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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Finally, to the millions of larvae that died in the making of this thesis, may they be reincarnated as something bigger with sharp teeth, much much higher in the food chain.

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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 diseased 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. Advanced stages of disease may have been associated with displacement of predominant members of the indigenous microbial community of larvae. Challenge tests utilising bacterial isolates from diseased larvae indicated that none could be considered particularly pathogenic when compared with the known pathogen *Vibrio tubiashii*. *Vibrio* population numbers peaked with the emergence of disease symptoms but remained less than 1% of the total population as indicated by prevalence in 16S rRNA gene clone libraries. 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. Results indicated that sub-lethal levels of copper could increase larvae susceptibility to bacterial pathogens under some conditions. Larvae behaviour was modified at copper levels as low as 2.5 ppb, which indicated that behaviour could be used as a sensitive biomarker of copper stress and might also be of use in assessment of other types of chemical stress and susceptibility to bacterial disease. The behavioural response to different concentrations of copper 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 to 48 h time period. A preliminary investigation into heat shock treatment indicated that heat shock could improve larvae performance under bacterial challenge.

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 2).

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 four 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. Both hypothesis B and C, 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 stress (environment) on susceptibility to disease incidence. Two different types of stress were examined: copper as a chemical stress and heat as a physiological stress. Heat shock is recognised in inducing stress responses in organisms and thus it is conceivable such a response may also serve to improve the resistance of larvae to bacterial challenge and thus represent a potential treatment to increase larvae resistance to disease.

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 copper 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)
C. That heat shock treatment may affect larvae susceptibility to bacterial disease	5. Determine whether heat shock can affect larvae mortality under bacterial challenge (Chapter 4)

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. Despite significant successes in overcoming disease problems along the way, disease 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 1.

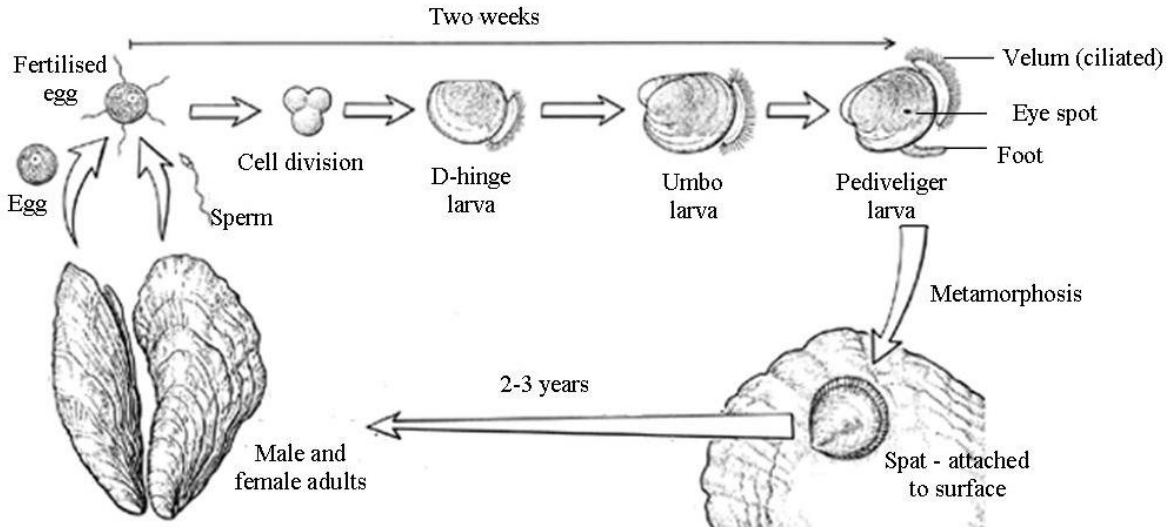


Figure 1 – Life cycle of oyster showing larval stages

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 4.2).

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*. Positive identification of pathogenic bacteria has been achieved using Koch's postulates, which is discussed further in section 3.2.

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 hemolysin and suggested that there may be additional unknown pathogencity factors (Elston et al 2008). Research by Milton (2006) demonstrated that exoproteins produced by a variety of marine *Vibrio* spp. (including those involved in pathogenesis) are controlled by a growth phase-dependent regulatory system (quorum-sensing).

Whilst the pathogenic agent has been identified, pathogenesis in oyster larvae described in detail, and mechanisms of pathogenicity partly elucidated, induction of the disease process

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 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.

Vibrio spp. are a diverse heterotrophic bacterial group indigenous to estuarine and seawater environments and have an important role in the decomposition of both particulate and dissolved organic matter (Thompson et al 2006). They have a broad range of lifestyles – particle-associated, free-living, oligotrophic and eutrophic and in a wide range of salinities (Rehnstam-Holm et al 2010). *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).

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).

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[1]	(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)

(1) 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) (discussed further in section 0).

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. 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 (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.

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 Chapter 2: Microbial ecology and temporal variability in a commercial oyster larvae tank

2.1 Abstract

A microbial ecological study of a commercial oyster hatchery in Bicheno, Tasmania was undertaken using both culture-dependent and culture-independent molecular tools, including 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 mass mortality events. 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. Temporal variability of microbial communities was measured as an indicator of system stability. Microbial communities of all compartments were dominated by *Alphaproteobacteria* including members of the Roseobacter clade. *Vibrio* spp. may have been enhanced in intensive culture conditions but remained below the level of detection using culture-independent methods. Altered culture conditions changed the microbial community of the WC but the larvae community was largely resistant to such change and remained closely related to the indigenous microbial community of the egg. The presence of predominant non-typical marine species, *Sphingomonas paucimobilis*$_{(97\%)}$ and *Ramlibacter tataouinensis*$_{(93\%)}$, in eggs and larvae samples, might have contributed towards community instability, and indicated a non-marine source of contamination occurring during spawning and fertilisation. Microbial communities of the larvae and water column varied primarily with larvae age and sampling period and the most likely cause of variability with sampling period was variability in the seawater.

2.2 Introduction

In the thesis Introduction it was noted that previous research into oyster hatchery epizootics has been undertaken with little or no understanding of the non-culturable microbial community, which comprises the vast majority of bacteria in the marine environment

(Ferguson et al 1984), and has therefore neglected the microbial environmental context in which disease has arisen. In doing so these studies have failed to take full account of the disease triangle conceptual model where disease is a three way interaction between host, pathogen and environment (McNew 1960). Research by a number of authors has indicated that characteristics of the microbial communities associated with larvae, other than simply the presence or absence of a proven bacterial pathogen, may be related to production success. In particular, the notion of microbial balance or stability may be of primary importance in hatchery production. Olafsen (2001) noted that measures to eliminate bacteria through water treatments such as filtration, ozone, UV, and sanitation of surfaces are often not met with successful production outcomes and suggested that these approaches may disturb the balance between microbial groups or favour proliferation of opportunistic bacteria or unpredictable development of bacterial communities. Schulze et al (2006) noted that aquaculture hatcheries contain diverse microbial communities that include pathogenic, innocuous and beneficial bacteria and suggested that the ability to maintain a proper balance of this microbiota may be the key to successful production. Tihn et al (2008) in agreement with the stability concept postulated that the inter-individual variation of microbial communities among healthy fish larvae may be considerably smaller than that among a total pool of larvae.

Verschuere et al (2000b) and Skjermo et al (1997), in support of the counter-argument, stated that hatchery practices such as discontinuous culture cycles, cleaning and sanitation of tank surfaces, and sudden increases in nutrients due to exogenous feeding make it unlikely that a stable microbial community can be achieved. Thus microbial stability may be a misnomer in the hatchery environment. Yet microbial stability in the hatchery might be a relative term that refers to avoidance of large fluctuations or rapid change within the microbial community.

Stability of an ecosystem can be defined in terms of its resistance and resilience to change. A microbial community is considered resistant if it experiences no change upon exposure to perturbation whereas a resilient microbial community is one that experiences change upon exposure but subsequently returns to its original composition (Allison & Martiny 2008). A microbial community that has experienced change may alter in its ecological function or

alternatively may experience no functionality change whatsoever. The degree to which functionality changes in response to a change in the community composition depends upon the degree of functional redundancy existing in the original community (Nogales et al 2010). In the case where a change in the microbial community alters functionality we can expect a change in the ecosystem services provided by the microbial community which usually implies a negative effect on ecosystem health (Nogales et al 2010). As such, a shift in the microbial community in association with larvae that affects the functionality, or range of services that the microbial community provides to the larvae, might affect larvae health with or without implication of a specific bacterial pathogen.

Seawater used by hatcheries, sourced from the coastal marine environment, is unlikely to be a stabilising influence on microbial communities within the hatchery. Many hatcheries use only minimal treatment of seawater such as filtration to 1-10 μm, which permits entry of most bacteria into the hatchery (Ferguson & Rublee 1975) and exposes oyster larvae to the full scale of microbial community variability encountered in the marine environment. Coastal marine water is highly variable in terms of microbial productivity (Nogales et al 2010) because of underlying variability in particulate and dissolved organic matter (POM and DOM, respectively) (Fukami et al 1983). Physical processes such as upwelling of nutrient-rich deep waters or aeolian and riverine deposition, algal blooms, or aggregation of particulate organic matter contribute to seawater nutrient variability (Lauro et al 2009). Variation is also known to occur temporally with predictable seasonal patterns, which may be modified by anthropogenic influences in coastal areas (Nogales et al 2010).

Changes in bacterial productivity with nutrient levels are also accompanied by changes in microbial community composition (Smith et al 1995). Oceanic conditions are generally oligotrophic and as such copiotrophic bacteria are frequently out numbered by their oligotrophic counterparts (Yanagita et al 1978). Oligotrophic species are able to maintain normal metabolic activities in nutrient-poor environments although they may not be able to respond to increases in nutrient availability when they do occur (Hirsch 1979). In contrast, copiotrophic species respond quickly to increases in nutrient level and may dominate in nutrient-rich environments such as those experienced in algal blooms (Smith et al 1995).

Microbial community composition of seawater taken up by hatcheries may also depend upon the quantity of suspended particles, which have a different composition compared to free-living communities (Bidle & Fletcher 1995). The quantity of particulate matter in the seawater may be increased by weather events and dredging operations of the sea floor. Thus water taken up by hatcheries may vary in both microbial abundance and species composition dependent largely upon seasonal factors, mediated by anthropogenic influences and local conditions that may increase uptake of suspended particles.

Practices within hatcheries may affect microbial stability including discontinuous cycles, cleaning and sanitation of tank surfaces, and eutrophication of culture conditions (Verschuere et al 2000b). A study by Verschuere et al (1997) 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. Of 19 culturable colony types isolated, 13 were present in only one of the four tanks and not one single morphological type was found in all four tanks. 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). Verschuere's study demonstrated the importance of remnant biofilm structures in determining the microbial composition of the culture water in aquacultural mesocosms and the potentially de-stabilising influence of cleaning and sanitation between water changes.

A number of authors have reported a shift in microbial community composition towards r-strategist species in hatcheries due to eutrophic conditions (McIntosh et al 2008; Verschuere et al 1997). The r/K species ratio is related to the stability of the system since r-strategist species can respond quickly to organic pertubations (Pianka 1970). The quantity of algae added and the manner in which it is delivered may contribute towards instability. Feeding beyond larvae requirements creates opportunities for rapid bacterial growth of r-strategist species. Similarly, Sandaa et al (2003) suggested that organic pulses in batch feeding of microalgae could shift the balance to r-strategist species.

Ecosystem stability, on average, increases with species diversity although according to McCann (2000) diversity is not the driver of this equation but rather ecosystem stability depends upon the ability of communities to contain species, or functional groups, that are capable of different responses. Hence stability of the microbial community of the larvae tank may be related to diversity although functional diversity may be more important than species diversity. A study by Lebaron et al (2000) showed that addition of organic matter to a mesocosm tank resulted in a reduction in microbial diversity; which may be indicative of 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. However the relationship between diversity and stability does not appear to have been investigated in aquacultural systems.

In this chapter a monitoring study of the Bicheno hatchery was undertaken to address the knowledge gap left by previous researchers limited to using culturable methods to study hatchery epizootics; little is known about the majority, non-culturable component of the microbial community associated with disease. Using microbiological tools that enable whole communities to be characterised the approach taken here is to widen the frame of view on disease incidence to describe the microbial ecology of the larvae tank and inputs into the tank; the broader microbial context in which disease occurs. Particular emphasis has been given to characterise temporal variability in microbial communities of the larvae tank as a measure of stability, which may be related to incidence of disease. Physiochemical data and nutrient analysis were also characterised as underlying environmental drivers of primary production.

2.2.1 Research objectives

The main objective in this chapter was to characterise the microbial ecology and temporal variability (stability) in the larvae tank with respect to tank inputs (research objective No. 1 as defined in Table 1). This main objective, which was addressed through a monitoring program at the Bicheno hatchery, was further divided into the following objectives:

(a) Characterise and compare microbial compartments of the larvae tank and inputs

(b) Characterise temporal variability in microbial communities of larvae and culture water

(c) Characterise differences in microbial communities between batch and flow-through production systems

Objective "c" was included because larvae were moved between different production systems during production runs for production reasons and thus needed to be accounted for in analysis, and also because comparison of the two systems provided an opportunity to observe the effect of changed culture conditions on larvae microbial communities. The monitoring program was conducted over a 12-month period and included eight production runs. To characterise microbial communities both culture based techniques and culture-independent molecular tools were used including 16S rRNA gene clone libraries and TRFLP analysis. The microbial environment was characterised by collection of physiochemical data and dissolved nutrient analysis.

2.3 Methodology

Both Chapter 2 and Chapter 3 were based on data from the same monitoring study. For this reason the methodology for both chapters is described here in Chapter 2.

2.3.1 The Bicheno hatchery – site details

The Shelfish Culture Ltd hatchery, where monitoring research was undertaken, is located on the Gulch in Bicheno (Figure 2). The hatchery location is shown at point D and a number of items of interest are also indicated. In particular, there are a number of potential sources of

anthropogenic pollutants including a boat slip (A) where boat maintenance works are conducted, an abalone farm (E), a jetty (B) used by commercial fishing boats and recreational fisherman, and a public boat ramp (F). Water is sourced from two different inlets, one located at point C, from a depth of approximately seven metres, and another from point G, at approximately 18 m.

Figure 2 – 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

2.3.2 Hatchery production systems

Two different production systems were used at the Bicheno hatchery including a flow-through and a batch production system. 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 26^{o}C heated and 1-10 µm filtered seawater, referred to as clean tank water (CTW) in the present study, at a rate of 0.6-3.0 L/min and IC tanks were changed every 24 h. The batch system was conducted in tanks of 11,500 L capacity ("Batch tanks") that were filled with CTW approximately 16 h prior to the addition of larvae, and used for a culture 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 water column (WC) to differentiate it from the CTW. Larvae were held at higher densities in the IC tank system (3-6 larvae/ml to 30-40 larvae/ml for larvae 10 d old 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 "F2" culture media and aerated with CO_2 enriched air (approximately 1% CO_2). Feed rates were based on assessments of larvae size, residual feed of larvae, 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 chemically induced. Spat were then transferred to a "nursery".

2.3.3 Monitoring program design

Three different monitoring programs were undertaken. The main monitoring program followed individual production runs to characterise the microbial environment and change occurring over the production run cycle. The second monitoring program involved collection of samples for comparison of microbial niches within the larvae tank and inputs; and the third involved monitoring of change in the microbial environment occuring in the larvae tank WC over a 48 h period.

2.3.3.1 Production run monitoring

Production run monitoring was undertaken to meet all objectives (a), (b) and (c) in section 2.2.1. Monitoring was focussed on compartments of the larvae tank (larvae, WC and biofilm) and inputs into the tank (algae, CTW and eggs), referred to as "sample types". Data collection included the following:

- Cultivation of samples from larvae, WC, CTW, and algae to determine microbial abundance including total viable and culturable (TVC) bacteria and *Vibrio* population No.s
- Physiochemical data on the larvae tank WC
- Nutrient analysis of the larvae tank WC
- TRFLP analysis of eggs, larvae and WC (analysis referred to as TRFLP-1)
- 16S rRNA gene clone libraries of all sample types
- Larvae production and health records

Monitoring was undertaken daily throughout five production runs and at three fixed time points throughout a further three production runs (2-5, 5-10, and > 10 days since spawning

(DSS)). Monitoring was conducted across three different time periods over a 12 month period from 16/01/09 to 01/02/10 (Summer 2009, Spring 2009, Summer 2010).

Production run independent variables are identified in Table 4. Variables DSS, time passed since the previous larvae tank water change (TSW), and tank type could not be strictly controlled because commercial operations took precedence over experimental design and disease incidence shortened some production runs. Disease status, although strictly speaking a dependent variable, was treated as an independent variable in order to separate the effect of disease in a multivariate environment. Three different levels of disease status were defined including diseased, not-diseased and moribund. Diseased larvae exhibited characteristic disease symptoms including damaged or detached vela, abnormal swimming behaviour, reduced activity, poor faecal consistency and necrotic soft tissues; conversely not-diseased larvae did not exhibit these symptoms. Moribund larvae were diseased larvae that had ceased swimming in the WC and formed dense aggregations on the tank bottom.

Table 4 – Production run independent variables

Independent variable	**Variable levels**
Sample type	• Larvae • WC • Biofilm • CTW • Eggs • Algae
Sampling period	• Summer 2009: 16/01/09 to 12/02/09 • Spring 2009: 07/09/09 to 03/10/09 • Summer 2010: 27/11/09 to 18/01/10
DSS	• 2-5 d (umbo to early veliger larvae) • 5-10 d (veliger larvae) • Greater than 10 d (pediveliger).
TSW	• 24 h • 48 h
Tank type	• Batch tank • IC tank
Disease status	• Diseased • Not-diseased • Moribund

TRFLP-1 analysis was undertaken selectively to achieve replication with sampling period and DSS in sample types larvae and WC; and also included egg samples. Diseased samples were included when incidence of disease occurred. Diseased instead of moribund larvae were included to characterise change in microbial communities associated with early departure from health rather than the more advanced stage of disease encountered in moribund larvae. This rationale was justified by a preliminary TRFLP trial, using samples from two production runs, which indicated a substantial difference between not-diseased and diseased larvae (data not reported).

Clone library analysis was undertaken selectively, using a total of 18 clone libraries, primarily to characterise microbial communities associated with disease incidence and as a secondary objective to characterise microbial communities associated with different sample types. Sample types were compared using samples from production run 070909 on the 15/09/09 and 16/09/09 (eggs were from 07/09/09), which was conducted in the IC tank, and included a total of six clone libraries. Production run 070909 was chosen for more detailed clone library analysis because disease onset and larvae mortality was sudden and resulted in 100% mortality. Two additional algae samples were included to characterise variability in microbial communities of algae samples over time. The remaining clone libraries were selected to characterise changes in the microbial community associated with emergence of disease symptoms in three different production runs: 070909, 280109 and 170909. Analysis of samples from production run 070909 indicated that there may be little difference between not-diseased and diseased larvae, although moribund larvae were quite different and shared similarities with the biofilm. From these observations clone libraries for production runs 280109 and 170909 included diseased larvae, moribund larvae and biofilm on days of disease incidence.

2.3.3.2 Sample type monitoring

Sample type investigation was undertaken to allow a comparison of the microbial composition of different sample types (larvae, WC, biofilm, algae, eggs, and CTW) to contribute towards objective (a) in section 2.2.1. Samples were collected for each sample type on the same day on six different occasions: 09/09/09, 15/09/09, 20/09/09, 24/09/09,

20/01/10 and 26/01/10, although eggs were sampled some days prior in each case. Comparisons among sample types were made using TRFLP analysis (referred to as TRFLP-2).

2.3.3.3 Forty-eight hour Batch tank monitoring

Monitoring of changes occurring in the microbial environment of the WC of a Batch tank was undertaken over a 48 h period, between water changes, to contribute towards objective (b) in section 2.2.1. Dependent variables measured over the 48 h period included bacteria counts (TVC and *Vibrio*), dissolved nutrients (phosphate, nitrite, nitrate, ammonia, and dissolved organic matter (DOC)), as well as microbial composition (using TRFLP, referred to as TRFLP-3). Samples were taken for bacterial enumeration at the beginning of the culture period, before and after addition of algae, and at the end of the culture period. Dissolved nutrient and microbial composition analysis (TRFLP-3) were undertaken at beginning and end points, peak bacteria counts, and before and after addition of algae. At the beginning of the 48 h period larvae age was 13 DSS. A summary of all variables monitored is given in Appendix 4.

2.3.4 Analytical procedures

2.3.4.1 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, 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 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.

2.3.4.2 Determination of microbial abundance

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 bag systems 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 total viable culturable (TVC) and those on TCBS were defined as presumptive *Vibrio* spp. (referred to as simply *Vibrio*). The ratio of these two counts were 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). Moribund larvae were collected from the tank bottom after water change had occurred using a sterilised spatula. Larvae samples were washed with 3 x 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 x 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).

Microbial abundance data were investigated with respect to the independent variables identified in Table 4 using the dataset in Appendix 2, which was highly unbalanced due to

having an uneven number of samples and additionally no data in certain combinations of variables. 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). In order to improve balance in statistical analyses, independent variables that did not appear to be significant were removed from the model except where the insignificant variable was of primary interest. A total of 11 statistical models based on microbial abundance data were used to investigate independent variables of Table 4, and a design summary of each model is given in Appendix 14 (labelled A – K). Another analysis, Statistical model P, was undertaken using SAS REG to examine relationships amongst microbial abundance variables in larvae and WC independently of the explanatory variables of Table 4.

