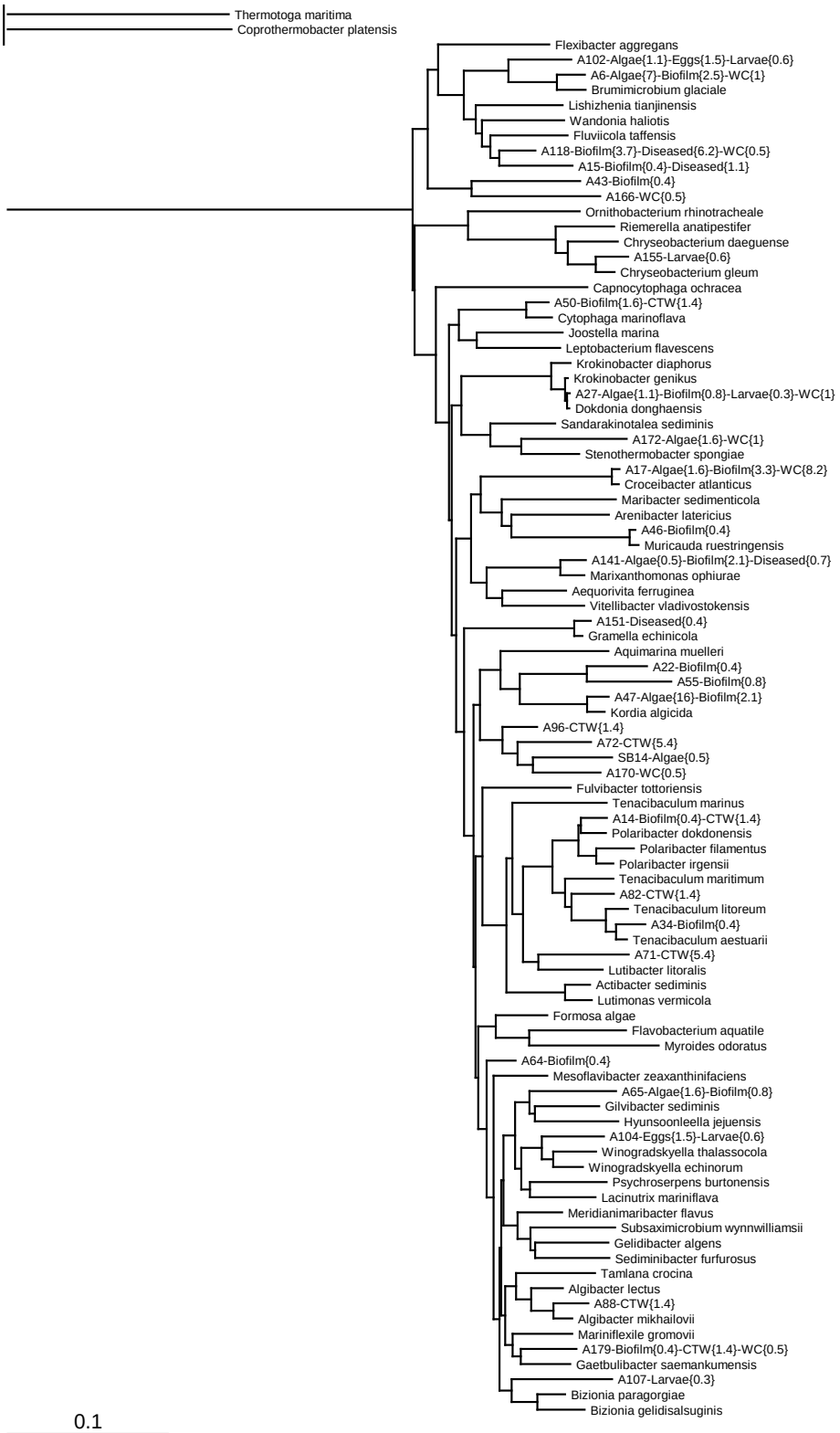
ID	Species (97%) or Genbank nearest match (Accession No.)	Genbank Accession No.	Genus (93%)	Family (90%)	Order	Class	Phylum
A97	*Lactobacillus maltaromicus*	JX486664	*Lactobacillus*	*Lactobacillaceae*	*Lactobacillales*	*Bacilli*	*Firmicutes*
A127	Seawater clone AB429662	JX486673				*Planctomycetacia*	*Planctomycetes*
A79	Seawater clone GU327810	JX486672				*Planctomycetacia*	*Planctomycetes*
A125		JX486589		*Aurantimonadaceae*	*Rhizobiales*	*Alphaproteobacteria*	*Proteobacteria*
A130		JX486590		*Aurantimonadaceae*	*Rhizobiales*	*Alphaproteobacteria*	*Proteobacteria*
A168	*Martelella mediterranea*	JX486598	*Martelella*	*Aurantimonadaceae*	*Rhizobiales*	*Alphaproteobacteria*	*Proteobacteria*
A20		JX486566	*Hoeflea*	*Phyllobacteriaceae*	*Rhizobiales*	*Alphaproteobacteria*	*Proteobacteria*
A25	*Hoeflea marina*	JX486568	*Hoeflea*	*Phyllobacteriaceae*	*Rhizobiales*	*Alphaproteobacteria*	*Proteobacteria*