2.3.4.3 Contribution of bacteria from tank inputs

Using microbial abundance data the total number of bacteria, and the total number of Vibrio spp., entering Batch larvae tanks through the CTW, larvae and algal feed was calculated to determine their relative contribution of bacteria to the larvae tank microbiota. Data used included samples from all stages of the production cycle. 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. The number of bacteria contributed from each source was calculated by multiplying TVC (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 undertaken for *Vibrio* counts. Statistical models L and M were undertaken for TVC and *Vibrio* counts, respectively.

2.3.4.4 DNA sample collection, extraction and purification

Larvae and eggs

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 for determination of microbial abundance. Fertilised egg samples were collected

in a similar fashion using 20 µm screens from a bucket in which fertilisation occurred. Larvae and egg samples were washed in 3 x 1 L of SSW on screens before being transferred into Eppendorf tubes and frozen at -18^{o}C until further processing. 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 CTAB (hexadecyltrimethylammonium bromide, 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 m/s for 1 min. Following bead beating, tubes were centrifuged at 16,000 x *g* for 5 min at 4^{o}C. The aqueous top layer was then 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 x *g* for 5 min at room temperature. The aqueous layer was removed to a new 1.5 ml microcentrifuge tube. Two volumes of PEG/NaCl precipitate solution was added and incubated at room temperature for 2 h. Precipitate was centrifuged at 18,000 x *g* for 10 min at 4^{o}C. The pellet was then washed in ice cold 70% ethanol and centrifuged again at 18,000 x *g* for 5 min at 4^{o}C. The pellet was then dried in vacuum desiccator for 10 min and pellet was resuspended in 10 mM Tris buffer.

WC and CTW

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 Falcon tube before being stored at -18^{o}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 Falcon 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^{o}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 x *g* for 15 min at 4^{o}C; and once with chloroform: isoamylalcohol (24:1) with centrifugation at 10,000 x *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 15 ml falcon tubes at 12,000 x *g* for 30 min at 4^{o}C. The pellet was washed in ice cold 70% ethanol and centrifuged again. The pellet was then dried in vacuum desiccator for 10 min and pellet was resuspended in TRIS buffer.

Biofilm

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 Falcon tube and frozen at -18^{o}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.

Algae

Extraction of microbial DNA from algae samples followed a modified protocol from Burke et al (2009). Algae samples of 50 ml were collected in the hatchery and frozen at -18^{o}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 then incubated at room temperature for 2 h with gentle agitation. The mixture was then centrifuged at 1,000 x *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.

2.3.4.5 TRFLP analysis

Amplification of the 16S rRNA gene was undertaken with PCR using labelled primers. A blocking primer developed by Powell (2011) was used to prevent amplification of algae DNA (907R_block). PCR reaction components included 10 µl of Immomix Mastermix (Bioline) with 0.2 µl BSA, 0.4 µl of primers 10F (GAGTTTGATCCTGGCTCAG) and 907R (CCGTCAATTCCTTTGAGTTT) and 0.8 µl of 907R_block (TGAGTTTCACCCTTGCGAGCG_C3 spacer) with 7.2 µl of water. 10F and 907R 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 *HaeIII*, *HinfI,* and *MspI* (New England Labs). Digestion reaction components included 1 µl of enzyme buffer, 3 µl of combined PCR reaction, 1 µl of water and 5 U of either *HhaI*, *HinfI* or *MspI* (New England Biolabs) and the digest was carried out at 37^{o}C for 3 h. The resulting digest was then diluted x 10 to 100 µl and 5 µl of each diluted digest was cleaned by ethanol precipitation into 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. TRFLP-2 laboratory procedures were carried out by Dr Shane Powell.

Initial data processing was carried out using CEQ software (Beckman Coulter). The raw data consisted of a chromatograph of absorbance intensity vs peak length for each digest reaction. For each sample replicate there were three digest reactions (one from each enzyme (*HhaI*, *HinfI* or *MspI*), from which a total of six chromatographs were generated (3 enzymes x 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 in both TRFLP-1 and TRFLP-2. Except where indicated otherwise, all TRFLP analyses 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). Except where indicated all statistical analyses were undertaken using abundamce data.

To visualize patterns in bacterial community structure based on the TRFLP data, MDS plots were constructed using the Bray-Curtis similarity matrix (Clarke 1993) (otherwise known as polar ordination), which is an distance-based ordination method where it is necessary for data to obey the triangle inequality (i.e. the distance between A and B plus the distance between B and C cannot exceed the distance between A and C). For each MDS plot a stress value is given and this is an indication of how well the data is represented in two dimensions. The lower the stress the better the data is represented with values of 0.2 indicating a potentially useful representation of the data that needs to be interpreted with caution (Clarke & Warwick 1994).

The statistical significance of differences among 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. PERMANOVA permits analysis of multivariate (or univariate) data in the context of more complex sampling structures. It adopts a more parametric approach that retains robustness by being totally resemblance and permutation-based. 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, which is not actually correct since different species can share the same TRF but nevertheless provides an estimate of species diversity.

Shannon-Weiner index (H') is a commonly used diversity index that combines both richness and evenness properties:

$$H' = - \sum_i p_i \log(p_i)$$

where p_i is the proportion of the total count arising from *i*th species.

Margalef's index (d) is a measure of the number of species present for a given number of individuals (species richness):

$$d = (S-1)/\log N$$

where S = the number of species and N = the number of individuals.

Pielou's evenness index (J') expresses how evenly the individuals are distributed among the different species (evenness):

$$J' = H'/H'_{max} = H'/\log S$$

where H'_{max} is the maximum possible value of Shannon diversity i.e. that would be achieved if all species were equally abundant (namely, log *S*).

Production run monitoring statistics (TRFLP-1 analysis)

TRFLP-1 analysis was undertaken using data from Appendix 1, a total of 131 replicates. MDS and PERMANOVA were used to compare samples with respect to independent variables identified in Table 4 while MVDISP and diversity analysis were used to further characterise differences among sample types (larvae, eggs and WC). With respect to these variables the dataset was unbalanced due to having an uneven number of replicates and additionally no data in certain combinations of variables (Appendix 1). PERMANOVA analysis is able to handle unbalanced data sets (Anderson et al 2008) but missing data required that the data be partitioned into a number of separate analyses. A total of 15 statistical models were reported and model design details are given in Appendix 14 (S – GG). Analyses were undertaken separately for different sample types and then the data were partitioned again by excluding some of the raw data to avoid missing data.

For analysis of tank type (S and X for larvae and WC, respectively) samples from Summer 2010 and samples from young larvae (2-5 DSS) were excluded because they did not include any samples from IC tanks. Analysis of disease status in both larvae and WC was conducted with all data (T and Y, respectively) and also with IC tank data excluded (V and AA, respectively). IC tanks were excluded in analysis T and AA due to missing data values.

Analyses T and Y included IC tank data but did not include tank type in the statistical model. Sampling period and DSS were included in all statistical models but were the primary variables of interest in U and W for larvae samples, and Z and BB for WC samples. Analysis was conducted with all data (U and Z) and with IC tank excluded (W and BB). Similarly to disease analysis, U and Z statistical models are irrespective of tank type.

For analysis of microbial composition variation with sample type, statistical analyses CC and DD were undertaken and all samples were included except Statistical model DD used only presence-absence data. No other variables were included in the model because they did not apply to eggs and the primary interest was sample type irrespective of independent variables. MVDISP and diversity indices were also generated for each sample type (larvae, eggs and WC). Diversity indices, including Shannon-Weiner (H'), Margalef's (d), and Pielou's evenness (J'), were exported from Primer and statistical analysis was undertaken using SAS GLM as described in section 2.3.4.2 (Statistical models EE, FF and GG, respectively).

Sample type monitoring statistics (TRFLP-2 analysis)

Samples of different type were compared using MDS, PERMANOVA and MVDISP based on data summarised in Appendix 3, a total of 86 replicates. PERMANOVA analysis included two statistical analyses: Statistical model Q included abundance data while Statistical model R included only non-abundance data (presence-absence). The design of both statistical models is given in Appendix 14.

Forty-eight hour monitoring statistics (TRFLP-3 analysis)

Samples included in TRFLP-3 analysis are identified in Appendix 4. MDS analysis was used to compare WC samples of different TSW (h). No trend was apparent so no further analysis was undertaken.

2.3.4.6 Clone libraries

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). The standard kit protocol was employed. Cultured clones were placed into a PCR reaction with the M13 primers (F: GTAAAACGACGGCCAG and R: CAGGAAACAGCTATGAC). M13 PCR reactions were then 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. Sequences were then sorted into class and phylogenetic trees were constructed for each class in which type strains were incorporated for identification purposes. A phylogenetic distance matrix was generated from each tree that was then imported into DOTUR software, which was used to group sequences into operational taxonomic units or phylotypes at 97% similarity. 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 phylotypes were grouped with type strains using DOTUR to species level (97% similarity). Where a species match could not be found phylotypes were matched to genus (93% similarity) or family (90% similarity) level. Sequences that could not be identified to 90% similarity to type strains were matched to unidentified clones in the Genbank database (GenBank, NCBI, Bethesda, MD, USA (http://www.ncbi.nlm.nih.gov/genbank/)) and the clone accession number is given. Where identified phylotypes are referred to in the Results and Discussion sections, the species name is followed by the percentage similarity to the type strain in brackets and subtitled (e.g. *Staphylococcus sciuri*$_{(97\%)}$).

Clone libraries were described in terms of percentage coverage (Good's formula), species diversity (Chao-1 estimator) and phylogenetic diversity (Faith's index). Percentage coverage was calculated using Goods formula (Good 1953), which provides an estimate of the percentage of total sample diversity that is represented in the clone library.

$$\text{Coverage (\%)} = (1 - ni/N) \times 100$$

where,

ni = the number of phylotypes that have been sampled once

N = the total number of individuals in the sample

Species diversity was estimated using the Chao-1 estimator (Chao 1984), which is a non parametric estimator for species richness that takes the form:

$$\text{Chao-1 estimate} = S_{obs} + (a^2/2b)$$

where,

S_{obs} = the number of phylotypes observed

a = the number of phylotypes observed just once

b = the number of phylotypes observed just twice

The Chao-1 estimator returns an estimate of the number of unique species in a sample.

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 among clone libraries was performed using the Unifrac analysis (http://128.138.212.43/unifrac; 01/01/2012), which uses phylogenetic information for comparisons (Lozupone & Knight 2005). The Unifrac distance metric was used to undertake Principal Component Analysis (PCA) to compare the similarity of a number of clone libraries simultaneously. This was undertaken using both abundance data (based on peak area) and presence-absence data. Pairwise comparisons were made between some clone libraries using the Unifrac significance test (Bonferroni correction). In addition to PCA analysis and Unifrac significance testing, clone library data were analysed to determine the number of common and unique phylotypes among libraries from production run 070909.

2.3.4.7 Dissolved nutrient analysis

Nutrient analysis included daily monitoring conducted during production runs 070909 and 170909 using field test kits, and analysis conducted for 48 h monitoring using laboratory analysis.

Daily assessments using field test kits (Production run monitoring)

Analysis of dissolved nutrients in larvae tank WC was undertaken on a daily basis for production runs 070909 and 170909 using test kits developed by Mars Fishcare (Hackettstown, USA). Ammonia, nitrate and nitrite were measured using "API saltwater liquid master test kit: Vendor item number 401M". Phosphate measurements were made using "API Freshwater/Saltwater Phosphate Test Kit: 63L". Test range and minimum detection levels are reported in Table 5.

Table 5 – Dissolved nutrient test range and minimum detection level

Dissolved nutrient	Test range	Minimum detection level
Ammonia	0 to 8 ppm	0.25 ppm
Nitrate	0 to 160 ppm	5 ppm
Nitrite	0 to 5 ppm	0.25 ppm
Phosphate	0 to 10 ppm	0.25 ppm

Forty-eight hour monitoring using laboratory analysis

Analysis of dissolved nutrients in larvae tank WC, undertaken for the 48 h monitoring program, was out sourced to Analytical Services Tasmania Pty Ltd (AST, Hobart, Tasmania) and conducted in accordance with NATA accreditation requirements (AST accreditation number: 5589). Water samples were filtered to 0.2 µm to remove particulate matter and frozen at -18^{o}C before being sent to AST. The following AST procedures were followed in analysis: ammonia, nitrate, nitrate, and phosphorous (dissolved reactive) using 1205-water; and DOC using 1404-water.

2.3.4.8 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 of Table 4 and two statistical models, Statistical model N and Statistical model O (Appendix 14), were reported to compare tank types with respect to pH and temperature, respectively.

2.3.5 Larvae-bacteria challenge tests

Bacterial isolates were obtained from diseased larvae samples during hatchery epizootics. Colonies were selected from both MA and TCBS plates based on dominance and uniqueness of colony morphology and grown on MA before being cryogenically preserved at -80^{o}C in a marine broth (per litre: 5 g peptone, 5 g yeast extract, 35 g Red Sea Salt) combined with 30% glycerol.

A total of eight isolates were used for bacterial challenges. Seven of these were isolated from moribund larvae from within the hatchery and the eighth species was a strain of *Vibrio tubiashii* obtained from Animal Health laboratories (Isolate ID: 09/2885-1 from Shellfish hatchery in SE Tasmania). Isolates were identified using 16S rRNA sequencing and matched to type strains in a phylogenetic tree. Type strains were identified from a phylogenetic tree developed by Yarza et al (2008) and an accession number was used to access the 16S rRNA sequence from a website developed by The National Center for Biotechnology Information advances science and health (NCBI) (http://www.ncbi.nlm.nih.gov/; 01/01/2012).

Preserved isolates were re-cultured on MA. After 48 h an individual colony was used to inoculate 10 ml marine broth and the culture was allowed to grow for 24 h at 25^{o}C before being washed twice in 10 ml SSW by centrifugation at 1500 x *g* for 10 min. The culture was resuspended in 10 ml SSW, plated on MA for enumeration and serially-diluted to concentrations of approximately 10^2, 10^4, 10^6, and 10^8 CFU/ml before being challenged against larvae.

Larvae six DSS were collected from Bicheno hatchery, from a commercial production run, and transported in an ice-cooled esky (approximate temperature 5 to 10^{o}C) to laboratories at the university in Sandy Bay, where bacterial challenge began six hours after pick-up. 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.

2.4 Results

2.4.1 Microbial abundance in the larvae tank and inputs

2.4.1.1 Microbial abundance and correlations

TVC and *Vibrio* levels were assessed in all larvae tank compartments and inputs in the production monitoring program, except for biofilm. Average TVC and *Vibrio* counts, expressed in log values, are presented separately for Batch tank and IC tank systems in Table 6. Regression analysis among microbial abundance data (Statistical model P) indicated that there was a significant correlation between the *Vibrio* population in the larvae and the WC (Larvae *Vibrio* and WC *Vibrio*: $Pr < 0.0001$), as represented in Figure 3. *Vibrio* abundance in the larvae was independent of bacterial abundance in the WC (Larvae *Vibrio* and WC TVC: $Pr < 0.52$).

Table 6 – Average TVC and *Vibrio* counts associated with different sample types in the Batch tank and IC tank

		Batch tank				IC tank			
		Larvae	CTW	Algae	WC	Larvae	CTW	Algae	WC
TVC	Average (log CFU/ml or /larvae)	2.75	3.65	6.50	5.38	3.25	3.75	6.55	5.20
	Stdev	2.99	3.94	6.36	5.38	3.67	4.26	6.33	5.34
	n	52	25	39	51	29	23	17	24
Vibrio	Average (log CFU/ml or /larvae)	1.83	1.77	1.70	2.71	2.45	1.22	1.76	4.16
	Stdev	2.52	1.74	2.13	3.05	3.08	1.24	2.12	4.41
	n	46	24	13	48	23	15	9	22
Vibrio/TVC	Average (%)	3.66	6.04	0.00	0.55	15.76	3.89	0.00	10.05
	Stdev	8.43	8.86	0.00	1.56	28.58	7.00	0.01	12.28
	n	48	24	12	46	24	15	8	22

2.4.1.2 Contribution of bacteria from tank inputs

Using microbial abundance data from the production run monitoring program the number of bacteria entering the Batch tank through the CTW, larvae and algal feed was assessed to determine their relative contribution of bacteria to the larvae tank WC over a 48 h period between water changes. Algae were the greatest source of TVC (11.51 +/- 0.38 log) followed by CTW (10.27 +/- 0.58 log) and then larvae (9.74 +/- 0.80 log) ($Pr < 0.0001$; Statistical model L) as indicated in Figure 4, which also includes total counts in the WC for comparison. Total *Vibrio* numbers were greater in association with the CTW (8.59 +/- 0.54 log) than with the larvae (7.38 +/- 1.1 log) and were not detected in the algae ($Pr < 0.0001$; Statistical model M). 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.

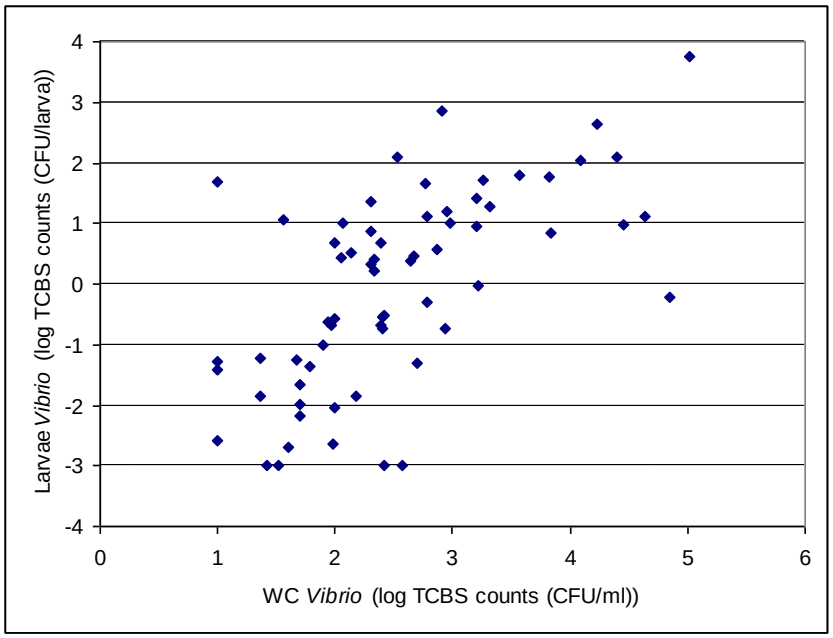


Figure 3 – Larvae *Vibrio* count and WC *Vibrio* count correlation

Regression: Pr < 0.0001 (Statistical model P)

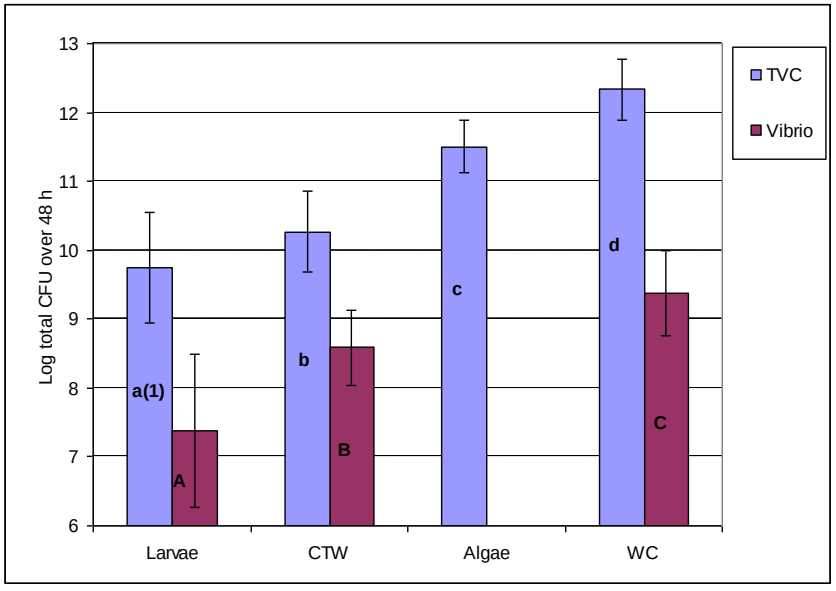


Figure 4 – Contribution of bacteria to the larvae tank from algae feed, CTW and larvae over a 48 h period

TVC (Sample type): Pr < 0.0001, LSD = 0.345, (Statistical model L). *Vibrio*: Pr < 0.0001, LSD = 0.457, (Statistical model M). (1) Letters on columns represent LSD groupings

2.4.2 Forty-eight hour monitoring between water changes in a Batch tank

Monitoring of bacterial levels in an 11,500 L Batch tank over a 48 h period showed an increase in TVC counts in the WC from 3.37 log CFU/ml at zero hours through to 5.81 log CFU/ml at 32 h when a plateau was reached (see Figure 5); which indicates an increase of 2.44 log CFU/ml. Growth of *Vibrio* spp. appeared slower initially and increased after 24 h to 3.26 log CFU/ml at 48 h, an increase of 2.26 log over the 48 h period. Stationary growth in the *Vibrio* population might not have been reached within the 48 h period, although the increase in *Vibrio* counts from 24 h to 48 h was less than one log unit. Following addition of algae there appears to have been an increase in TVC counts, especially earlier in the 48 h period at points A, B and C, when TVC counts were lower, and the increase seems to have been more pronounced in *Vibrio* than in TVC counts, even though algae contained high levels of bacteria but no *Vibrio*. No trend in the microbial community structure over the 48 h period was observed in Figure 6, which was constructed using TRFLP-3 analysis.