A10	*Ruegeria mobilis*	JX486563	*Ruegeria*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A103		JX486586	*Roseisalinus*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A12	*Shimia marina*	JX486564	*Shimia*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A122	*Seohicola saemankumensis*	JX486588	*Seohicola*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A124	*Thalassobacter oligotrophus*	JX486587	*Thalassobacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A158		JX486597	*Thalassobacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A174	*Maritalea myrionectae*	JX486599	*Maritalea*	*Hyphomicrobiaceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A177		JX486601	*Paracoccus*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A181	*Roseobacter denitrificans*	JX486600	*Roseobacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A26	*Oceanibulbus indoliflex*	JX486569	*Oceanibulbus*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A32		JX486571		*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A36	*Tropicibacter naphthalenivorans*	JX486572	*Tropicibacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A48	*Antarctobacter heliothermus*	JX486574	*Antarctobacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A49		JX486575	*Roseovarius*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A56	*Roseovarius nubinhibens*	JX486576	*Roseovarius*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A68		JX486579	*Roseovarius*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*

ID	Species (97%) or Genbank nearest match (Accession No.)	Genbank Accession No.	Genus (93%)	Family (90%)	Order	Class	Phylum
A69		JX486577	*Sulfitobacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A70		JX486578	*Sulfitobacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A73	*Nautella italica*	JX486580	*Nautella*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A76		JX486584	*Marivita*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A85		JX486583	*Thalassobacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A99	*Phaeobacter arcticus*	JX486585	*Phaeobacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