Dissolved nutrient analyses over the 48 h period, including ammonia, phosphorus, nitrate and nitrite, are summarised in Figure 7. Levels of all nutrients followed a relatively similar pattern, increasing to a peak level at the 32.5 h assessment followed by subsequent decline. Nutrient peaks at approximately 32.5 h were coincidental with maximum bacterial density as indicated by TVC counts in Figure 5. The increase was greatest for nitrate, which increased almost one order of magnitude from approximately 0.02 ppm at time zero (tank water prior to the addition of algae or larvae) to 0.18 ppm. Concentrations of phosphorus, nitrate and nitrite increased sharply following the addition of algae, which was assessed either side of the addition of algae feed at time point C (Figure 7). Conversely, DOC concentration, which was assessed more frequently, appeared to decrease when algae was added at time points C, D and E. Maximum levels of nutrients were all within the normal range for seawater (see Table 2) as recorded in a study by Harris et al (1991), in which nutrient levels were assessed at a location in Storm Bay (south east Tasmania) at 10 m below sea level, over a period of four years from 1985 to 1989.

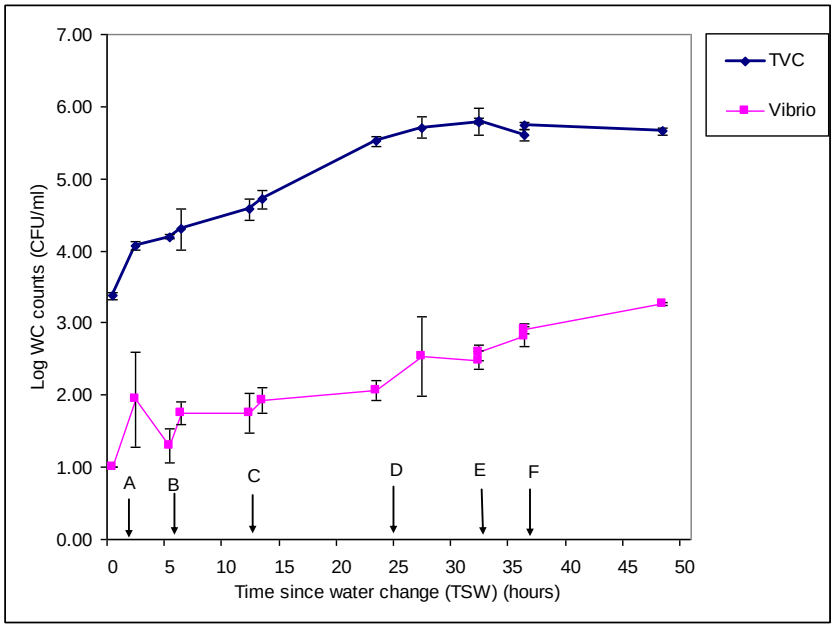


Figure 5 – Bacterial counts in a Batch tank over a 48 h period

Arrows indicate addition of algae feed. A = 40 L Chaetoceros calcitrans ("cal") + 30 L mixed flagellate algae species ("bag feed"). B = 30 L bag feed. C = 80 L bag feed. D = 20 L cal + 40 L bag feed. E = 40 L bag feed. F = 20 L cal + 40 L bag feed

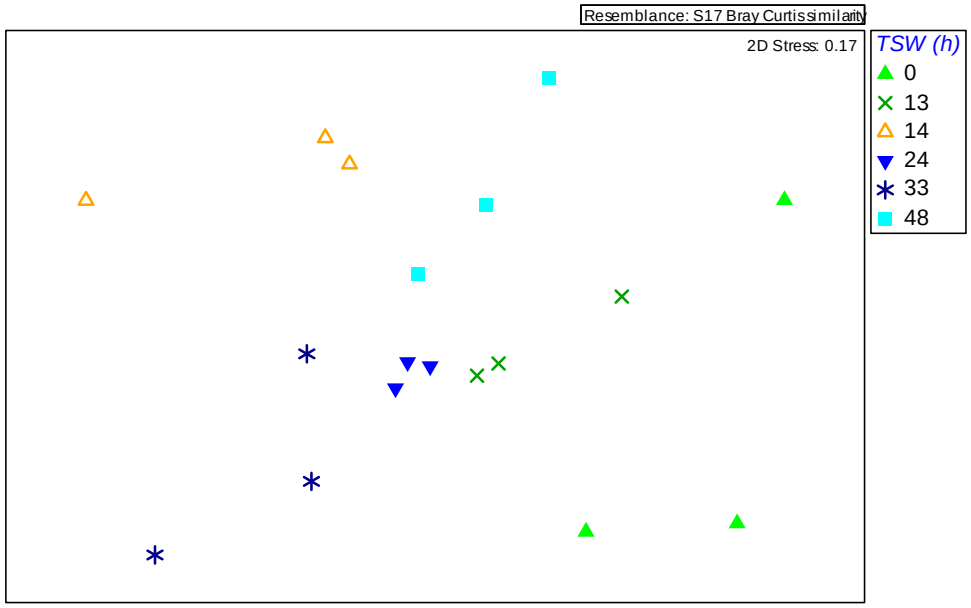


Figure 6 – MDS plot showing changes in WC microbial community composition over a 48 h period

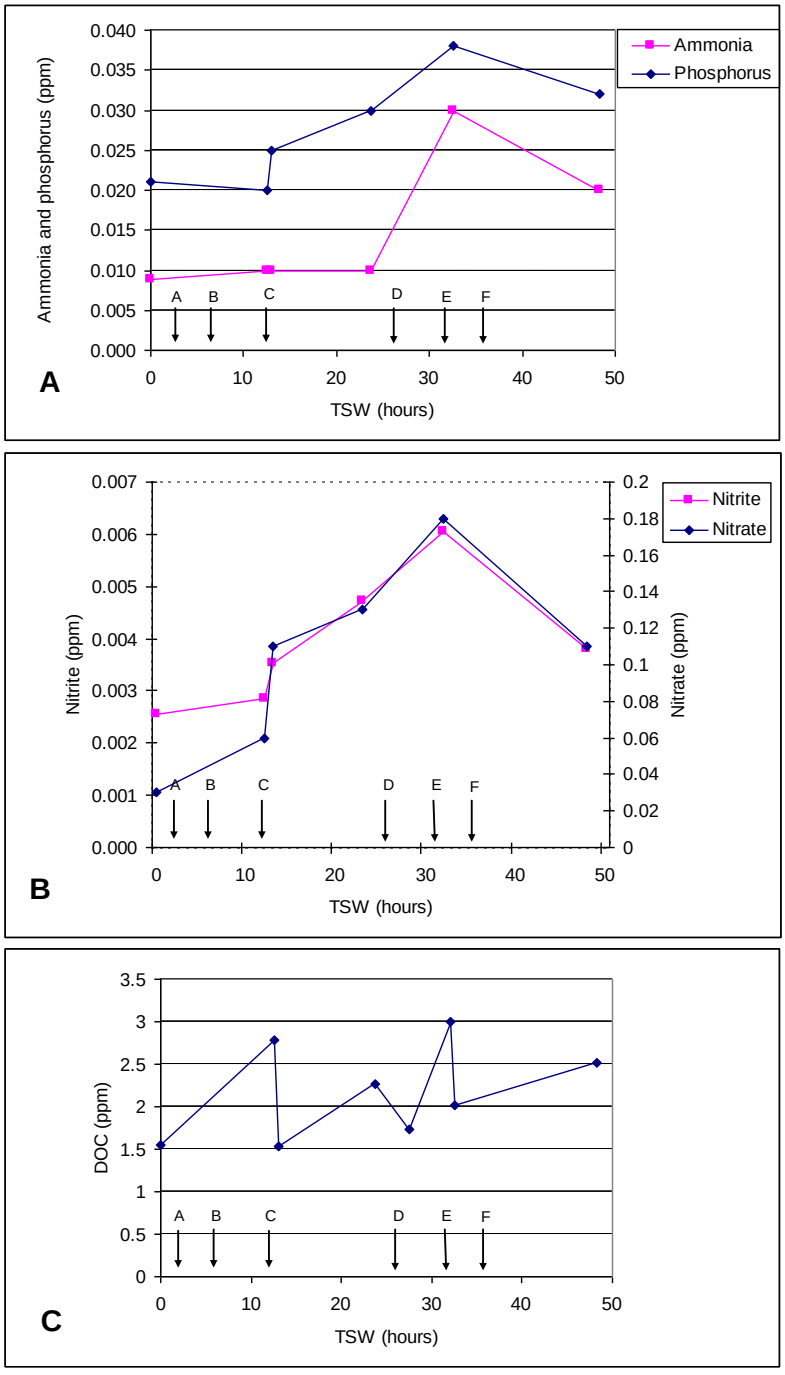


Figure 7 – Dissolved nutrients in the WC over 48 h: A – Ammonia and phosphorus; B – nitrate and nitrite; C – DOC

(1) Arrows indicate addition of algae feed. A = 40L *Chaetoceros calcitrans* ("cal") + 30L mixed flagellate algae species ("bag feed"). B = 30L bag feed. C = 80L bag feed. D = 20L cal + 40L bag feed. E = 40L bag feed. F = 20L cal + 40L bag feed

Table 2 – Comparison of observed nutrient levels with natural seawater

Nutrient species	Maximum level observed	Normal seawater range
Ammonia	0.03	Not reported
Phosphorus	0.038	0.019 - 0.076 ppm
Nitrate	0.18	<0.005 to 0.37 ppm
Nitrite	0.006	0.092 ppm
DOC	3.0	0.6 – 2.4 ppm

2.4.3 Tank type and associated microbial communities

Microbial communities of the WC differed significantly between the two tank types as indicated by statistical analyses in Table 7. TRFLP-1 analysis revealed different microbial composition of the WC between the Batch and IC tanks ($Pr < 0.0001$) as illustrated in Figure 8. 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; $Pr < 0.0001$) as illustrated in Figure 9. By contrast, larvae microbial communities were much less affected by tank type (Table 7). Larvae had similar microbial composition in both tank types (TRFLP-1 analysis) 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) and pH was lower in IC tanks compared to Batch tanks (7.95 and 8.06, respectively).

Table 7 – Summary of statistical analyses with tank type

Dependent variable	Statistical model	Pr	Batch tank mean	IC tank mean
Larvae				
TVC count	Not significant so removed from model[1]			
Vibrio count	Not significant so removed from model			
Vibrio/TVC	Statistical model C	0.0276	3.66 % (+/- 8.43%)	15.75 % (+/-28.58%)
Microbial composition	Statistical model S	0.1869	-	-
WC				
TVC count	Statistical model D	0.0495	5.38 log CFU/ml (+/-5.38)	5.20 log CFU/ml (+/-5.34)
Vibrio count	Statistical model E	0.0017	2.71 log CFU/ml (+/- 3.05)	4.16 log CFU/ml (+/- 4.41)
Vibrio/TVC	Statistical model F	< 0.0001	0.55% (+/-1.56)	10.05% (+/- 12.28)
Microbial composition	Statistical model X	< 0.0001	-	-
pH	Statistical model N	0.0002	8.06 (+/- 0.11)	7.95 (+/- 0.05)
Temperature	Statistical model O	< 0.0001	23.41 ^{o}C (+/- 1.25)	25.89 ^{o}C (+/- 1.82)

Notes: (1) Non significant variables were removed from statistical models to address the problem of unbalanced data.

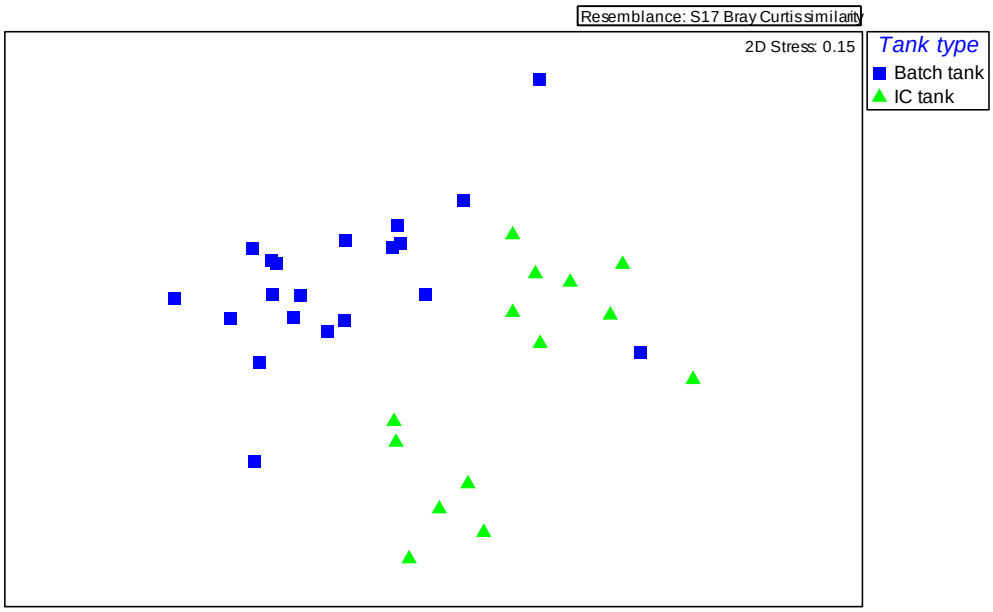


Figure 8 – MDS plot showing microbial composition similarity of WC samples from different tank types

Pr < 0.0001 (Statistical model X)

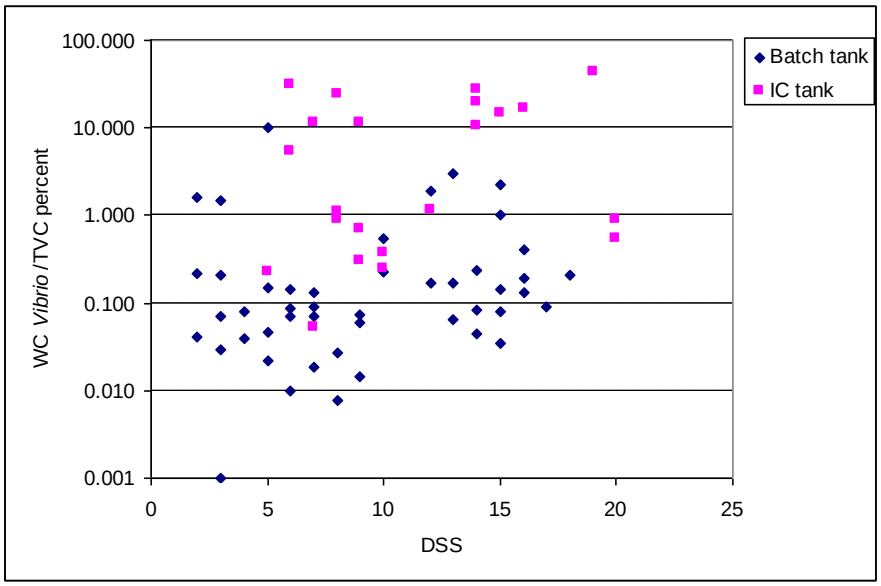


Figure 9 – WC *Vibrio*/TVC percentage over time (DSS) for different tank types

Pr < 0.0001 (Statistical model F)

2.4.4 Compartmentation and variability within larvae tank microbial communities

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. Comparisons of the different sample types using TRFLP-2, represented in Figure 10, indicated that associated microbial communities were significantly different to each other ($Pr < 0.0001$; Statistical model Q). Pair-wise PERMANOVA tests presented in Table 8, using both abundance data and presence-absence data, indicated that all sample types were significantly different except for larvae and eggs ($Pr < 0.0001$ or 0.0002 except for larvae and eggs). The test for larvae and eggs was almost significant using abundance data ($Pr = 0.0647$). 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.

MVDISP based on TRFLP-2 data provided an indication of the magnitude of variability encountered in microbial communities among different samples of the same type. MVDISP data indicated that variability may have differed among sample types in order of decreasing variability: biofilm (1.44), larvae (1.15), WC (0.95), algae (0.94), CTW (0.55) and eggs (0.29); although fewer samples were collected for eggs (Appendix 3) and so the comparison with eggs should be treated with caution.

Unifrac analysis of clone library data indicated that there was no significant difference between an egg sample (E_070909) and both seven DSS larvae (L_140909) and nine DSS larvae (L_160909) from production run 070909 ($Pr = 0.39$ and 1.0, respectively – using only presence-absence data). The relatedness of these samples is represented in Figure 17.

TRFLP-1 analysis, which included a greater number of larvae, WC and egg samples than used in TRFLP-2 analysis, allowed a more in-depth analysis of variability and diversity in these compartments. TRFLP-1 analysis, presented in Figure 11, supports the observation made from TRFLP-2 analysis above that bacterial communities associated with different sample types were different ($Pr < 0.0001$; Statistical model CC). Pairwise PERMANOVA

comparisons given below in Table 9, using both abundance and presence-absence data, indicated that the difference between larvae and WC using TRFLP-1 data was statistically significant and, in contrast to TRFLP-2 and clone library analysis, indicated that there was significant difference between larvae and eggs. Again the differences between sample types were both in species composition and relative abundance.

Table 8 – PERMANOVA pairwise comparisons among sample types using TRFLP-2

Pair-wise comparisons	Abundance data[1]	Presence-absence data[2]
Biofilm, Algae	< 0.0001	< 0.0001
Biofilm, CTW	< 0.0001	< 0.0001
Biofilm, Larvae	< 0.0001	< 0.0001
Biofilm, WC	< 0.0001	< 0.0001
Biofilm, Eggs	< 0.0001	< 0.0001
Algae, CTW	< 0.0001	< 0.0001
Algae, Larvae	< 0.0001	< 0.0001
Algae, WC	< 0.0001	< 0.0001
Algae, Eggs	< 0.0001	0.0002
CTW, Larvae	< 0.0001	< 0.0001
CTW, WC	< 0.0001	< 0.0001
CTW, Eggs	< 0.0001	< 0.0001
Larvae, WC	< 0.0001	< 0.0001
Larvae, Eggs	0.0647	0.1035
WC, Eggs	0.0002	< 0.0001

(1) Statistical model Q (2) Statistical model R

Table 9 – PERMANOVA pair-wise comparisons among sample types using TRFLP-1

Pair-wise comparison	Abundance data[1]	Presence-absence data[2]
Larvae, WC	< 0.0001	< 0.0001
Larvae, Eggs	< 0.0001	0.0002
WC, Eggs	< 0.0001	< 0.0001

(1) Statistical model CC (2) Statistical model DD

MVDISP based on TRFLP-1 data was greatest in the larvae (1.183), followed by the WC (0.811), and then the eggs (0.452) in similar order as was observed using TRFLP-2 analysis. Average similarities of microbial communities within sample types, calculated in PERMANOVA analysis, was lowest in larvae (29.0%), followed by WC (40.8%), and then the eggs (51.5%), which supports MVDISP data in the observation that microbial communities of the larvae were more variable than microbial communities of the WC and the eggs.

Limited analysis of the differences among the composition of bacterial community associated with larvae, WC and CTW was undertaken using culture-based techniques. The *Vibrio*/TVC ratio in each compartment was compared using multivariate statistics, which indicated that sample type was not significant (Pr = 0.1016; Statistical model K). The analysis showed interaction between sample type and tank type although the interaction was not of high significance (Pr = 0.0112). Average values of *Vibrio*/TVC were given in Table 6. In general it appears that *Vibrio* levels were not enhanced, as a proportion of the TVC population, in the larvae or the WC relative to the CTW.

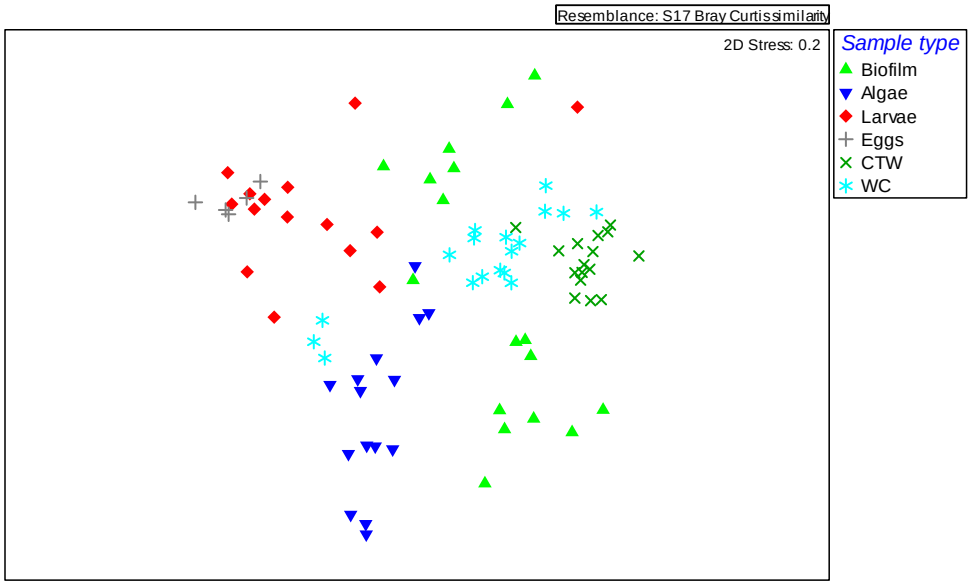


Figure 10 – MDS plot showing microbial composition similarity of sample types using TRFLP-2 data

Pr < 0.0001 (Statistical model Q)

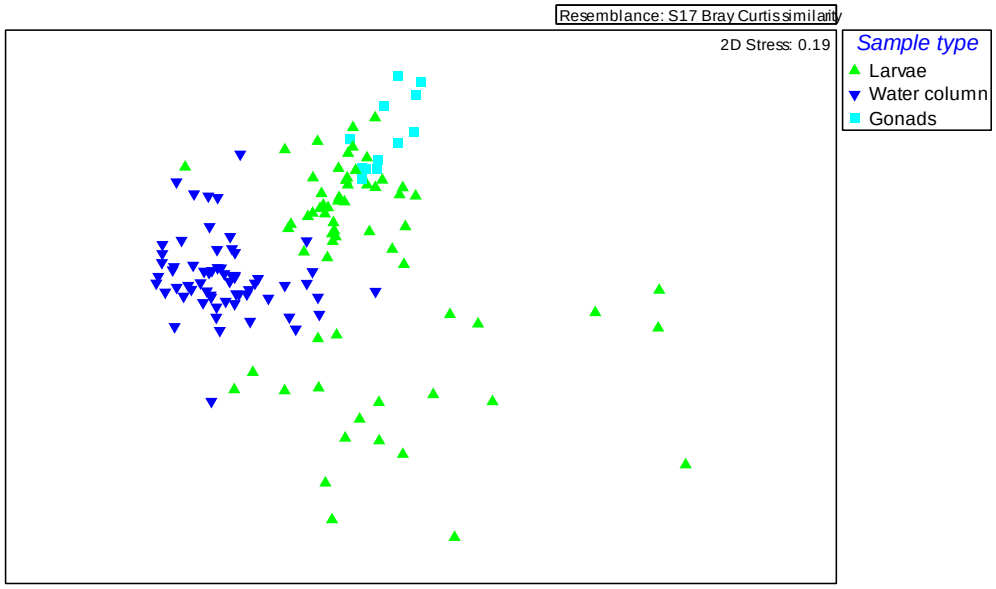


Figure 11 – MDS plot showing microbial composition similarity of larvae, WC and eggs samples using TRFLP-1 data

Pr < 0.0001 (Statistical model CC)

2.4.5 Temporal variability in microbial communities of larvae and WC

Temporal variability in the microbial communities associated with larvae and WC was assessed for sampling period, DSS, and TSW using TRFLP-1 and microbial growth data. Larvae and WC results are reported separately as different environments.

2.4.5.1 Larvae

A number of statistical analyses, summarised in Table 10, were carried out to assess temporal variability in the larvae microbial community using microbial abundance and TRFLP-1 data.

Table 10 – Analysis of temporal variation in microbial abundance and composition in association with larvae

Data source	Independent variable	Statistical model	Pr
Microbial abundance			
TVC counts	Sampling period	Non-significant so removed from model[1]	
	DSS	Statistical model A	0.0064
	TSW	Statistical model G	0.2181
Vibrio counts	Sampling period	Non-significant so removed from model	
	DSS + Disease status	Statistical model B	0.0485
	TSW	Statistical model H	0.964
Vibrio/TVC	Sampling period	Non-significant so removed from model	
	DSS	Statistical model C	0.6717
	TSW	Not tested	
Microbial composition			
TRFLP-1	Sampling period	Statistical model U	< 0.0001
	DSS	Statistical model U	0.0003
	TSW	Not tested	

(1) Non-significant results were removed from microbial growth statistical models due to unbalanced data

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; Statistical model U). MDS plots of sampling period and DSS are presented in Figure 12 and Figure 13,

respectively. Microbial composition of larvae of all sampling periods was different from all other sampling periods as indicated by pair-wise PERMANOVA tests (Table 11). Pair-wise PERMANOVA tests of DSS groupings in Table 11 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.

Table 11 – Pair-wise PERMANOVA tests between microbial communities of larvae from different sampling periods and different DSS

Independent variable	Tested pairs	Pr[1]
Sampling period	Summer 2009, Summer 2010	< 0.0001
	Summer 2009, Spring 2009	< 0.0001
	Summer 2010, Spring 2009	< 0.0001
DSS	> 10 DSS, 2-5 DSS	< 0.0001
	> 10 DSS, 5-10 DSS	0.0063
	2-5 DSS, 5-10 DSS	0.0926

(1) Analysis undertaken using Statistical model U

Microbial growth, as indicated by TVC counts per larvae, was shown to increase with DSS (Pr = 0.0064, Table 10), which may be easily explained by increasing surface area with larvae growth. *Vibrio* counts were on the limits of statistical acceptability (Pr = 0.0485, Table 10) with a statistical model including both sampling period and disease status. Whilst the global statistic was significant the influence of these two variables on Larvae *Vibrio* counts could not be separated.

TSW was not significant for TVC, *Vibrio* or *Vibrio*/TVC (Table 10), which indicates that there was no difference in the density of TVC bacteria or presumptive *Vibrio* bacteria associated with larvae between 24 and 48 h following water change in the Batch tank system.

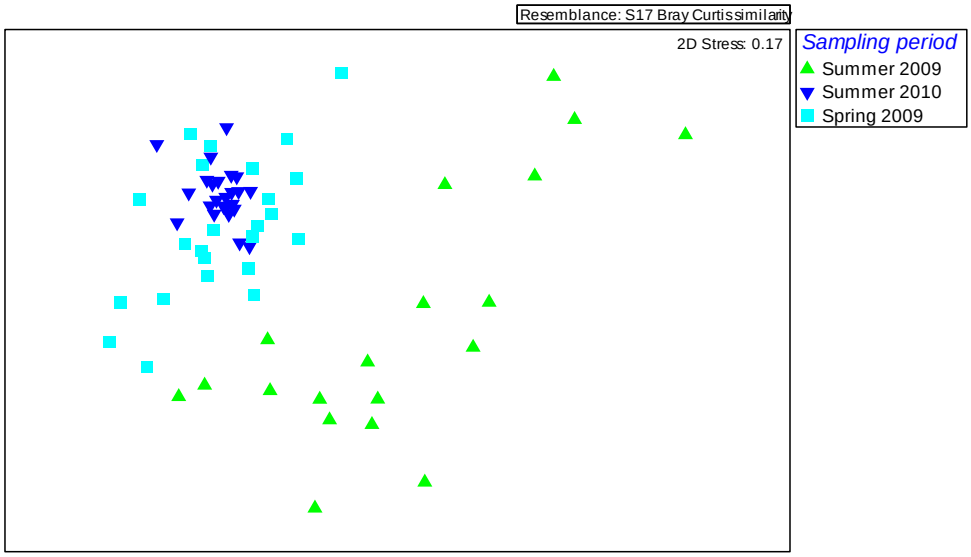


Figure 12 – MDS plot showing microbial composition similarity of larvae samples from different sampling periods

Pr < 0.0001 (Statistical model U)

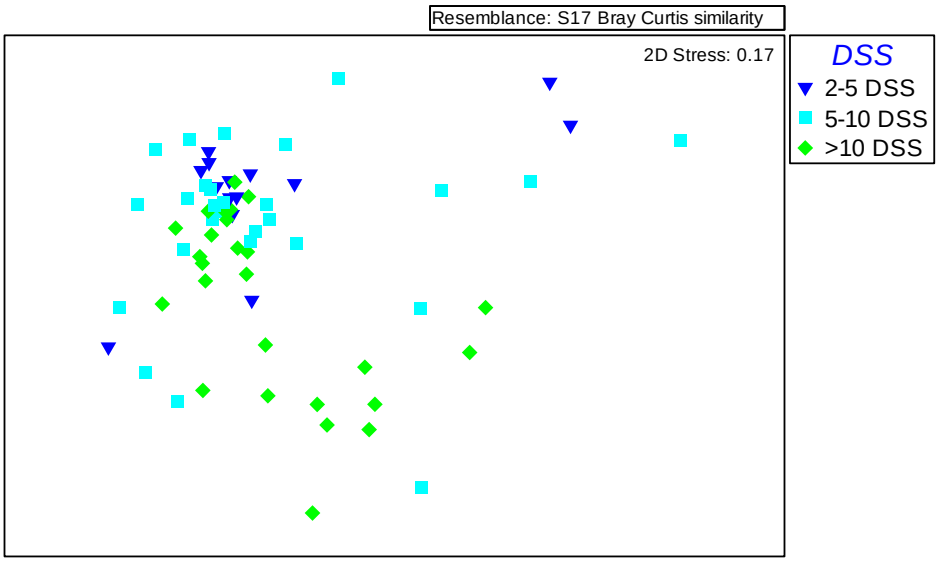


Figure 13 – MDS plot showing similarity of microbial communities of larvae samples from different DSS groupings

$F_{2,66}$ = 3.13, Pr = 0.0003 (Statistical model U)

2.4.5.2 Water column (WC)

A summary of statistical analyses carried out to assess temporal variability in the WC microbial community is given in Table 12.

Table 12 - Analysis of temporal variation in microbial abundance and composition in the WC

Data source	Independent variable	Statistical model	Pr
Microbial abundance			
TVC counts	Sampling period	Statistical model D	0.0084
	DSS	Statistical model D	0.0384
	TSW	Statistical model I	0.1478
Vibrio counts	Sampling period	Non-significant so removed from model[1]	
	DSS	Statistical model E	0.6871
	TSW	Statistical model J	0.1034
Vibrio/TVC	Sampling period	Non-significant so removed from model	
	DSS	Statistical model F	0.7338
	TSW	Non-significant so removed from model	
Microbial composition			
TRFLP-1	Sampling period	Statistical model Z	< 0.0001
	DSS	Statistical model Z	< 0.0001
	TSW	Not tested	

(1) Non-significant results were removed from microbial growth statistical models due to unbalanced data

Microbial composition of WC samples was affected by both sampling period and DSS (Pr < 0.0001 for both variables). The statistical model used excluded IC tank samples from the analysis because tank type was shown to be a significant factor in influencing microbial communities in the WC and the data were insufficiently balanced to include all variables of interest in the model. MDS plots of sampling period and DSS are given in Figure 14 and Figure 15, respectively, which also exclude IC tank samples.

Microbial composition of WC of all sampling periods was different from all other sampling periods as indicated by pair-wise PERMANOVA tests (Table 13). Pair-wise PERMANOVA tests of DSS groupings in Table 13 showed that all DSS groups were

different although the difference between 2-5 DSS and 5-10 DSS was more highly significant.

Table 13 – Pair-wise PERMANOVA tests between microbial communities of larvae from different sampling periods and DSS

Independent variable	Tested pairs	Pr[1]
Sampling period	Summer 2009, Summer 2010	< 0.0001
	Summer 2009, Spring 2009	< 0.0001
	Summer 2010, Spring 2009	< 0.0001
DSS	> 10 DSS, 2-5 DSS	0.014
	> 10 DSS, 5-10 DSS	0.0033
	2-5 DSS, 5-10 DSS	< 0.0001

(1) Analysis undertaken using Statistical model Z

Microbial growth as indicated by TVC counts was shown to differ with both Sampling period (Pr = 0.0084) and DSS although DSS was only marginally significant (Pr = 0.0384). TVC counts were greatest in the sampling period Spring 2009 (5.45 log +/- 5.43 log), followed by Summer 2009 (5.17 log +/- 5.24 log) and Summer 2010 (5.06 log +/- 4.93 log). LSD groupings indicated that Spring 2009 was significantly different from Summer 2010.

TSW was not significant for TVC, *Vibrio* or *Vibrio*/TVC (see Table 12), which indicates that there was no difference in the abundance of TVC or *Vibrio* bacteria associated with the WC between 24 h and 48 h following water change in the Batch tank system. These results are in agreement with results obtained in 48 h monitoring (section 2.4.2) for TVC counts although appear to differ with *Vibrio* counts, as the 48 h monitoring showed an increase in *Vibrio* counts of one order of magnitude between 24 h and 48 h (Figure 5). However, the analysis given in Table 12 should be given more weight due to statistical rigour and so it is accepted that *Vibrio* counts did not increase significantly between 24 h and 48 h.

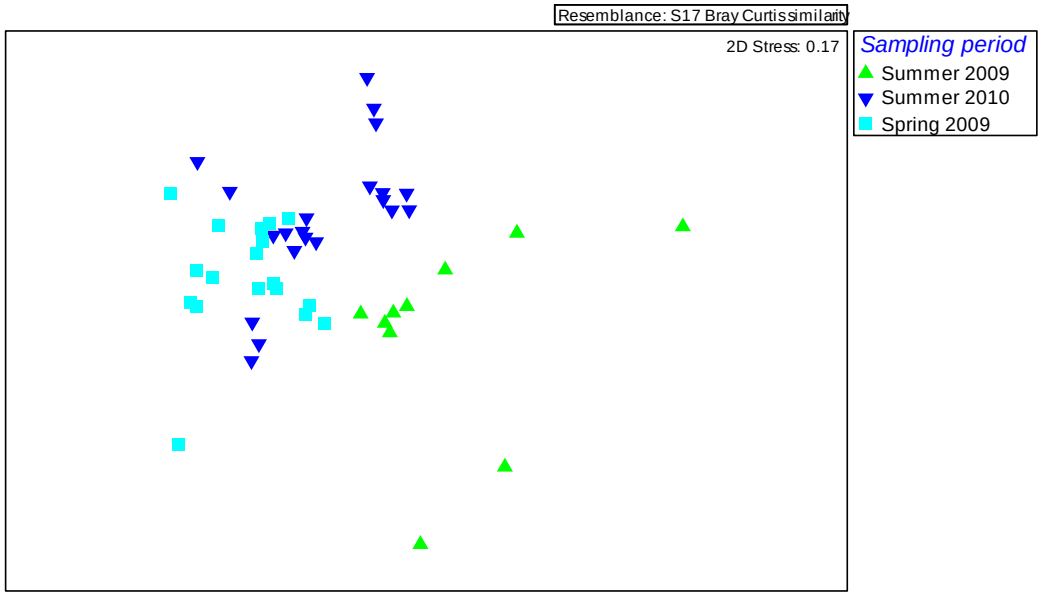


Figure 14 - MDS plot showing similarity of microbial communities of WC samples from different sampling periods

Pr < 0.0001 (Statistical model Z)

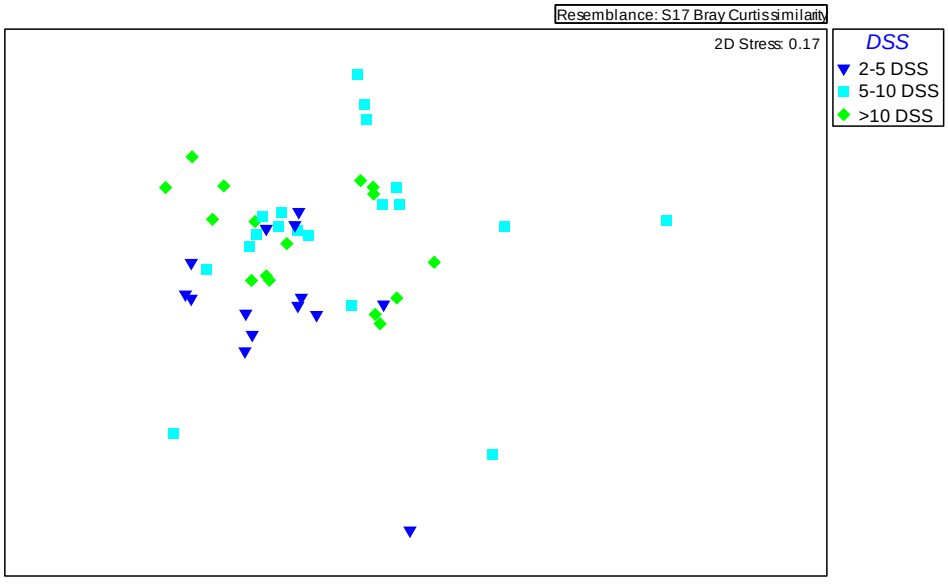


Figure 15 - MDS plot showing similarity of microbial communities of WC samples from different DSS groupings

Pr < 0.0001 (Statistical model Z)

2.4.6 Characterising microbial communities of sample types

Identification of bacterial species associated with different sample types was undertaken using clone libraries. A total of 18 clone libraries were generated including algae (3), CTW (1), eggs (1), biofilm (3), WC (2) and larvae (8), from both IC and Batch tank systems. Clone library details including number of clones, number of phylotypes, coverage, species diversity and phylogenetic diversity are given in Table 14.

Table 14 – Clone library details including coverage and species diversity

Sample source	Sample name and date	Tank type	No. of clones	No. of phylotypes	Coverage (%) (Goods formula)	Species diversity: Chao-1 estimator (+/- StDev)	Phylogenetic diversity: Faith's index
Algae	A_101209	-	56	17	88	21.1 (+/-3.4)	78
Algae	A_150909	-	64	14	95	15.5 (+/-1.9)	61
Algae	A_200110	-	67	11	94	15 (+/-4.3)	66
Biofilm	B_031009	Batch	58	15	84	35.3 (+/-14)	70
Biofilm	B_100209	IC	93	34	81	74.5 (+/-19.8)	117
Biofilm	B_160909	IC	92	15	93	33 (+/-15.2)	74
CTW	C_150909	-	74	29	78	93 (+/-33)	103
Moribund larvae	D_031009	Batch	114	14	96	18.2 (+/-4)	72
Moribund larvae	D_100209	Batch	73	15	90	23.2 (+/-6.5)	71
Moribund larvae	D_150909	IC	88	10	94	16.3 (+/-5.9)	54
Larvae	L_030209	Batch	62	10	89	22.3 (+/-9.6)	47
Larvae	L_050209	IC	40	4	98	4.5 (+/-1.1)	24
Egg	E_070909	-	65	11	91	29 (+/-15.2)	74
Larvae	L_140909	IC	83	22	88	32 (+/-6.7)	91
Diseased larvae	L_160909	IC	75	18	92	27 (+/-7.7)	90
Larvae	L_260909	Batch	90	17	91	27.7 (+/-7.9)	64
WC	W_140909	IC	94	20	88	80.5 (+/-37.7)	80
WC	W_160909	IC	102	21	90	71 (+/-32.6)	87

Percentage coverage, according to Goods formula for all clone libraries was relatively high (Table 14). The smallest coverage value of 78% was recorded for C_150909, which indicated that 78% of the species diversity was captured in the clone library. Other clone library coverage values ranged from 81 to 98%. Thus all clone libraries captured most of the sample diversity though rare taxa may not have been identified.

Species composition data from clone libraries is summarised by class for the different sample types in Figure 16. Bacterial community composition in all sample types was dominated by *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.

A total of 120 phylotypes among the 1390 clones obtained across all 18 clone libraries (detailed in Appendix 5). Phylogenetic trees were constructed for all classes encountered and were used to identify phylotypes to the nearest matching type species or genus and are shown in Appendix 7- Appendix 13. Species matches of the ten most predominant phylotypes for each sample type are given in Appendix 6 with percentage similarity and relative predominance in other sample types.

The most commonly encountered bacterial species in all sample types, as presented in Appendix 6, were typical marine heterotrophs. *Ramlibacter* and *Sphingomonas* sp., which were the two most prevalent species in the egg clone library and were also common in larvae clone libraries, are not typical marine species, being more commonly associated with soil or fresh water. 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. Similarly *Janthinobacterium*, which was only encountered in the biofilm, is more typically found in soil samples. Members of the *Roseobacter* clade were particularly common in all parts of the system while SAR 11 species were absent.

Sample types with more ubiquitous phylotypes (present in at least 4/5 of the algae, WC, CTW, biofilm and larvae) included the WC, biofilm and algae with 7, 6 and 5, respectively (Appendix 6), whereas larvae, CTW and eggs had a smaller number of ubiquitous phylotypes with 3, 2 and 2, respectively. Although comparisons among these sample types using clone library summaries need to be made cautiously due to unequal numbers of libraries in each type as well as differences in sampling date, this data agrees with the Unifrac PCA analysis (proceeding section 2.4.8) that larvae and CTW had more distinctly different bacterial communities than WC, biofilm and algae.

2.4.7 Diversity of bacterial communities and sample type

Bacterial community diversity of different sample types was evaluated using clone libraries and TRFLP-1 data.

Clone library

Species diversity as indicated by species richness varied substantially among clone libraries of all samples from as low as 4.5 (L_050209) to 93 (C_150909) using Chao-1 estimator as shown in Table 14. Phylogenetic diversity also varied substantially from 24 (L_050209) to 117 (B_100209) using Faith's index.

Due to differences in number of clones, sampling dates and production run factors, comparisons among clone libraries need to be made cautiously. Nevertheless it is worth noting that none of the eight larvae clone libraries were as species rich as the two WC samples or the CTW sample (Table 14). It is also noteworthy that differences in species diversity between larvae and WC samples were greater than differences in phylogenetic divergence.

TRFLP

Diversity of the bacterial communities associated with larvae and WC was assessed using TRFLP-1 analysis in which three diversity indices (Shannon-Weiner (H'), Margalef's (d), and Pielou's evenness (J') were generated as summarised in Table 15.

Table 15 – Species diversity indices from TRFLP-1 data comparing larvae, WC and egg samples

	Shannon-Weiner index (H')		Margalef's richness index (d)		Pielou's evenness index (J')	
	Average	LSD grouping	Average	LSD grouping	Average	LSD grouping
Larvae	3.54 (+/- 0.47)	A	8.46 (+/- 2.34)	A	0.894 (+/- 0.029)	A
WC	3.85 (+/- 0.36)	B	11.16 (+/- 3.12)	B	0.902 (+/- 0.018)	A
Eggs	3.21 (+/- 40)	C	5.76 (+/- 2.36)	C	0.904 (+/- 0.020)	A
LSD	0.21		1.90		0.0191	
Pr	<0.0001		<0.0001		0.2101	
Model	Statistical model D		Statistical model E		Statistical model F	

The Shannon-Weiner index (see Table 15) showed 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'): $Pr = 0.6273$)).