SA2	*Marivita cryptomonadis*	JX486594	*Marivita*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
SB32	*Sulfitobacter donghicola*	JX486595	*Sulfitobacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
SB5		JX486654	*Marinicella*	*Hyphomonadaceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A2	*Thalassospira lucentensis*	JX486559	*Thalassospira*	*Rhodospirillaceae*	*Rhodospirillales*	*Alphaproteobacteria*	*Proteobacteria*
A1	*Erythrobacter aquimaris*	JX486558	*Erythrobacter*	*Erythrobacteraceae*	*Sphingomonadales*	*Alphaproteobacteria*	*Proteobacteria*
A139		JX486591	*Sphingomonas*	*Shingomonadaceae*	*Sphingomonadales*	*Alphaproteobacteria*	*Proteobacteria*
A144	*Sphingomonas paucimobilis*	JX486592	*Sphingomonas*	*Shingomonadaceae*	*Sphingomonadales*	*Alphaproteobacteria*	*Proteobacteria*
A145	*Sphingomonas aestuarii*	JX486593	*Sphingomonas*	*Shingomonadaceae*	*Sphingomonadales*	*Alphaproteobacteria*	*Proteobacteria*
A184	*Citromicrobium bathoceanense*	JX486602	*Citromicrobium*	*Shingomonadaceae*	*Sphingomonadales*	*Alphaproteobacteria*	*Proteobacteria*
A39		JX486573		*Shingomonadaceae*	*Sphingomonadales*	*Alphaproteobacteria*	*Proteobacteria*
A5		JX486562	*Sphingopyxis*	*Shingomonadaceae*	*Sphingomonadales*	*Alphaproteobacteria*	*Proteobacteria*
SB51		JX486596		*Erythrobacteraceae*	*Sphingomonadales*	*Alphaproteobacteria*	*Proteobacteria*
A16	Seawater clone AY664177	JX486565				*Alphaproteobacteria*	*Proteobacteria*
A74	Seawater clone GQ350388	JX486581				*Alphaproteobacteria*	*Proteobacteria*
A75		JX486582				*Alphaproteobacteria*	*Proteobacteria*
A100		JX486603	*Ramlibacter*	*Comamonadaceae*	*Burkholderiales*	*Betaproteobacteria*	*Proteobacteria*
A59	*Janthinobacterium lividum*	JX486605	*Janthinobacterium*	*Oxalobacteraceae*	*Burkholderiales*	*Betaproteobacteria*	*Proteobacteria*