Hence it appears that the clone library and TRFLP data are in agreement that species richness was lower in the larvae and the egg samples compared to the WC. Clone library data indicates that there may be no difference between larvae and WC in terms of phylogenetic diversity.

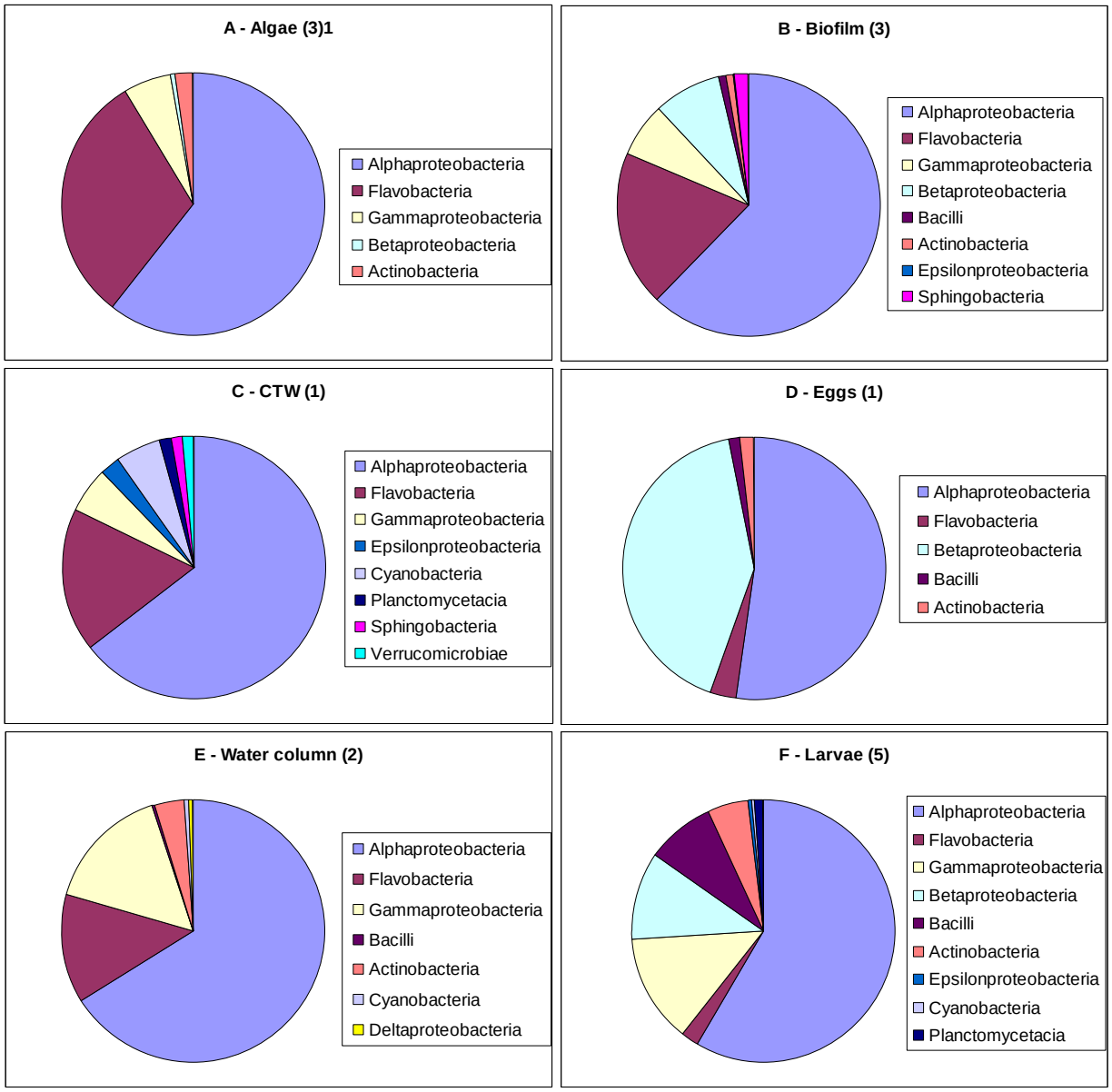


Figure 16 – Summary of clone libraries for different sample types:

Notes: (1) Numbers in brackets indicates the number of clone libraries represented
A = Algae (3 sample, 187 clones); B = Biofilm (3 sample, 243 clones); C = CTW (1 sample, 74 clones); D = Eggs (1 sample, 65 clones); E = WC (2 samples, 196 clones); F = Larvae (8 samples, 350 clones).

2.4.8 Relationships among microbial niches of the larvae tank

Comparisons using clone library data provided insight into the relationships among sample types. To eliminate production run variability, comparisons in this section were made among clone libraries from only one production run (070909). It should be noted that production run 070909 was conducted in the IC tanks and so caution needs to be taken in extrapolating findings to Batch tanks, especially since microbial communities associated with the WC were shown to be different (section 2.4.2). Larvae microbial communities, on the other hand, were not different between tank types and differences in the biofilm communities between tank types were not assessed. Algae and CTW were not expected to differ between tank types as they were obtained from the same source and differed only in using different delivery lines. Clone library data are summarised in Table 14 and samples used include the following: A_150909, L_070909, L_140909, L_160909, C_150909, B_160909, W_140909, and W_160909.

For clone library comparison, a phylogenetic distance based analysis was conducted using the Unifrac PCA function, which is presented in Figure 17. PCA analysis indicated that WC, algae and biofilm were similarly related (as evidenced from clustering) when only presence-absence data were analysed but less similar when abundance data were included. Conversely CTW was distinctly different from WC, algae, and biofilm when only presence-absence data were used but similar when abundance data were included. This suggests that the WC, algae and biofilm shared many of the same (or phylogenetically similar) bacterial species but in different proportions whereas CTW species composition was different but may have had some similar predominant species. Note that diseased larvae are also included in the figure and are discussed in Chapter 3.

In support of TRFLP-2 data (Figure 10) larvae and eggs plotted closely in the PCA plots (Figure 17), using both abundance and presence-absence data, which indicated a similarity in species composition and relative species abundance, but were distinctly different from the other sample types regardless of whether abundance data were used or not.

In addition to PCA analysis and Unifrac significance testing, clone library data were analysed to determine the number of common and unique phylotypes among libraries from production run 070909. Two separate comparisons were made: one including all the different libraries of the larvae tank (Table 16) sampled on 15/09/09 and 16/09/09; and a second analysis between larvae and eggs (Table 17).

Table 16 – Unique and common phylotypes amongst samples from production run 070909

Sample type	Clone library ID	No. of unique phylotypes[1]	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

Notes: (1) No. of unique clones is in comparison to all other samples combined.

Analysis of unique phylotypes in Table 16 indicates that all samples had a number of phylotypes that were not found elsewhere in the larvae tank, which would contribute to the difference observed among sample types in Unifrac analysis (see (A) in Figure 17). CTW had the largest number of unique phylotypes (22/29), followed by larvae (10/18), biofilm (6/15), WC (4/21) and algae (3/14). The WC may be considered a subset of the other sample types since only 4/21 phylotypes were unique and it had the highest number of shared phylotypes with each sample type.

CTW had the greatest number of unique phylotypes with 22/29 (76%) not found elsewhere in the larvae tank, which may indicate that the majority of CTW bacteria were not well suited to the conditions of the larvae tank. Of the 6 phylotypes CTW shared with the WC, three were absent from the algae.

Only 3/14 phylotypes of the algae were unique, which may suggest that algae bacteria were well suited to the conditions in the larvae tank with a large number of phylotypes persisting in the WC (10/14) and to a lesser extent in the biofilm (6/14). These bacteria could be less suited to colonisation of larvae (4/14 similar phylotypes). Of the 10 phylotypes algae shared with the WC, seven were absent from the CTW.

Comparison of the number of unique and similar phylotypes in the clone libraries of eggs and larvae (E_070909, L_140909 and L_160909) in Table 17 provided further insight into the relationship between the two sample types.

Table 17 - Unique and common phylotypes between clone libraries of larvae and eggs (from production run 070909)

	No. of phylotypes:		
Larvae age (DSS)	**Present in both egg and larvae**	**Unique to Larvae**	**Unique to eggs**
7	9	13	2
9	9	9	2

Data in Table 17 shows that approximately 50% (9/21 and 9/18 for seven DSS and nine DSS larvae, respectively) of phylotypes found in larvae clone libraries were also encountered in egg samples. The small number of phylotypes unique to eggs (2/11 in both comparisons) indicates that the majority of bacteria in egg samples (9/11) persisted as significant members of the larvae bacterial community till at least nine DSS.

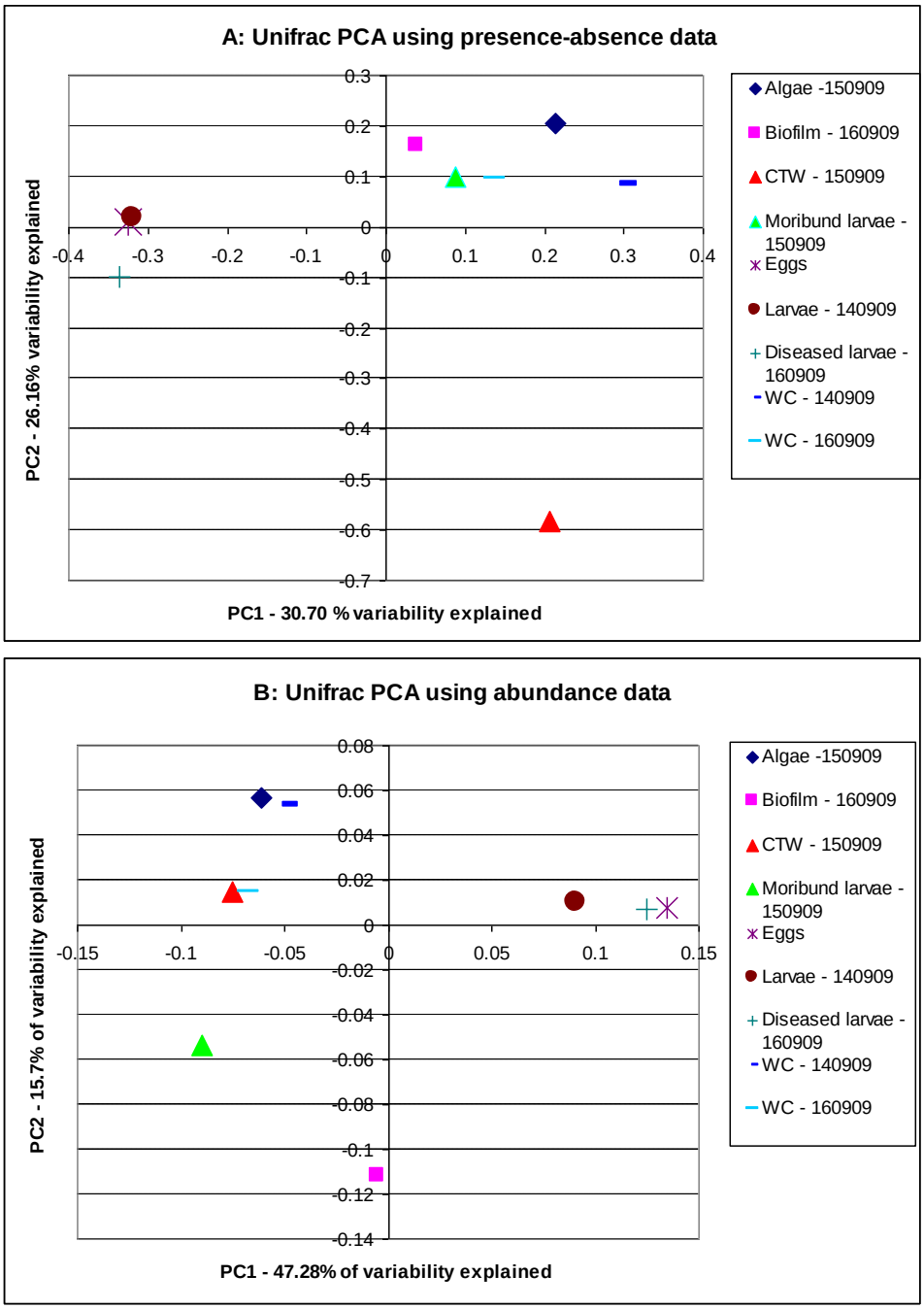


Figure 17 - Unifrac PCA analysis of clone libraries of different sample types from production run 070909

(A) Only presence-absence data were used in this analysis and two vectors are shown, which represent 57.8% of the variability encountered. (B) Abundance data were used in this analysis and two vectors are shown, which represent 63.0% of the variability encountered

2.5 Discussion

2.5.1 Response of larvae and WC microbial communities to culture conditions

Microbial communities of the WC differed significantly between the two tank types (section 2.4.3), which indicates that WC microbial communities can be substantially influenced by conditions of culture. TRFLP-1 analysis revealed different microbial composition of the WC between the Batch and IC tanks ($Pr < 0.0001$). 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 ($Pr < 0.0001$). By contrast, larvae microbial communities were much less affected by tank type. Larvae had similar microbial composition in both tank types (TRFLP-1 analysis) 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 in the WC caused by altered culture conditions.

Culture conditions in the IC tank differed from the Batch tank with higher densities of larvae, continuous feeding, and continuous water exchange as described in section 2.3.2. Temperature was slightly higher ($P < 0.0001$) and pH slightly lower in IC tanks ($P = 0.0002$) compared to the Batch tanks. Elevated total levels of organic matter, higher temperature, and lower pH in the IC tank may have favoured *Vibrio* spp., which are known to play an important role in the decomposition of both particulate and dissolved organic matter (Thompson et al 2006) and respond rapidly to nutrient pulses (Rehnstam-Holm et al 2010). For example, Larsen (1985) reported a 1,000-fold increase in *V. anguillarum* in seawater off the Danish coast due to discharge of carbohydrate-containing waste water. It was noted in the 48 h monitoring study (section 2.4.2), that the *Vibrio* population responded more rapidly to additions of algae than the TVC population, although such additions did not appear to be associated with elevated levels of dissolved nutrients in the WC, which remained within a normal range for east coast Tasmanian seawater.

Flow-through systems are assumed to avoid organic pulses by allowing organic matter to be removed from the tanks at a constant rate and by continuous addition of algae rather than

batch feeding (Andersen et al 2000). This is thought to favour development of a microbial community dominated by slow growing (k-selected) species, which have been proposed to be less likely to cause disease (Skjermo & Vadstein 1999). The present study did not support these findings as the IC system (flow-through) favoured *Vibrio* spp., which are fast-growing (r-selected) species. However, the comparison between tank types is limited due to the differences in density in the different tank types. The increase in *Vibrio* spp. might relate simply to the higher level of organic matter (particulate or DOC) in the IC tank.

2.5.2 Relationships among microbial niches of the larvae tank

Within the respective production systems culture conditions in the larvae tank, which were controlled and optimised for larvae growth, were consistent among production runs and seasons, and could not account for variability encountered in larvae and WC microbial communities. In contrast, the major bacterial inputs into the larvae tank, including eggs, seawater and algae (Elston 1984), were not controlled and consequently may have caused variability within microbial communities of the larvae tank. Although not assessed in this study, algae starter-cultures are often inoculated with particular strains of bacteria that have a mutualistic relationship with algae species, but otherwise bacterial inoculation is left to chance (Nicolas et al 2004). Seawater was used as a culture medium for algal cultures but pasteurisation at 80^{o}C for 60 min reduced the associated microbial diversity such that this was not considered a major route for bacteria into the larvae tank. Treatment of seawater for larviculture involving filtration to 1-10 µm is unlikely to have excluded many bacteria, which are mostly free-living cocci less than 0.5 µm (Ferguson & Rublee 1975). While heating of water 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. Similarly 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).

TRFLP data indicated that microbial community composition of the larvae, eggs, WC, biofilm, CTW and algae were all different (section 2.4.4). Relationships among microbial

niches of the larvae tank are depicted schematically in Figure 18. While the eggs, algae and seawater microbial communities originated from outside the larvae tank and were subject to independent influences, the communities of the WC, biofilm and larvae were dependent upon these inputs. Accordingly, clone library analysis (section 2.4.8) indicated that microbial communities associated with the IC tank WC at nine DSS, had only 4/21 unique phylotypes and may have been a subset of other sample types. During the early stages of larvae development, from fertilisation through to umbo stage (approximately two DSS), larvae were not fed xenic algae, instead receiving only very small amounts of axenic diatom algae (approximately 20 L of diatom culture in 11,500 L Batch tanks in the first 24 h following spawning). Hence during spawning and the first two days of a production run algae-associated bacteria were absent from the larvae tank and so Figure 18 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 indicated 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 2.4.4). Clone library comparisons of samples from production run 070909 showed that the majority of phylotypes in fertilised egg samples (9/11) were also present in larvae samples from seven and nine DSS, which indicates that the indigenous microbial community of the fertilised eggs was resistant. This finding is consistent with section 2.5.1 in which larvae microbial communities were resistant to altered microbial communities of the WC under different culture conditions. 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, and persists throughout much of the larval

period (Olafsen 2001). Elston (1989) supports this idea and adds that broodstock can be a major source of contamination of bivalve culture systems. Significantly, in the present study formation of the indigenous microbial community occurred prior to the addition of xenic algae.

Algae-associated bacteria mostly survived in the IC tank WC at nine DSS with 10/14 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 2.4.8). In contrast the majority of phylotypes (22/29) detected in the CTW were not detected in the WC (three of the six phylotypes CTW shared with the WC were absent from the algae), which indicates that the majority of CTW bacterial species were not competitive in the cultural conditions of the IC tank. Unifrac PCA analysis, using presence-absence data indicated that algae, WC and biofilm microbial communities were similar, which suggests a primary role of algae in determining the species composition of the WC and biofilm in the IC tank (Figure 17). Although when abundance data were used CTW plotted close to algae and WC samples in the Unifrac PCA plot, which suggests 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 system over 48 h at 13 DSS showed that algae were the largest source of bacteria ($Pr < 0.0001$; section 2.4.1.2). 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 suggests a primary role of algae in influencing Batch tank microbial communities (after two DSS), as was 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 must be dependent upon feed demand, which was greatly reduced at the beginning of the production run when the influence of CTW on larvae tank microbial communities at the beginning of the production run would have been greater.

Algae microbial communities may have affected larvae communities directly through concentration of algae and associated bacteria in the larvae intestinal tract. There was not significant evidence that this occurred since comparison of clone libraries from production run 070909 indicated that only 4/14 of larvae phylotypes were also found in the algae, which had a total of 18 phylotypes (section 2.4.8). Unifrac PCA analysis indicated that larvae and algae were not similar using either presence-absence or abundance data (Figure 17). These results indicate that algae-associated bacteria mostly did not persist in the larvae's digestive system or were numerically overwhelmed by bacteria consumed through continuous drinking or bacteria already resident in the larvae. A study by McIntosh et al (2008) made similar findings with only 2/15 bacterial phylotypes associated with feed-rotifers 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.

While the influence of biofilm on microbial community variability in the larvae tank was not investigated in the present study, work by Verschuere et al (1997), described in the Introduction (section 2.2), demonstrated that biofilm can influence the WC. Analysis of the number of shared phylotypes (section 2.4.8) in samples from production run 070909 showed that the biofilm and WC shared 8/15 phylotypes identified in the biofilm, which indicates a relationship between the biofilm and WC communities that is likely to be interdependent. As Verchuere's work showed the influence of biofilms is dependent largely upon cleaning and sanitation practices between water changes.

Due to the intimate association of larvae and WC it seems likely that their associated microbial communities may have been related. The correlation between larvae *Vibrio* counts and WC *Vibrio* counts ($Pr < 0.0001$; section 2.4.1.1) supports this idea; yet the question may be asked whether one had greater influence over the other. 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. Gatesoupe further states that the intestinal microbial composition may change rapidly with the intrusion of bacteria coming from water and food. By numbers alone the present study supports the primacy of the WC microbial community as it was shown in

section 2.4.1.2, that the average total number of TVC bacteria associated with the WC at 48 h (TSW) out-numbered those directly associated with the larvae by 176-fold in the Batch tank ($Pr < 0.0001$). Although larvae were resistant to the full scale of change experienced in the WC (section 2.5.1), it seems that WC and larvae microbial communities may have had an unequal relationship in which the larvae-associated community was influenced by the WC community rather than vice versa.

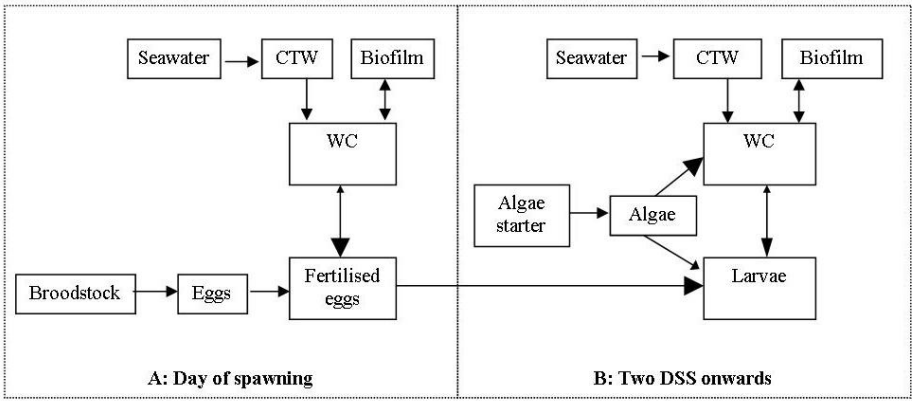


Figure 18 – Relationships among microbial niches of the larvae tank

A: On the day of spawning fertilization occurs in a bucket before being transferred to a Batch tank after approximately one hour. B: After two DSS xenic algae are added to the feed mix and the quantity of this addition is dependent upon larvae feed demand

2.5.3 Variability in microbial communities of the larvae and WC between water changes

Analysis of microbial change in the Batch tank over 48 h indicated a rapid increase in the WC TVC and *Vibrio* population over the first 24 h followed by no significant change in the TVC population, *Vibrio* spp., and microbial composition between 24 h and 48 h (TSW), indicating that a relatively stable microbial community was reached after 24 h and maintained until 48 h, when the culture period finished and larvae were moved to new tanks (section 2.4.5). A similar study was undertaken by Verschuere et al (1997) in an *Artemia* tank in which a strong and rapid increase in the TVC count to $10^8 – 10^9$ CFU/ml was observed followed by a two-log reduction over the six day culture period. A proportional

increase in r-strategists in the culture water was observed with sampling time including an increase in *Vibrio* spp. and *Pseudomonas* spp., which Verschuere et al attributed to the eutrophic conditions of the *Artemia* tank. No such reduction in TVC or increase in *Vibrio* spp. was seen in the present study although the culture period was much shorter (two days compared to six).

2.5.4 Sampling period variability in microbial communities of the larvae and WC

Sampling period affected the microbial composition of the larvae and the WC equally (Larvae: $Pr < 0.0001$. WC: $Pr < 0.0001$; section 2.4.5) and in both larvae and WC the sampling period, Summer 2009, was the most divergent, which indicates that sampling period variability in both larvae and WC may have been due to a common influence. The close relationship between the larvae and WC microbial communities (section 2.5.2), in which the WC community had influence over the larvae community, suggests that the influence of sampling period on larvae may have been mediated through the WC. However, the larvae microbial community was shown to be resistant to the full scale of change occurring in the WC microbial community (section 2.5.1), which does not explain the equal influence of sampling period on WC and larvae microbial communities if mediated through the WC. The larvae microbial community was shown to be related to the indigenous microbial community of the fertilised eggs, which was influenced by the initial egg microbial community and the WC. Thus, sampling period may have exerted influence on the larvae microbial community through the WC during formation of the indigenous microbial community when the developing eggs were prone to greater influence from the WC. Differences among the initial egg microbial communities of different production runs may have contributed towards sampling period variability in the larvae but not in the WC since larvae communities are unlikely to have substantially influenced WC communities (section 2.5.2).

Since algae associated microbial communities had a significant effect on the WC microbial community (section 2.5.2) it follows that variability in the algae microbial community might

explain sampling period variability in the WC microbial community. The existence of such a relationship may be more tenable if the temporal variability in the algae were similar to sampling period variability observed in the WC; that is relatively consistent over the period of weeks and divergent over the period of months. In a related study in the same hatchery, change in the microbial communities of algal cultures was observed to occur on a monthly basis (Powell & Tamplin 2011). Variability of algae microbial communities over the time frame of weeks was not assessed but may have been minimised by cultural practices of keeping continuous flow algae cultures at stationary growth phase for periods up to three months and using 10-15 individual cultures to produce a composite harvest. Monthly variation in algae microbial communities may have occurred in response to changing temperature as the algae production facility was not temperature controlled and therefore prone to fluctuations with season. Additionally, algal culture nutrient mix was halved during the monitoring period between Spring 2009 and Summer 2010 and this may have contributed to observed monthly variability. Thus it appears that variability in the algae microbial community may have contributed towards sampling period variability in the WC. However, since algae did not contribute towards formation of the indigenous microbial community of the larvae and larvae communities were resistant to change in the WC, it seems unlikely that variability in the algae microbial community could account for the equal influence of sampling period in both the WC and the larvae if mediated through the WC. Algae microbial communities may have alternatively influenced larvae directly through larvae ingestion of algae and concentration of algae associated bacteria in their digestive tract. However, there was little evidence that this occurred (section 2.5.2) and thus it seems unlikely that variability in algae microbial communities could have caused sampling period variability in the larvae.

Seawater microbial community composition is known to vary temporally with predictable seasonal patterns (Nogales et al 2010) and thus may be suspected of causing sampling period variability in the larvae and WC microbial communities. The CTW microbial community is likely to have reflected seawater variability (section 2.5.2) and may have had primary influence in formation of the indigenous microbial community of larvae. Thus seawater variability may have accounted for the equal influence of sampling period on

larvae and WC microbial communities. Evidence section 2.5.2 did not indicate that the CTW had a large effect on the WC microbial community relative to the algae in later stages of the production cycle but at earlier stages of the production run, when feed demand was low, the influence of CTW would have been greater.

While only limited investigation was undertaken to characterise the relationship between biofilm structures and WC microbial communities, the propensity for biofilms to influence WC microbial communities has been demonstrated by Verschuere et al (1997), as detailed in the Introduction (section 2.2). Evidence from Verschuere, indicated that rigorous cleaning and sanitation could cause much variation within the remnant biofilm and as such, it seems unlikely that biofilm could account for the relative stability in microbial communities of the larvae and WC observed within production runs in the present study. Since no assessment was made of remnant biofilm structures following cleaning and sanitation, their influence on microbial communities of the larvae tank remains speculative.

2.5.5 DSS variability in microbial communities of the larvae and WC

DSS was a significant factor causing 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 2.4.5). Larvae and WC were subject to a different set of influences (Figure 18), none of which could singularly account for equal variation with DSS in both sample types. Any influences mediated through the WC, such as seawater variability and biofilm influence, could not account for equal variation in the larvae due to the resistance of larvae microbial communities to changes occurring in the WC (section 2.5.1).

Algae microbial communities had a significant effect on the tank WC (section 2.5.2) and it follows that variation in the quantity and quality of algae added to the larvae tank may explain variation in microbial communities of the WC and the larvae. In general the quantity of xenic algae added to the tank increased with larvae feed demand over the production run. Changes in algae species composition in the feed-mix due to changing larvae dietary requirements over the production period may have also affected WC

microbial communities since different algae species may harbour different microbial species (Sandaa et al 2003). Such changes in the WC microbial community are likely to have altered the resistant larvae microbial community to a lesser degree. Algae microbial communities might have affected larvae communities directly through concentration of algae in the larvae intestinal tract but as discussed above (section 2.5.2) there was little evidence that this occurred.

The larvae microbial community would have been subjected to age dependent influences that may not have affected the WC, such as microbial succession. TRFLP-1 MDS plots of the different DSS groupings did not present clear evidence of a characteristic microbial community developing with larvae age (Figure 13), although samples older than ten DSS moved slightly downwards in the MDS plot (Figure 15), which may indicate occurrence of a common change. In a study of a Great scallop *(Pecten maximus)* hatchery, Sandaa et al (2003), found a high degree of stability in the microbial community composition of scallop larvae throughout the larval period. In contrast, McIntosh et al (2008) observed a clearly defined and reproducible microbial succession in cod larvae with age. Both studies used DGGE analysis, which is less sensitive than TRFLP so may not detect changes occurring in less dominant parts of the community. Typical predominant genera of the intestinal contents of bivalves, marine fish and crustaceans include primarily *Pseudomonas* and *Vibrio* (Gatesoupe 1999). However, bacterial cultivation data indicated that *Vibrio* counts in larvae were not increased as a proportion of the TVC population with larvae age (section 2.4.5). Similarly, in a study of Tropical rock lobster it was observed that *Vibrio* did not significantly increase in relative proportion of the total microbial community in the hepatopancreas until day 18 (Webster et al 2006). The relative absence of these genera in the larvae samples in the present study may indicate that the microbial community of larvae intestines remained in an early successional stage throughout the larval period.

2.5.6 Microbial diversity and stability

Reduced species diversity was observed in the larvae microbial community in comparison to the WC as measured using both TRFLP-1 and clone library data (section 2.4.7). Since diversity of microbial populations is usually positively correlated with stability, it may be

postulated that the microbial community of the larvae environment was more variable than the community of the WC environment due to observed differences in species richness. While difference in species diversity was observed between the larvae and WC microbial communities, no such difference was observed in phylogenetic diversity (Faith's index). As discussed in the Introduction (section 2.2) it is functional diversity that is of primary importance to ecosystem function. According to Lozupone et al (2008) phylogenetic diversity is a better predictor of functional diversity than species diversity, and so it follows that phylogenetic diversity may be a better indicator of ecosystem stability. However, in the present study species diversity and not phylogenetic diversity was negatively correlated with variability, which does not support the notion of phylogenetic diversity being a better indicator of stability. It is worth noting that the comparison made here between the diversity and stability of different microbial niches may not be valid since both environments are subject to a different set of influences.

2.5.7 The indigenous microbial community and stability

Verschuere et al (1997) suggested that the length of time that the indigenous microbial community may persist and the degree of change experienced may be dependent upon how well the indigenous microbial community is adapted to the conditions of culture. That is to say indigenous communities poorly adapted to the conditions of culture may be less persistent and therefore inherently less stable. In the present study, significant species common to both eggs and larvae included *Ramlibacter* sp., *Sphingomonas paucimobilis, Roseisalinus* sp., *Sphingopyxis* sp., *Phaeobacter articus*, *Martelella mediterranea* and *Microbacterium oxydans* (in order of decreasing abundance in the egg sample). Whilst many of these species are typical marine heterotrophs, the most abundant species are not. Both *Ramlibacter* (class *Betaproteobacteria*) and *Sphingomonas paucimobilis* (class *Alphaproteobacteria*) 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 resistance of the indigenous microbial community to change. Following on from Verschuere's idea of suitability of the indigenous community to the environment being linked to stability, it may

be postulated that the oyster larvae in this monitoring study were inherently less stable due to an indigenous microbial community that was poorly adapted to the conditions of culture.

The presence of non-typical marine species in association with eggs may be explained by the conditions of culture of the broodstock or contamination from numerous sources during spawning and fertilisation such as fresh water used for washing, water splashing off the hatchery floor, human skin, and equipment used such as buckets and meshes. 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 and as such may have supported non-typical marine species. Olafsen (2001) affirms that the microbial population of the egg incubator may differ considerably from that of the sea. Verschuere et al (1997) stated that the indigenous microbial community of the eggs is influenced by deterministic and stochastic factors. The microbial community of the broodstock environment and the egg incubator were not investigated in this study, although the same water used for larvae tanks (CTW) was used in egg incubators.

2.5.8 Predominant microbial communities of the larvae tank

The water chemistry within a larval rearing environment is highly eutrophic compared to the oligotrophic oceanic environment, and consequently the microbial composition of the larvae tank may differ substantially from that of the raw seawater (Bourne et al 2004). As discussed in section 1.5.1.1, eutrophication generally favours fast-growing, r-selected species, which may explain the absence of SAR11 species from clone libraries in the present study. 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 surface bacterioplankton communities (Bruhn et al 2007).

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

Gammaproteobacteria (76% of phylotypes) with only 7% contribution of *Alphaproteobacteria* (Schulze et al 2006), although characterisations were limited to culturable bacteria. In the present study *Gammaproteobacteria* were less common but were still the second most predominant group associated with larvae and WC samples, with 13.4 and 15.4%, respectively. Nakase (2007) conducted a study in a Red Sea bream hatchery that showed predominance of *Alphaproteobacteria* and members of the phylum *Bacteroidetes* 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.

The relative absence of *Vibrio* spp. from clone libraries (three *Vibrio* spp. clones detected among 1,390 clones across 18 clone libraries) indicates that the *Vibrio* population represented less than 1% of the total bacteria population, which is of interest due to their notoriety as pathogens of bivalve larvae and the high incidence of mortality during the monitoring period. Using culturable methods, average *Vibrio*/TVC levels were 15.8% in IC tank larvae and 3.7% in Batch tank larvae. Hence *Vibrio* levels appear to be over-represented in culture counts. 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. Non-detection of *Vibrio* spp. using clone libraries and TRFLP in the present study indicates a limitation of these methodologies in being biased towards abundant species (discussed further in section 2.5.9).

A number of hatchery studies have reported *Vibrio* spp. as a predominant component of the microbial communities under conditions of disease as well as successful production. However, in no shellfish hatchery study have *Vibrio* levels been reported as a percentage of the total population using culture-independent methods, instead being expressed as a percentage of the TVC population. In a survey of several bivalve hatcheries from locations on the Atlantic and Pacific coasts of North America, Hawaii and the Marshall Islands, Elston et al (2008) reported average WC *Vibrio* levels of 1.06×10^3 CFU/ml and *Vibrio*/TVC levels of 35%; maximum *Vibrio* levels were recorded at 3.28×10^4 CFU/ml. These *Vibrio* levels are in the same order of magnitude as those encountered in the present

study with average values of 5.12 x 10^2 CFU/ml and 1.45 x 10^4 CFU/ml in the Batch tank and IC tank, respectively, although IC tank values appear to have been higher (see Table 6). Elston et al (2008) found that the average *Vibrio* levels in the raw seawater at Netarts Bay, over an 18-month survey, varied between 1.95 x 10^4 CFU/ml and 2.74 x 10^4 CFU/ml (24.2% to 47.6% *Vibrio/*TVC) depending on water temperature and tidal direction. These levels of *Vibrio* were vastly greater than those encountered in the present study, when compared to *Vibrio* levels in the CTW, which was shown in a related study at the same hatchery to be similar to raw seawater (Powell & Tamplin 2011). Average CTW *Vibrio* levels in the Batch tank were 58 CFU/ml, which represented 5.09% of the TVC population (Table 6). Hence it appears that *Vibrio* and TVC counts of the raw seawater were much lower in the present study than those encountered by Elston, which may reflect oligotrophic conditions.

Members of the *Roseobacter* clade were particularly common in all parts of the hatchery. *Roseobacter* spp. 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); and have been identified as a potential probiotic species for aquacultural use (Porsby et al 2008). Members of the *Roseobacter* clade dominate among marine algae-associated bacteria (Buchan et al 2005) and possess a number of specialist adaptations that allow them to colonise algae including 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), and 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 of the larvae tank. Colonisation of the larvae may require specialised adaptations such as adaptations for mucosal adhesion such as cell surface hydrophobicity or expression of adhesion receptors (Hansen & Olafsen 1999). This may explain the relative absence of algae-associated bacteria from the larvae microbial communities.

Also relatively common in the algae, CTW, WC and biofilm were members of the class *Flavobacteria*. *Flavobacteria* was the second most predominant class in algae, biofilm, and

CTW (30.0%, 19.3%, and 17.8%, respectively). Similarly to *Roseobacter* spp. these bacteria often predominate in algae rich waters in the marine environment. In general, *Alphaproteobacteria* and *Flavobacteria* are important in phytoplankton cultures (Nakase et al 2007). *Kordia algicida* was the most prevalent species of the three combined clone libraries of algae and is noteworthy since it has algicidal properties. An excreted exudate appears to be involved that has high protease activity and its production is dependent on the cell density of *K. algicida* and may be mediated by quorum factors (Paul & Pohnert 2011). The algicidal principle appears to be algae-host specific and was demonstrated to be active against marine diatoms *Skeletonema costatum, Thalassiosira weissflogii* and *Phaeodactylum tricornutum* but was not active towards *Chaetoceros didymus* (Paul & Pohnert 2011). None of these diatom species were used in the hatchery although the presence of *K. algicida* in high proportions in the algae might indicate algicidal activity in cultivation. Such activity could potentially release DOC and nutrients from algal cells and cause rapid growth in heterotrophic bacterial populations such as those following the collapse of algal blooms in the marine environment.

2.5.9 Limitations of monitoring study

The present study was undertaken at a single hatchery and any conclusions from this study may not be applicable to other hatcheries. It is apparent that there are considerable differences among the microbial composition of different hatcheries due to differences in ocean seawater, anthropogenic influences and complexities within each hatchery environment. Significant influence of management and stochastic factors are expected and cannot be adequately accounted for. The differences among hatcheries make generalizing difficult and it is for that reason that Sainz-Hernandez and Maeda-Martinez (2005) recommends case studies of hatcheries, such as the present study.

This study relied upon PCR-based 16S rRNA amplification for generation of clone libraries and TRFLP data. Biases exist in DNA extraction, PCR amplification and cloning procedures (Wintzingerode et al 1997). For this reason abundance data are particularly prone to inaccuracy and it is desirable to confirm abundance estimates with other methods (Lozupone & Knight 2008). Both abundance and presence-absence data were used in

statistical analysis of clone libraries and TRFLP in this study. TRFLP includes extraction and PCR bias and additionally includes restriction enzyme and capillary electrophoresis bias. Hartmann and Widmer (2008) found that although these biases impaired detection of real community structures, relative changes in predominant structures and diversities were reliably reflected in TRFLP profiles. TRFLP data were not used to identify specific bacterial species in this study due to the difficulty in accurately identifying TRF's. Wherever possible clone library, TRFLP and culture-based methods were used in conjunction to avoid problems associated with each methodology. TRFLP and clone libraries are biased towards abundance and so minor but important components of the population such as *Vibrio* spp. may have fallen below detections limits.

Measurement of species diversity using clone libraries and TRFLP has risks inherent in the methodologies. TRFLP is limited in the assessment of diversity metrics in complex communities as the number of peaks detected will grossly underestimate the actual richness of any community with a long-tailed rank abundance distribution (Bent et al 2007). This risk may be not be high in this study given that the communities of the larvae tank for which diversity was determined (eggs, larvae and WC) appear to have a relatively reduced diversity. Assessment of diversity using clone libraries was undertaken using Chao-1 estimator, which tends to underestimate true species richness at low sample sizes (Hughes et al 2001). That clone library and TRFLP diversity metrics were in agreement may increase confidence in the final outcome.

Confidence in TRFLP data was achieved by using a large number of samples across eight production runs to identify general trends. Clone library data were used to provide species information to complement TRFLP analysis, although samples were not always optimally selected to enhance TRFLP analysis because clone libraries were selected chiefly to target disease incidents. As replication of samples was not possible using clone libraries due to expense, caution has been taken in drawing conclusions from clone library data alone.

Finally, statistical analysis was complex largely due to the inclusion of too many factors into the analysis, which was difficult to avoid given the commercial nature of the operation and

management changes that introduced additional factors that then needed to be included in to the analysis. This problem was largely circumvented by partitioning statistical analyses.

2.6 Summary and conclusions

The microbial community of the larvae tank was primarily composed of typical marine heterotrophic species. Of a total of 1390 bacterial clones analysed from different compartments within the larvae tank, 120 different phylotypes were identified. All compartments were dominated by *Alphaproteobacteria* and members of the *Roseobacter* clade in particular. *Vibrio* spp. may have been enhanced in intensive culture conditions but remained below the level of detection using culture-independent methods, comprising less than 1% of the total population. It appears that the larvae tank may have favoured fast-growing bacterial species with adaptations such as attachment to algae surfaces, biofilm formation, antibiotic production, and utilisation of algal exudates.

Comparison of the two tank types demonstrated that altered culture conditions could change microbial communities of the WC, but larvae communities were shown to be largely resistant to change experienced in the WC. Algae had primary influence on microbial communities of the WC as xenic algae inputs increased with larvae feed demand but evidence did not indicate a large effect of algae microbial communities on larvae communities, which added further evidence to larvae communities being resistant. Larvae microbial communities were closely related to the indigenous microbial communities of the eggs, which may be influenced by the microbial community of the unfertilised egg and the rearing water. 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 spawning and fertilisation. Moreover, their poor suitability to the saline conditions might have contributed towards community instability.

Microbial communities of the larvae and WC varied primarily with DSS and sampling period. The most plausible explanation for equal sampling period variability in the larvae and WC, may be seasonal variability in the seawater since variability in the initial egg

microbial community could not account for variability in the WC, and variability in the algae microbial community could not account for variability in the larvae. DSS also affected microbial communities of the larvae and WC equally but could not be wholly accounted for by a single factor.

3 Chapter 3: Disease etiology in a commercial oyster hatchery

3.1 Abstract

Microbial communities of a commercial Pacific oyster larvae tank in Bicheno, Tasmania, were characterised during eight different production runs. Seven runs included larvae possessing disease symptoms consistent with bacillary necrosis. Using a combination of bacterial cultivation and culture-independent microbiological tools including TRFLP and 16S rRNA gene clone libraries it was determined that while microbial communities of the larvae and the water column changed with the emergence of disease symptoms there was no characteristic microbial community associated with disease, and no recognised bacterial pathogens were detected. Larvae-bacteria challenge trials with predominant bacterial species of diseased larvae indicated that predominant bacterial species were not highly pathogenic when compared with the known pathogen *Vibrio tubiashii*. Advanced stages of disease were associated with displacement of predominant members of the microbial community associated with the larvae prior to disease. Disease incidents were accompanied by population peaks in *Vibrio* spp. and despite representing less than 1% of the total population using 16S rRNA clone libraries, the total number of *Vibrio* spp. may have been sufficient to cause disease. Larvae aggregative behaviour near the tank bottom prior to the development of definitive disease symptoms occurred in 80% of disease incidents and may indicate a non-microbiological primary cause of disease, or a microbiological etiology that occurred below the detection limits of 16S rRNA gene-based analyses used. This study is the first to characterise microbial communities associated with bacillary necrosis in a bivalve hatchery using culture-independent methods.

3.2 Introduction

Hatchery studies implicating specific bacterial pathogens in oyster larvae epizootics have been conducted broadly in accordance with Koch's postulates, which are detailed as follows:

1. The pathogen must be regularly associated with the disease and its characteristic lesions.
2. The pathogen must be isolated from the diseased host and grown in culture.
3. The disease must be reproduced when a pure culture of the pathogen is introduced into a healthy, susceptible host.
4. The same pathogen must be re-isolated from the experimentally infected host.