SA53	*Ralstonia pickettii*	JX486606	*Ralstonia*	Burkholderiaceae	Burkholderiales	*Betaproteobacteria*	Proteobacteria
A126	*Iodobacter fluviatilis*	JX486604	*Iodobacter*	Neisseriaceae	Neisseriales	*Betaproteobacteria*	Proteobacteria
A167		JX486674				*Deltaproteobacteria*	Proteobacteria

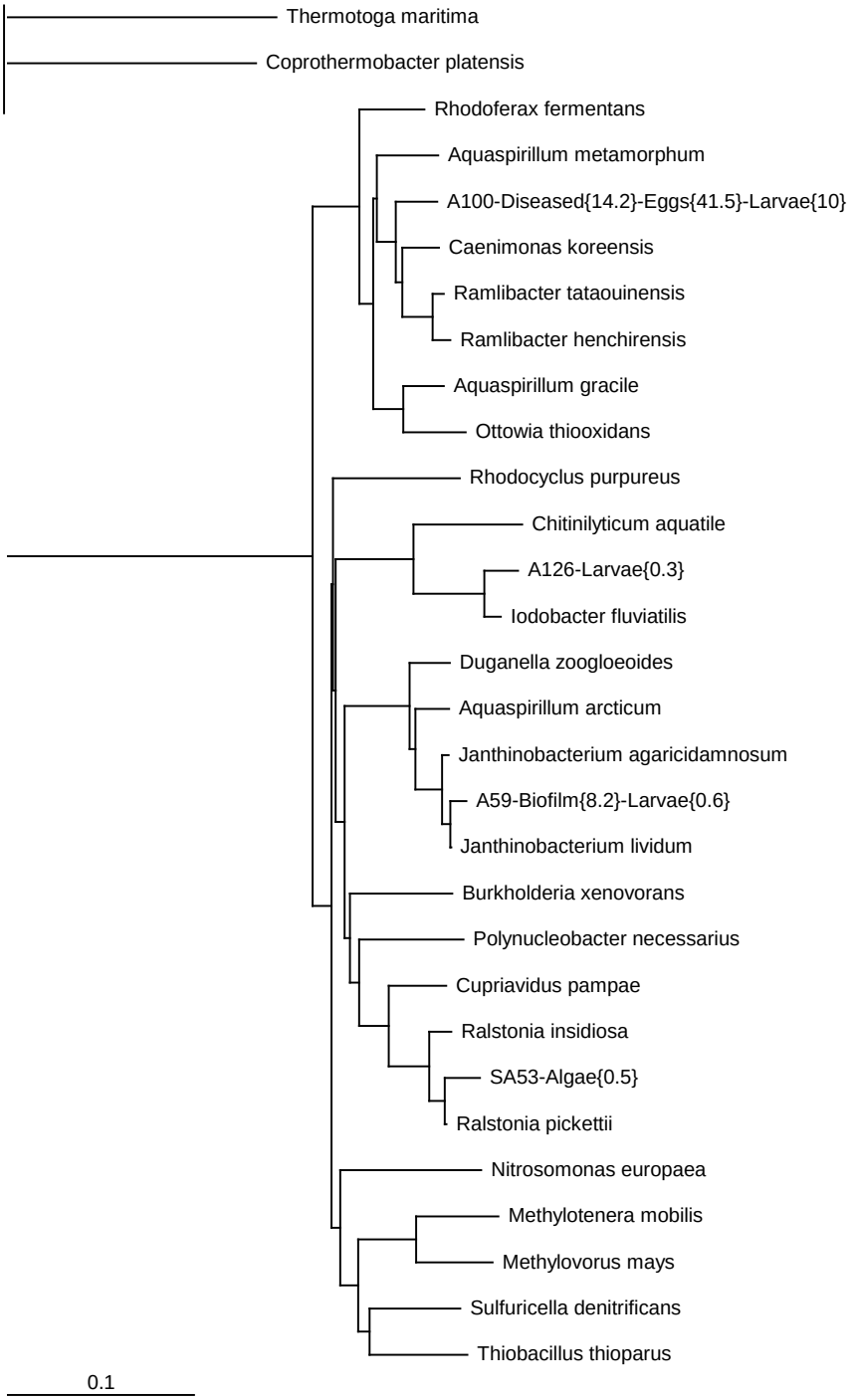
ID	Species (97%) or Genbank nearest match (Accession No.)	Genbank Accession No.	Genus (93%)	Family (90%)	Order	Class	Phylum
A138		JX486669	*Arcobacter*	Campylobacteraceae	Campylobacterales	Epsilonproteobacteria	Proteobacteria
A91		JX486668	*Arcobacter*	Campylobacteraceae	Campylobacterales	Epsilonproteobacteria	Proteobacteria
A160	*Pseudoalteromonas marina*	JX486648	*Pseudoalteromonas*	Pseudoalteromonadaceae	Alteromonadales	*Gammaproteobacteria*	Proteobacteria
A21		JX486567	*Marinovum*	Alteromonadaceae	Alteromonadales	*Gammaproteobacteria*	Proteobacteria
A3	*Marinovum algicola*	JX486560	*Marinovum*	Alteromonadaceae	Alteromonadales	*Gammaproteobacteria*	Proteobacteria