All published studies in which pathogenic species have been implicated in disease epizootics (Table 2) have involved isolation of the pathogen from diseased larvae and reintroduction of the pathogen to healthy individuals as per Koch's postulates. For example, Elston et al (2008) isolated *V. tubiashii* from moribund larvae, characterised the pathogen, and conducted detailed histology on oyster larvae tissue. The pathogenicity of the *V. tubiashii* isolate was then confirmed *in vitro* with larvae-bacteria challenge trials in which similar disease symptoms were observed; although, the pathogen was not re-isolated in accordance with Koch's fourth postulate. Another study by Brown (1981) followed a similar methodology to implicate *V. anguillarum* and whilst the study did not include detailed histology, Koch's fourth postulate was satisfied with re-isolation of the bacterial pathogen. It is also interesting to note that in proposing a protocol for testing the pathogenicity of bacterial strains against Pacific oyster larvae Estes et al (2004) does not include re-isolation of the pathogen from the host in the protocol. Thus it appears that Koch's postulates has been followed broadly in identifying pathogens of bivalve larvae but perhaps not rigorously.

Exact fulfilment of Koch's postulates may not be necessary to establish causation in all cases. Paillard et al (2004) states that in the case of bacterial pathogens always associated with disease, isolation, biochemical and/or molecular characterisation should be sufficient to diagnose that a disease is due to the pathogenic microorganism. Cases of bacillary necrosis

must fall into this category since there is substantial evidence implicating *Vibrio* spp. such as *V. tubiashii* and *V. anguillarum* as the etiologic agent (see Table 2). Yet while pathogenic species have been identified, a number of studies have demonstrated co-existence of pathogenic species and healthy larvae (Bourne et al 2004; Brown 1981; Elston et al 2008; Schulze et al 2006) and induction of the disease process remains poorly understood (Elston et al 2008).

A commonality in studies of hatchery epizootics is that implication of bacterial pathogens has been achieved with little or no understanding of the non-culturable microbial community in which the pathogen has arisen; primarily because appropriate microbiological tools had not been developed until more recently. Moreover, bacteria-larvae challenge tests have been conducted within *in vitro* conditions utilising sterile water, whereas disease epizootics in the hatchery occur in mixed bacterial populations. Thus these studies appear to have neglected the microbial environmental context in which disease has occurred and therefore have failed to take full account of the disease triangle conceptual model (McNew 1960).

Isolation of the pathogen and host in bacteria-larvae challenge tests in the laboratory environment appears to be a requirement of Koch's postulates, which points towards a limitation of the methodology. Paillard et al (2004) argues that Koch's postulates take 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. Given the complexity of the microbial environment of the hatchery and microbial relationships that may affect larvae growth and survival (section 1.5.1.4) it might be that characteristics of the microbial environment influence disease incidence either by bearing directly on the larvae-pathogen relationship or indirectly through affecting larvae health and disease susceptibility.

Given the importance of context in disease incidence, this study has endeavoured to characterise microbial communities associated with disease within the hatchery context. In a monitoring study of commercial production runs, microbial communities of the larvae tank were characterised in detail during disease incidents. With respect to the disease

triangle concept, information on larvae health and larvae tank cultural conditions was also collected.

3.2.1 Research objectives

The main objective in this chapter, as defined in Table 1 (research objective No. 2) was to characterise the microbial environment associated with the emergence of disease symptoms and identify the microbial cause(s) of disease. This objective was addressed through the 12-month hatchery monitoring program described in Chapter 2 and larvae-bacteria challenge trials. Of the eight production runs monitored a total of seven runs terminated in disease incidence. To characterise microbial communities both culture based techniques and culture-independent molecular tools were used including 16S rRNA gene clone libraries and TRFLP analysis. The microbial environment was characterised by collection of physiochemical data and dissolved nutrient analysis.

3.3 Methods

The methods are described in full in Chapter 2 as data for both chapters was obtained from the same monitoring study. Much of the same data from the analysis identified as TRFLP-1 are reported in this chapter but with a different statistical analysis that incorporates disease as an independent variable to separate the effects of disease from other variables including sampling period, DSS and tank type, which were addressed in Chapter 2. Some clone library data are reported here again for comparison with clone libraries from moribund larvae, which were not reported in Chapter 2. Microbial growth information, larvae performance data, and larvae-bacteria challenge data are also reported in this chapter and not in Chapter 2.

In this chapter, broad trends associated with changes in the microbial community with emergence of disease symptoms have been examined using TRFLP-1 data, and three production runs (280109, 070909 and 170909) are examined in detail to identify microbiological changes on a production run basis.

3.4 Results

3.4.1 Larvae microbial communities and disease incidence

3.4.1.1 Diseased larvae

The microbial community composition in diseased larvae, which were still swimming, and not-diseased larvae was compared using TRFLP-1 analysis. An MDS plot was generated to visually compare the similarity of microbial communities associated with larvae of differing disease status (Figure 19). The absence of clustering of diseased larvae samples in the MDS plot indicates that there may not have been a characteristic microbial community associated with diseased larvae.

Statistical analysis indicated that there may be a small difference between microbial communities of diseased and not-diseased larvae (Pr = 0.0633; Statistical model T) although the P-value was beyond the usual limits of acceptability (0.05). Statistical model T pooled data from both IC and Batch tanks. This approach was validated since Statistical model S indicated that tank type made no difference to the microbial communities of larvae (Pr = 0.1869). Nevertheless when IC samples were removed from the analysis in Statistical model V, disease status was significant (Pr = 0.0026) although the interaction between sampling period and disease status was also significant (Pr = 0.0007). The interaction indicates that the difference associated with disease status might have been dependent upon the sampling period. Thus it appears as though there may have been a change in the microbial community of larvae associated with emergence of disease symptoms, at least in the Batch tanks, but the difference may have been largely dependent on sampling period and was much smaller than the change experienced with sampling period and DSS (compare P-values in Statistical model V: sampling period: Pr < 0.0001; DSS: Pr < 0.0001; disease status: Pr = 0.0026). Note that statistical analysis with three-way interaction with disease status, tank type and sampling period was not possible due to missing data (see Appendix 1).

Evidence from cultivation of bacteria also provided weak evidence of a change in microbial community composition with disease status. Although neither TVC nor *Vibrio* counts were

significantly different between diseased and not-diseased larvae, the percentage *Vibrio* (*Vibrio*/TVC), was on average higher in diseased larvae with averages of 18.2% and 2.4% for diseased and healthy larvae, respectively (Pr = 0.0102; Statistical model C).

3.4.1.2 Moribund larvae

Moribund larvae were not included in the TRFLP analysis but three clone libraries were constructed for moribund larvae from three different production runs (D_150909, D_280109 and D_031009). Unifrac PCA analysis of all clone libraries using presence-absence data indicated that microbial community composition associated with moribund larvae was different from communities associated with diseased and not-diseased larvae and appeared to have been more similar to the community composition of biofilm, algae and WC, which grouped together (Figure 20). Although some caution should be taken in drawing conclusions from this figure since only 30.8% of encountered variability could be represented in the two-dimensional format due to inclusion of a large number of clone libraries. When only samples from a single production run (070909) were plotted in PCA analysis the same trend was apparent but more of the variability, 56.9%, was represented in the figure (see Figure 17 (A) in Chapter 2).

Clone library composition analysis (summarised to class level in Figure 21) showed that moribund larvae of production run 070909 were dominated by *Alphaproteobacteria* and also had a high proportion of *Flavobacteria*. Production run 170909 moribund larvae were dominated by *Alphaproteobacteria* and *Betaproteobacteria* whereas production run 280109 moribund larvae were dominated by *Bacilli* and *Gammaproteobacteria*. Unifrac PCA analysis (Figure 20) and clone library composition analysis indicated a high degree of phylogenetic divergence among moribund larvae of different production runs. As such there appears to have been no characteristic bacterial community associated with moribund larvae common to all production runs.

Bacterial cultivation data of larvae samples, including both TVC and *Vibrio* counts, showed elevated levels in moribund larvae compared to not-diseased larvae, using only data from

larvae older than 9 DSS, with TVC and *Vibrio* counts being approximately 6-fold and 75-fold higher, respectively (Table 18).

Table 18 – Cultivation of bacteria from moribund and not-diseased larvae

	Average TVC (CFU/ml)	**Average *Vibrio* (CFU/ml)**
Moribund larvae	5199 (+/- 5724), n = 5	754 (+/- 751), n = 3
Not-diseased larvae	837 (+/- 635), n = 6	10 (+/- 8.9), n = 5

3.4.2 WC microbial communities and disease incidence

From the MDS plot of TRFLP-1 data in Figure 22, the emergence of disease symptoms in larvae does not appear to have been associated with a characteristic microbial community in the WC across different production runs, as would be evidenced by clustering. Instead, diseased samples in Figure 22 were as widely dispersed as not-diseased samples, although the centre of the distribution appears to be closer to the top of the MDS plot.

Multivariate statistics indicated that there was a difference between microbial communities associated with the WC of diseased and not-diseased larvae, using Statistical model Y (Pr = 0.0117), which included data pooled from both the IC tank and Batch tank. Disease status was similarly significant when IC tank data were excluded from the analysis in Statistical model AA (Pr = 0.0062), which may be a more rigorous statistical assessment as tank type was shown to affect WC microbial composition in Statistical model X (Pr = 0.0001). In any case both models show that disease status was significant and additionally show interaction between disease status and sampling period (Statistical model Y: Pr = 0.0033. Statistical model AA: Pr < 0.0001), which indicates that the change in microbial composition may be dependent on sampling period. Furthermore the magnitude and the significance of the difference with disease status was much smaller than sampling period and DSS as indicated by P-values from both Statistical model Y and Statistical model AA (Statistical model Y: Disease: Pr = 0.0117. Sampling period: Pr < 0.0001. DSS: Pr = 0.0002).

Bacterial abundance data including TVC, *Vibrio* and *Vibrio*/TVC provided no evidence of any change in the WC microbial community with disease status (TVC: Pr = not significant so removed from Statistical model D; *Vibrio*: Pr = 0.0984, Statistical model E; *Vibrio*/TVC:

Pr = 0.391, Statistical model F). Evidence from clone library data were presented for production run 070909 (two clone libraries: W_140909 (not-diseased) and W_160909 (diseased)) and indicated no significant change in the WC as disease symptoms emerged (more detailed results in section 3.4.3.2 for production run 070909).

Daily nutritional analysis undertaken for WC before and during disease incidents indicated that samples remained below minimum detections levels at all times; although minimum threshold values were well in excess of those encountered in natural seawater, as indicated in Table 19. In Table 19 maximum natural levels of nutrients were taken from a four year survey of water quality in Storm Bay by Harris et al (1991). Minimum detection limits of nitrate, phosphorous, and nitrite were greater than maximum levels encountered by Harris et al (1991) by a factor of 13.5, 3.3 and 2.7, respectively.

Table 19 – Minimum detection limits of daily monitoring compared to maximum levels encountered in seawater

	Maximum natural level (ppm)[1]	**Minimum detection level (ppm)**	**Exceedance factor[2]**
Phosphorus	0.076	0.25	3.3
Nitrate	0.37	5.0	13.5
Nitrite	0.092	0.25	2.7

(1) Maximum natural levels encountered in Storm Bay survey (Harris et al 1991) (2) Minimum detection level/Maximum natural level

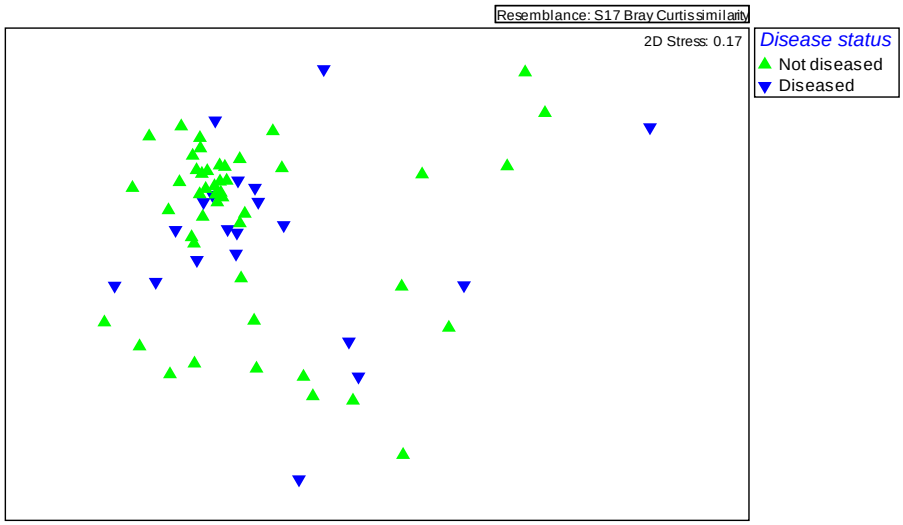


Figure 19 – MDS plot showing microbial composition similarity of diseased and not-diseased larvae from seven runs

Pr = 0.0633 (Statistical model T)

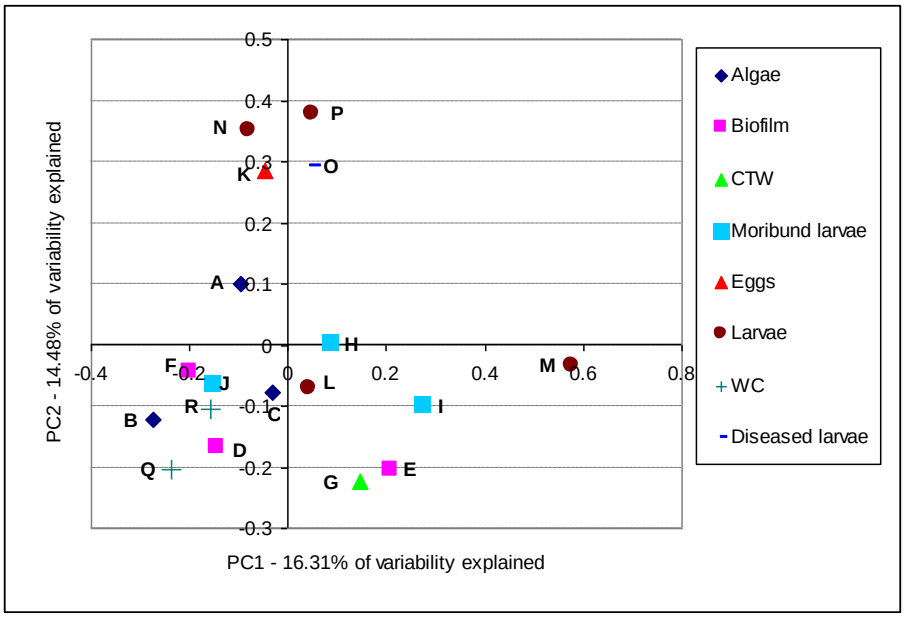


Figure 20 – Unifrac PCA analysis of all clone libraries using presence-absence data

Clone library identities: A = A_101209; **B** = A_150909; **C** = A_200110; **D** = B_031009; **E** = B_100209; **F** = B_160909; **G** = C_150909; **H** = D_031009; **I** = D_100209; **J** = D_150909; **K** = E_070909; **L** = L_030209; **M** = L_050209; **N** = L_140909; **O** = L_160909; **P** = L_260909; **Q** = W_140909; **R** = W_160909

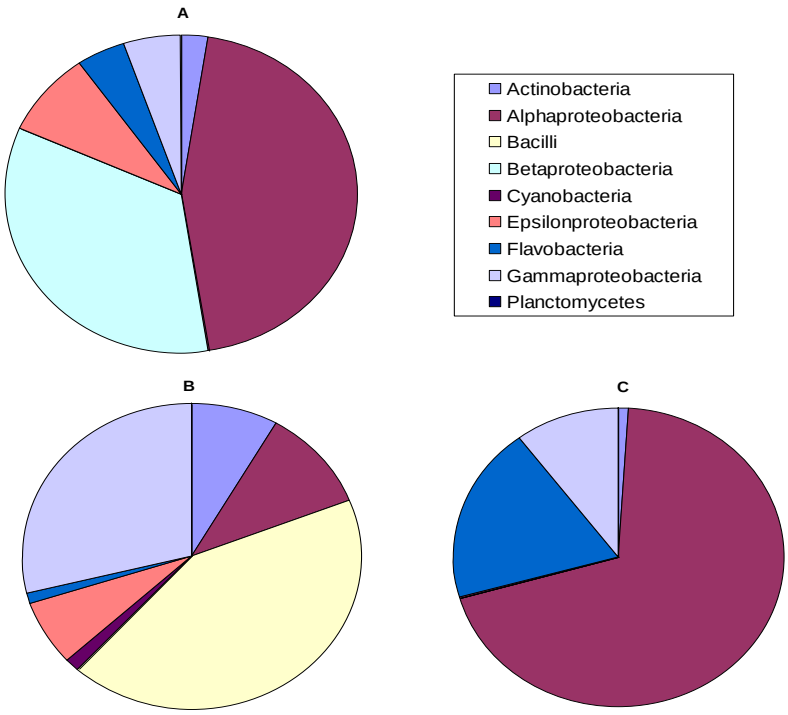


Figure 21 – Clone library data from moribund larvae from different production runs

A = Production run 170909 (sample D_031009); **B** = Production run 280109 (sample D_100209); **C** = Production run 070909 (sample D_150909)

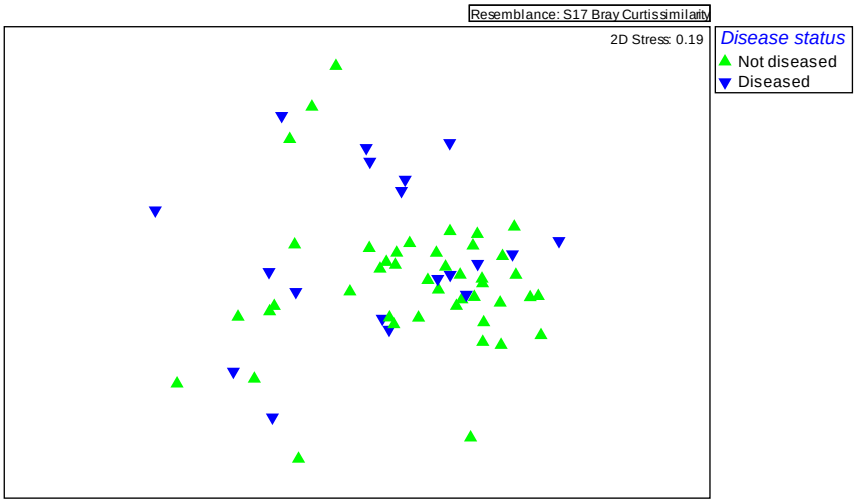


Figure 22 - MDS plot showing disease status of WC samples including diseased WC from seven runs

Pr = 0.0117 (Statistical model Y)

3.4.3 Disease incidence by production run

Since TRFLP analyses of microbial communities in the larvae and WC indicated that change experienced with disease incidence may have been different in each production run, three disease-affected production runs are reported here in detail to identify commonalities and differences in each disease incident.

3.4.3.1 Production run 280109

Production run 280109 commenced in the Batch tanks until seven DSS when the larvae were moved to the 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. Obvious disease symptoms emerged at 14 DSS, from which time, health deteriorated rapidly (see health score in Figure 23) and terminated in mass mortality at 16 DSS. Following an initial decline in velum condition larvae began releasing large amounts of dark mucus-like faeces into the WC and black spotting appeared on the inside of the tank at the water line. Activity levels remained high but swimming behaviour was erratic. Necrotic internal regions were noted in moribund larvae.

TVC and *Vibrio* counts, as well as *Vibrio*/TVC percentage, are graphed against DSS for both larvae and WC in Figure 24. The most notable feature in Figure 24 is a sharp increase in the *Vibrio*/TVC percentage in larvae coincident with the onset of disease symptoms. *Vibrio*/TVC percentage rose to 23.6% and 25.1% in the WC and larvae, respectively, on the first day of disease (14 DSS). On the following day *Vibrio*/TVC percentage rose to 100% in the larvae and was steady in the WC at around 16.6%. Mass mortality occurred at 16 DSS with larvae *Vibrio*/TVC percentage still at 100% and the culture was abandoned. A single colony morphology was observed on TCBS plates from moribund larvae samples and the species was identified through 16S rRNA sequencing as *Staphylococcus sciuri*$_{(97\%)}$[1].

[1] Percentages in brackets and in subscript indicate the minimum dissimilarity of the phylotype to the type strain

Clone libraries were constructed for several samples including moribund larvae (D_100209) and tank biofilm (B_100209) at 14 DSS when disease symptoms first emerged, and not-diseased larvae at seven DSS (L_030209) and nine DSS (L_050209) prior to the emergence of disease symptoms. Clone library data are summarised in Table 14 of Chapter 2. All clone libraries had good coverage with at least 81% (Goods formula). Clone library analysis of not-diseased larvae samples from seven DSS and nine DSS indicated a high degree of variability. Seven and nine DSS larvae did not share a single phylotype and Unifrac PCA analysis confirmed that the samples were phylogenetically divergent (Figure 20). Moribund larvae had a total of 15 phylotypes and were different from both larvae samples sharing only three and two phylotypes with seven DSS (total phylotypes = 10) and nine DSS larvae (total phylotypes = 4), respectively. Moribund larvae shared 8 phylotypes with biofilm, which had a total of 34 phylotypes. The predominant bacteria of moribund larvae were *Bacilli* (42.5%) and *Gammaproteobacteria* (28.8%) as indicated in Figure 21.