A4		JX486561		Alteromonadaceae	Alteromonadales	*Gammaproteobacteria*	Proteobacteria
A51	*Alteromonas macleodii*	JX486639	*Alteromonas*	Alteromonadaceae	Alteromonadales	*Gammaproteobacteria*	Proteobacteria
A60	*Shewanella baltica*	JX486640	*Shewanella*	Shewanellaceae	Alteromonadales	*Gammaproteobacteria*	Proteobacteria
A148		JX486646		Ectothiorhodospiraceae	Chromatiales	*Gammaproteobacteria*	Proteobacteria
A121	*Serratia proteamaculans*	JX486645	*Serratia*	Enterobacteriaceae	Enterobacteriales	*Gammaproteobacteria*	Proteobacteria
SA20	*Alcanivorax borkumensis*	JX486653	*Alcanivorax*	Alcanivoracaceae	Oceanospirillales	*Gammaproteobacteria*	Proteobacteria
A81	*P. fluorescens*	JX486642	*Pseudomonas*	Pseudomonadaceae	Pseudomonadales	*Gammaproteobacteria*	Proteobacteria
A164	*Vibrio crassostreae*	JX486651	*Vibrio*	Vibrionaceae	Vibrionales	*Gammaproteobacteria*	Proteobacteria
A154		JX486647	*Stenotrophomonas*	Xanthomonadaceae	Xanthomonadales	*Gammaproteobacteria*	Proteobacteria
A163		JX486650				*Gammaproteobacteria*	Proteobacteria
A37		JX486638				*Gammaproteobacteria*	Proteobacteria
A80		JX486641				*Gammaproteobacteria*	Proteobacteria
A86		JX486643				*Gammaproteobacteria*	Proteobacteria
A95		JX486644				*Gammaproteobacteria*	Proteobacteria
A90		JX486671				*Verrucomicrobiae*	Verrucomicrobia

Appendix 3 – Alpha proteobacteria phylogenetic tree

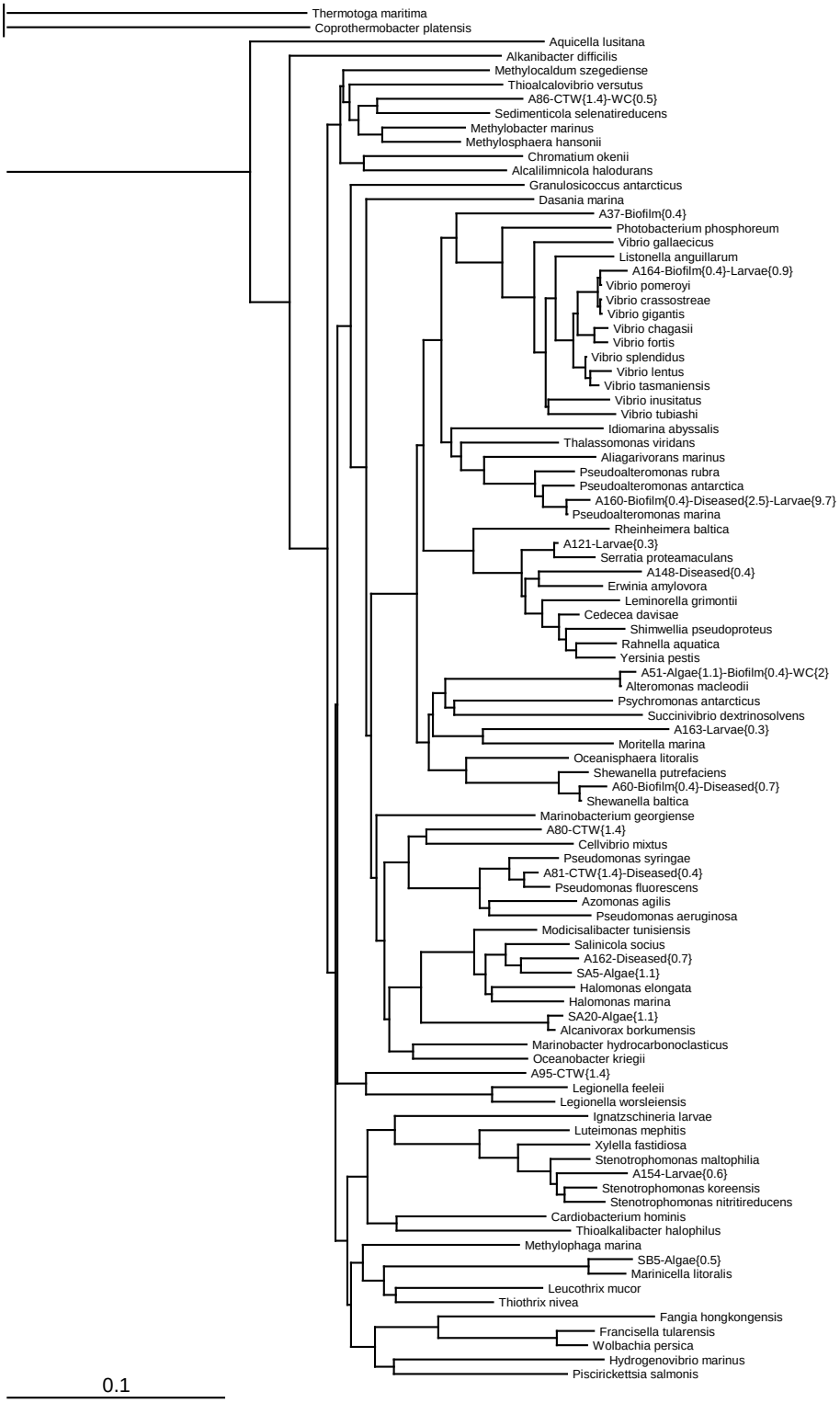
Appendix 4 – *Flavobacteria* phylogenetic tree



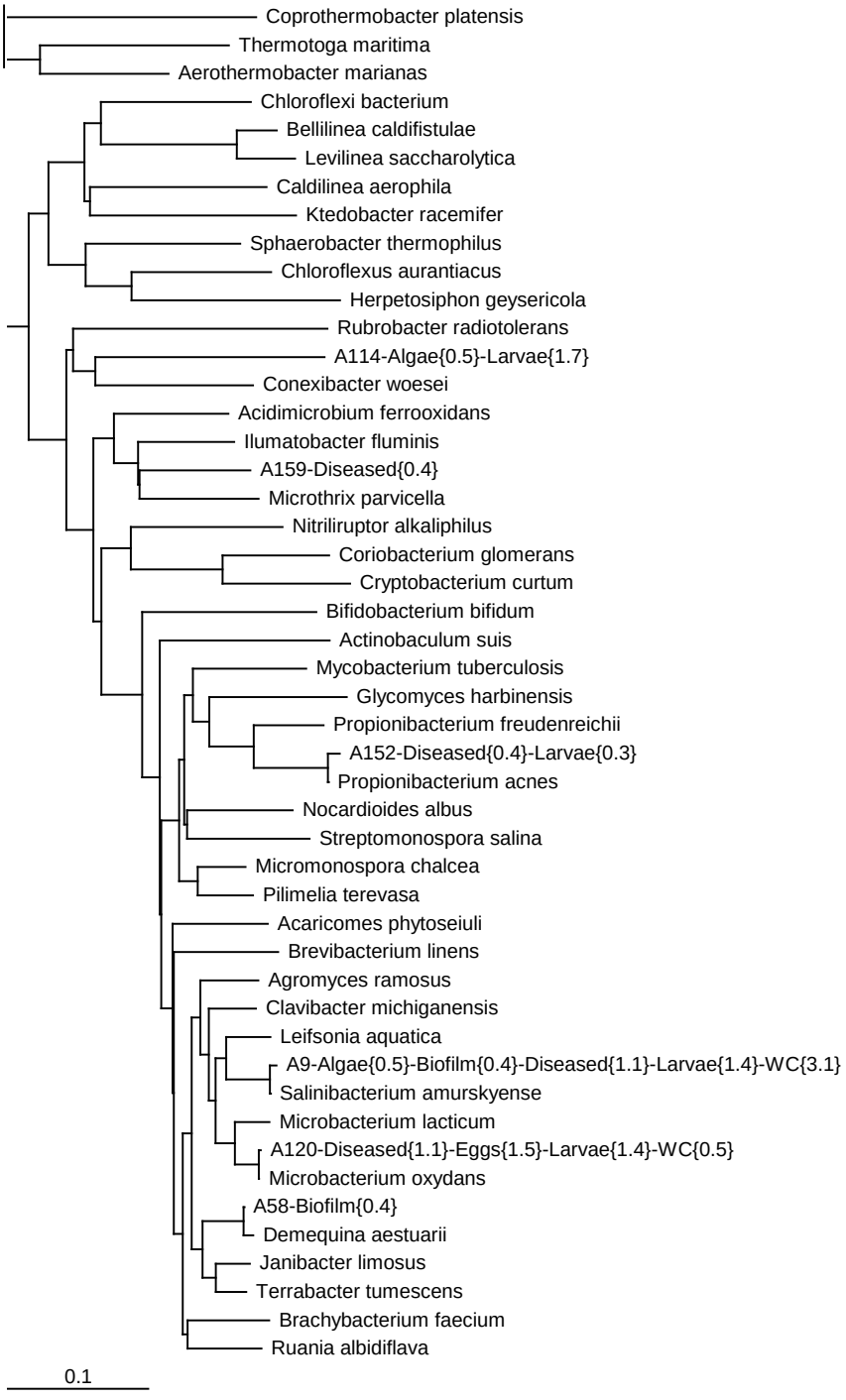
Appendix 5 – Beta proteobacteria phylogenetic tree



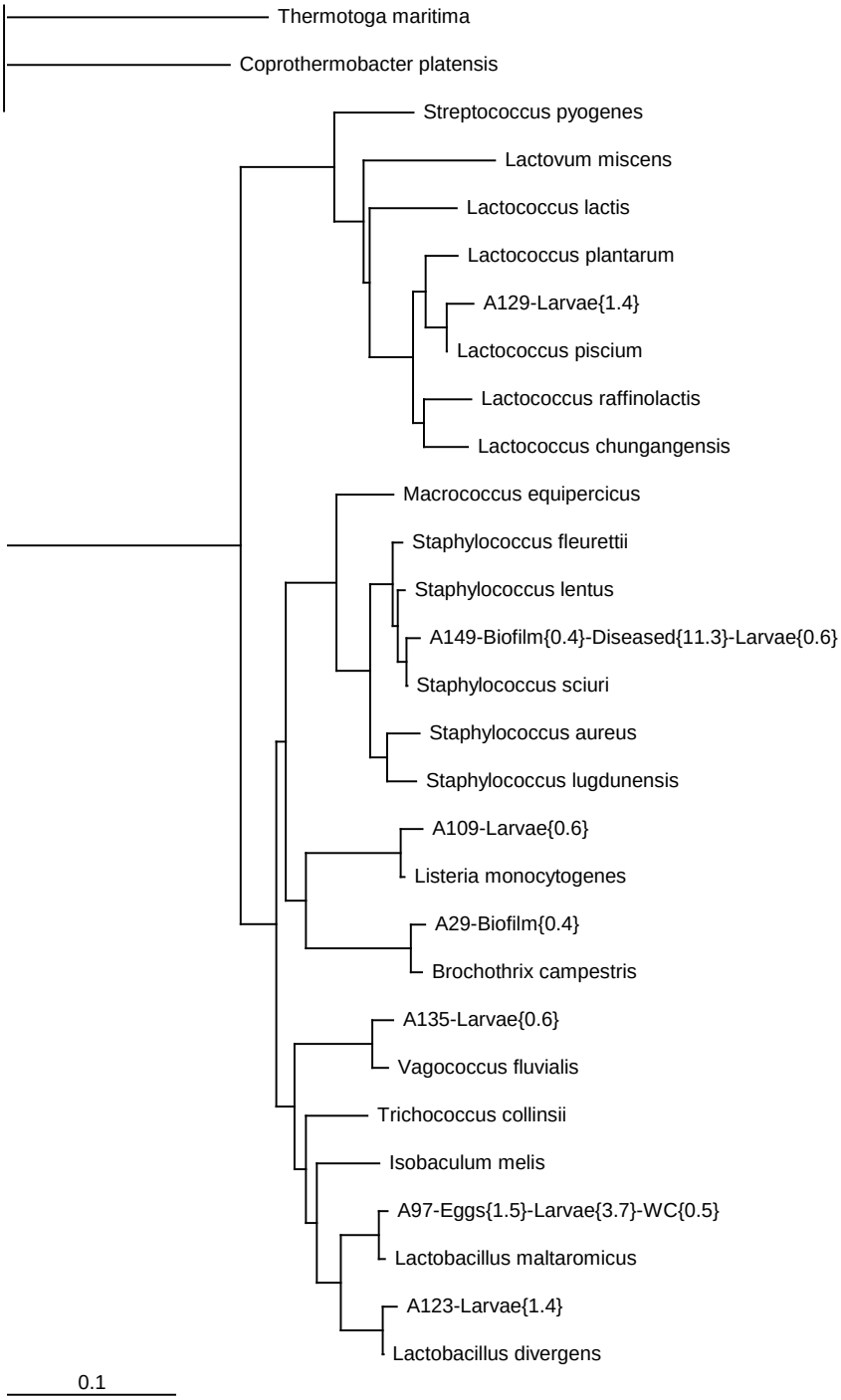
Appendix 6 – Gamma proteobacteria



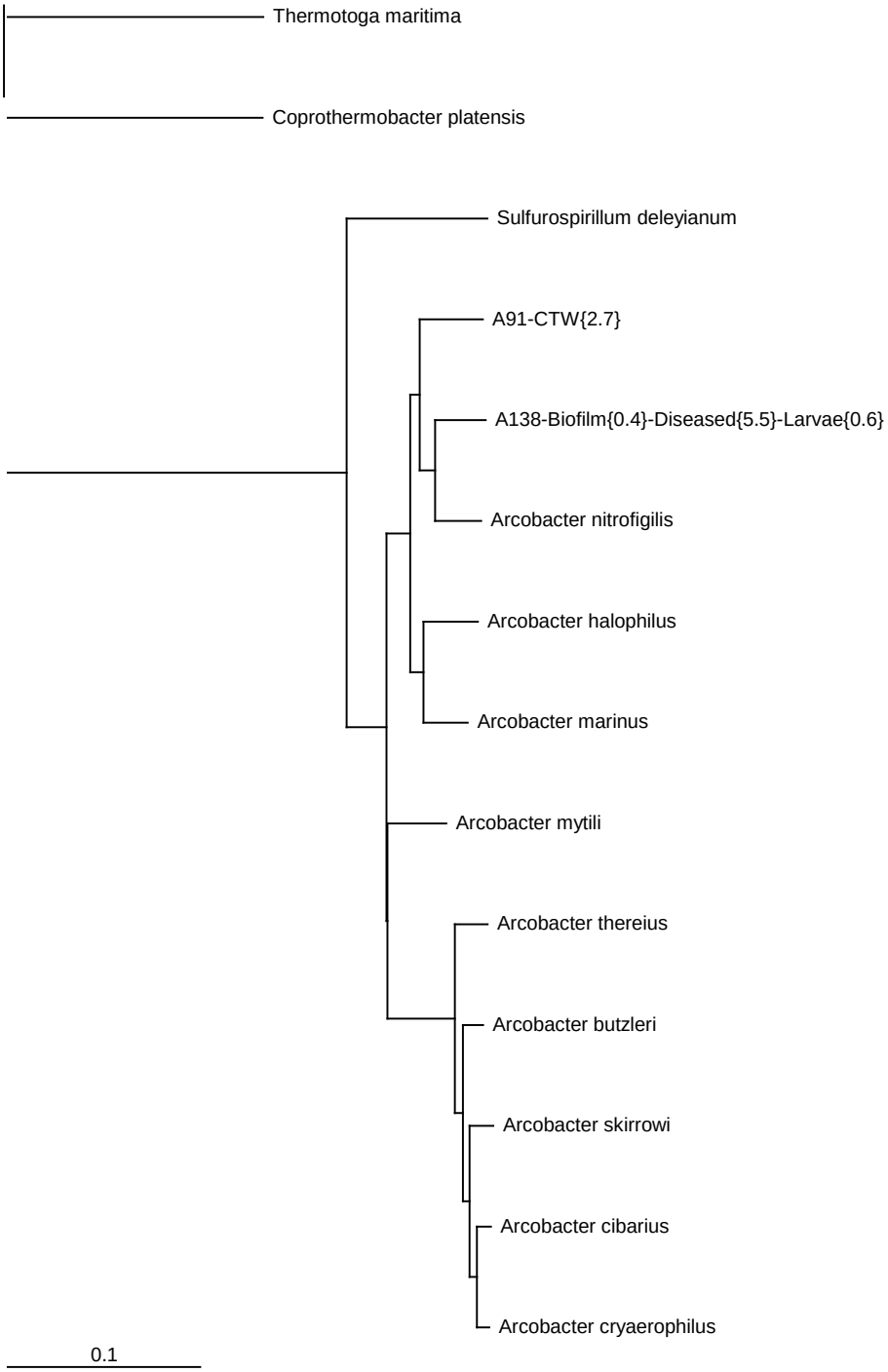
Appendix 7 – Actinobacteria phylogenetic tree



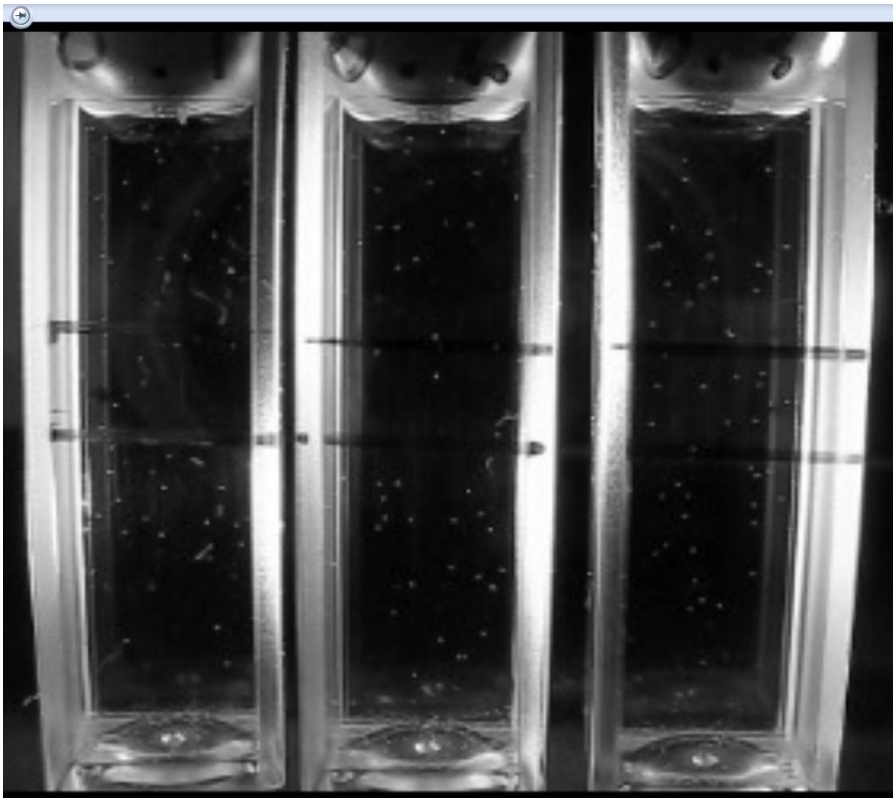
Appendix 8 – Bacilli phylogenetic tree



Appendix 9 – Epsilon proteobacteria phylogenetic tree



Appendix 10 – Larvae behaviour observations in 4.5 ml cuvettes



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