Phylotypes present in each clone library are given in Figure 25. Both seven DSS and nine DSS larvae were dominated by a single species. Seven DSS larvae had 82% *Shimia marina*$_{(97\%)}$ while nine DSS larvae had 85% *Pseudoalteromonas marina*$_{(97\%)}$. The most predominant phylotype of moribund larvae was *Staphylococcus sciuri*$_{(97\%)}$ with 42.5% of all clones identified, followed by *Marinovum algicola*$_{(97\%)}$ with 21.9%, *Arcobacter nitrofigilis*$_{(93\%)}$ at 6.8%, and *Pseudoalteromonas marina*$_{(97\%)}$ with 6.8%.

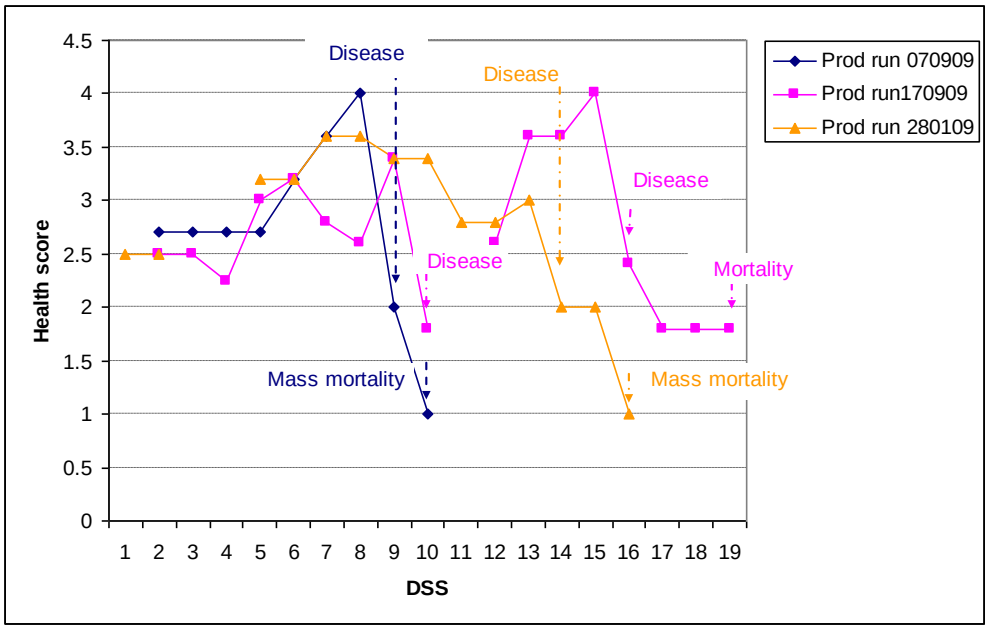


Figure 23 – Health scores of larvae from production runs 070909, 170909 and 280109 vs DSS

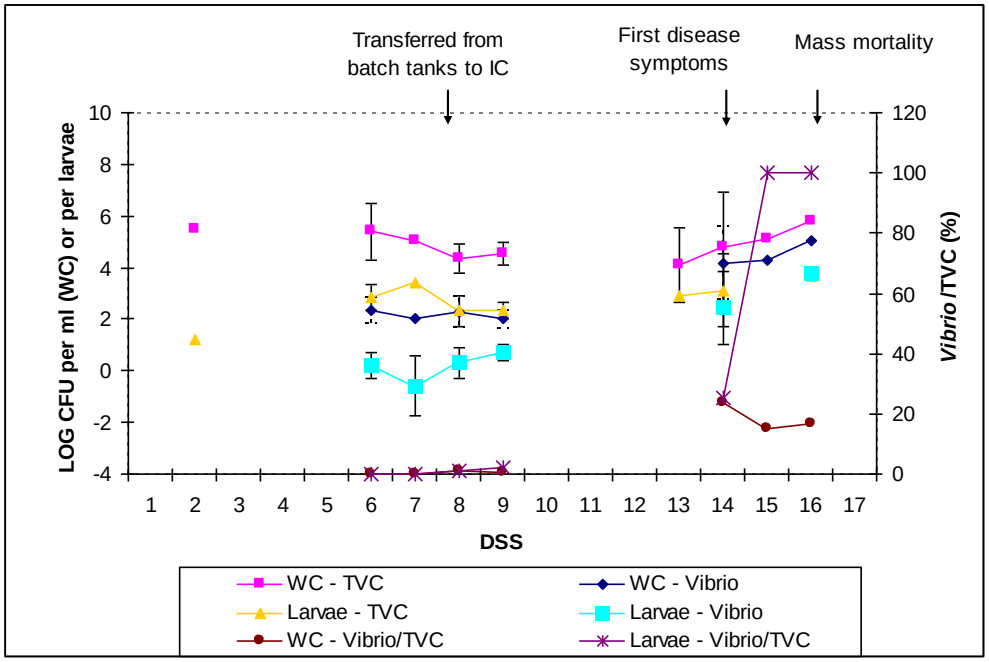


Figure 24 – TVC counts, *Vibrio* counts, and *Vibrio*/TVC percentage for larvae and WC of production run 280109

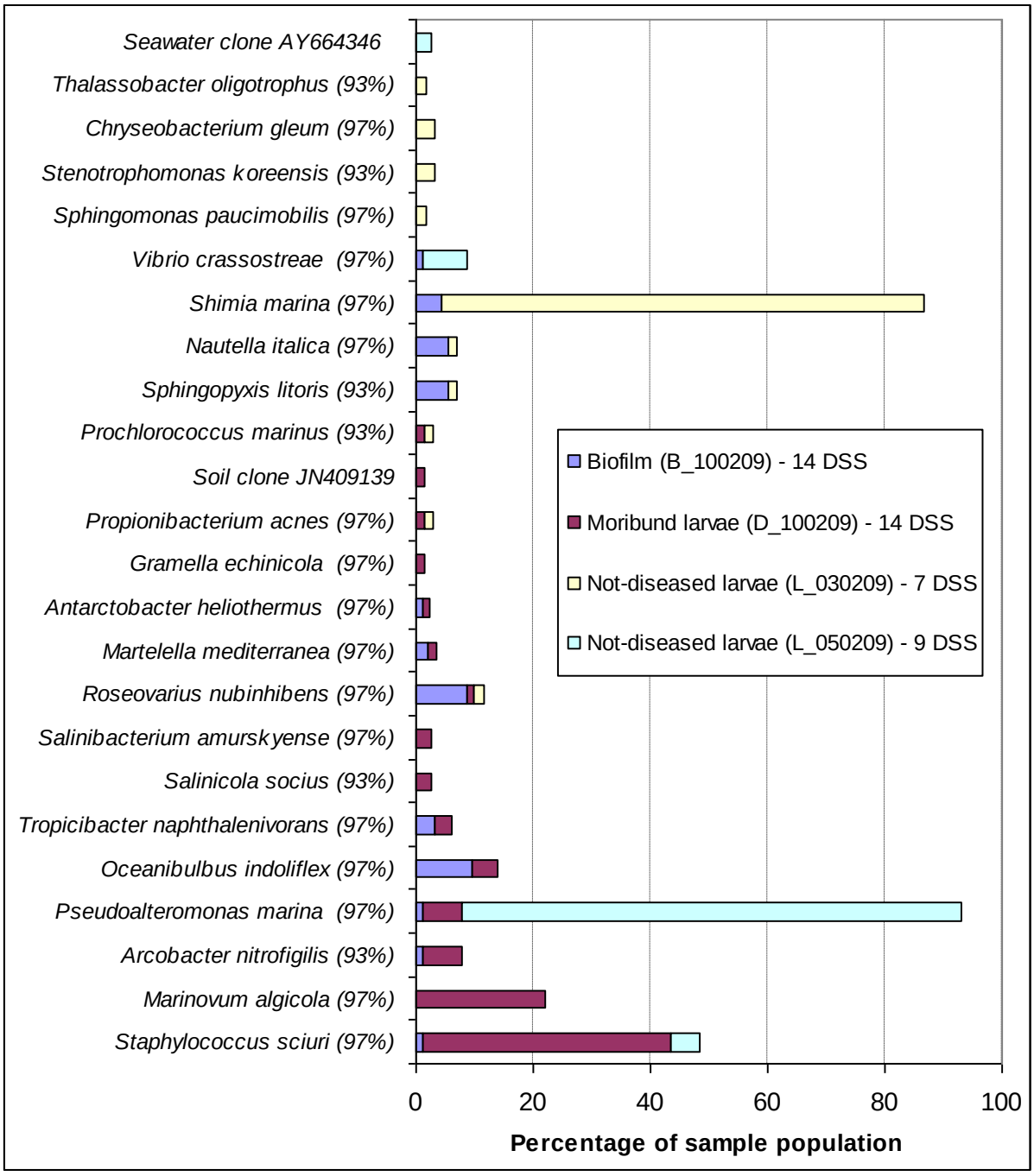


Figure 25 – Summary of clone libraries for production run 280109

Note: (1) Phylotypes of biofilm samples are only shown where they are also present in a larvae sample. (2) Percentage indicates minimum percentage similarity to type strains

3.4.3.2 Production run 070909

Production run 070909 commenced in the Batch tanks until six DSS when the larvae were moved to the IC 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 microscope 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 and recovered upon agitation with a plunger. At six DSS the larvae were transferred to the IC tanks. By eight DSS larvae were in excellent health and growing well. At nine DSS clear symptoms of disease emerged. Microscopic observations were as follows: guts lightened-off and faeces not compact; algae not digested and passed through the gut with mucus entrail; activity was reduced and progressively worse throughout the day. The emergence of disease symptoms was described by an abrupt decline in health score as shown in Figure 23. At 10 DSS the culture crashed and was abandoned. Larvae had erratic swimming behaviour, damaged or detached velum, and moribund larvae had necrotic internal regions.

Incidence of drop-out and aggregative behaviour at three and five DSS were accompanied by an increase in larvae TVC from the previous day of approximately 1.3 and 1.7 log at three and five DSS, respectively (Figure 26). No concomitant change in *Vibrio* counts in the larvae or WC was observed. From five DSS to eight DSS *Vibrio*/TVC percentages steadily increased in the larvae and WC from close to zero to peaks of 24.1 and 35.4% in the WC and larvae, respectively, which was coincidental with excellent larvae health (Figure 23). At nine and 10 DSS, as disease and mass mortality occurred, the *Vibrio*/TVC percentage declined rapidly to less than 1% in both by 10 DSS.

A larger number of clone libraries were undertaken for production run 070909 compared to other production runs to enable a more detailed examination of disease etiology. Clone libraries were undertaken for eggs (E_070909), not-diseased larvae (L_140909) and WC (W_140909) at eight DSS (coincidental with excellent larvae health and high *Vibrio*/TVC

percentages), and samples from all compartments of the larvae tank during disease incidence at nine and 10 DSS including moribund larvae (nine DSS, D_150909), CTW (nine DSS, C_150909), diseased larvae (10 DSS, L_160909), biofilm (10 DSS, B_160909), WC (10 DSS, W_160909) and algae (nine DSS, A_150909). Clone library data are summarised in Table 14 of Chapter 2. All clone libraries had good coverage with at least 78% (Goods formula).

Unifrac PCA analysis indicated that there was little difference among eggs, not-diseased larvae at eight DSS prior to the emergence of disease symptoms, and diseased larvae at 10 DSS, when mass mortality occurred (Figure 20 of Chapter 3 and Figure 17 of Chapter 2[2]). Moribund larvae were different from not-diseased and diseased larvae and may have been more closely related to algae, WC and biofilm in terms of species composition as indicated by Unifrac PCA analysis using presence-absence data (Figure 20). Moribund larvae were predominated by *Alphaproteobacteria* (69.3%) and *Flavobacteria* (19.3%). Unifrac PCA analysis of WC samples at eight and 10 DSS indicated that the difference in the WC microbial communities as disease symptoms emerged may not have been substantial.

Species composition analysis (Figure 27) showed that predominant members of the egg, not-diseased and diseased larvae microbial communities were no longer present in moribund larvae including *Sphingomonas paucimobilis*$_{(97\%)}$, *Ramlibacter tataouinensis*$_{(93\%)}$ and *Roseisalinus antarcticus*$_{(93\%)}$. Moribund larvae also had predominant bacterial phylotypes that were not present in not-diseased or diseased larvae including the two most predominant phylotypes: *Nautella italica*$_{(97\%)}$ at 61.4% and an unidentified *Flavobacteria*$_{(>10\%)}$ at 19.3% of the total population. *Nautella italica*$_{(97\%)}$ was ubiquitous in the larvae tank being found in the CTW, biofilm and WC at 10 DSS and was also present in the WC at eight DSS. The third most predominant species in moribund larvae was *Marinovum algicola*$_{(97\%)}$ at 9.1%, which was present in larvae from at least eight DSS. All of the six most predominant

[2] Both Figure 20 and Figure 17 include Unifrac PCA analysis of samples from 070909 but Figure 20 includes samples from other production runs and therefore less of the total variability can be represented in the two dimensional plot

bacterial species of moribund larvae were also present in the biofilm. These results indicate that advanced stages of disease may be associated with displacement of indigenous microbial species by new predominant species.

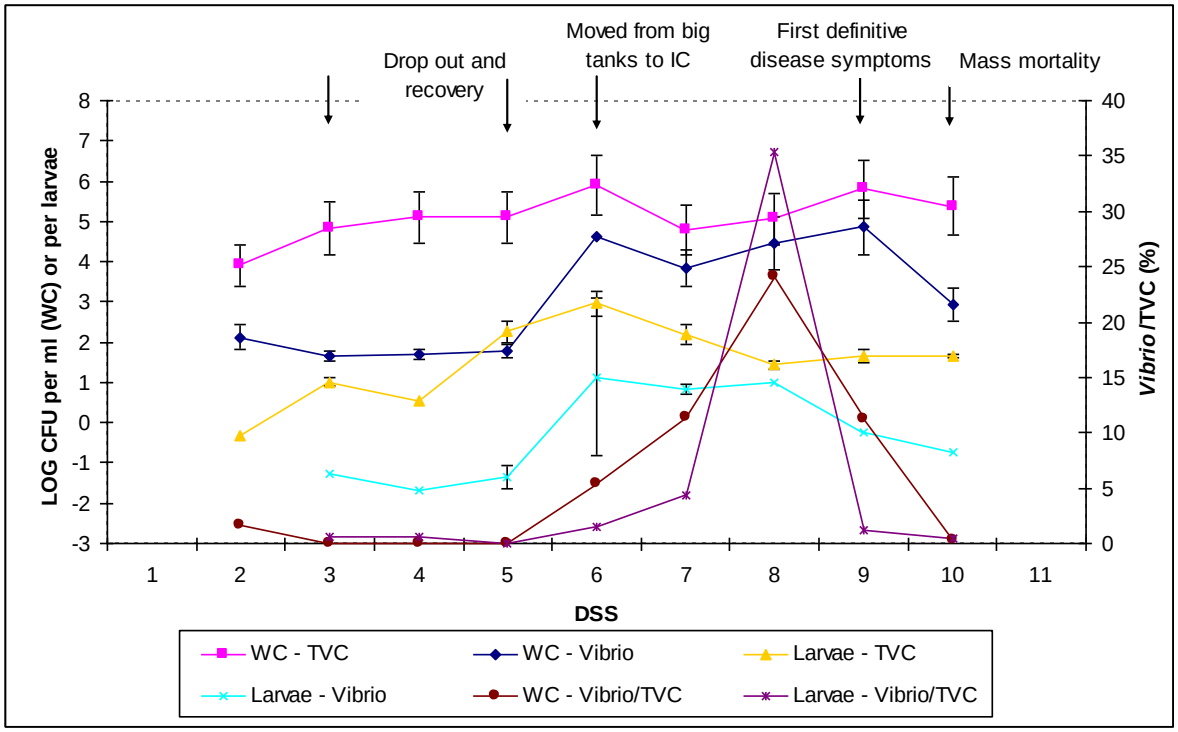


Figure 26 – TVC counts, *Vibrio* counts, and *Vibrio*/TVC percentage for larvae and WC of production run 070909

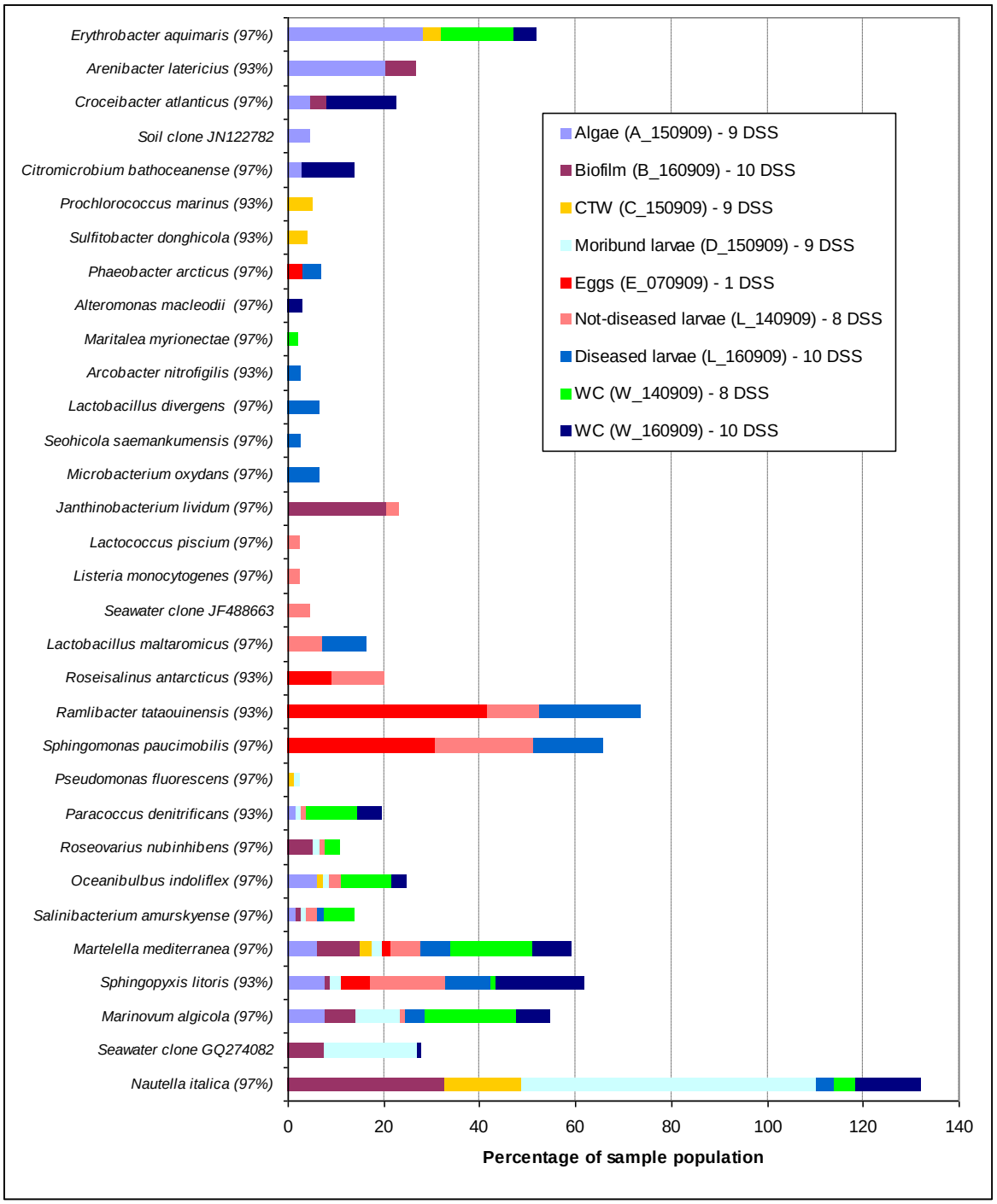


Figure 27 - Summary of clone libraries for production run 070909

Note: (1) Phylotypes of biofilm, algae and CTW are only shown where they are also present in another sample. (2) Phylotypes less than 2% are not shown except in moribund samples (3) Percentage indicates minimum percentage similarity to type strains

3.4.3.3 Production run 170909

Production run 170909 was conducted entirely in the Batch tanks. At four DSS larvae dropped out in all tanks and formed dense aggregations near the bottom of the tank. Under 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 to a lesser degree than at four DSS and larvae recovered following addition of algae feed. Again there were no other symptoms indicating disease. At 10 DSS, larvae health declined (Figure 23) with a noted reduction in activity levels, minor velum abnormalities and reduced gut contents; 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 gut content decreased, non-compact faeces, poor development and some mortality; and velum and activity levels were relatively normal. At 17 DSS larvae health had deteriorated further with reduced activity and velum damage noted. Larvae were sent to set 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.

Drop out and recovery on four and six DSS was accompanied by a small increase in WC TVC count compared to the previous day of 0.7 and 1.1 log for four and six DSS, respectively, but no concomitant increase in *Vibrio* counts was observed (Figure 28). From six DSS, both TVC and *Vibrio* counts increased. Larvae *Vibrio* counts increased exponentially to 10 DSS when the first definitive disease symptoms occurred; at which point larvae *Vibrio*/TVC percentage had risen to 9.6%. Larvae *Vibrio* counts subsided from 12 DSS as larvae health improved. The emergence of disease symptoms at 16 DSS was again associated with a rise in larvae *Vibrio* counts. From this point larvae did not recover and mortality at 19 DSS was associated with a sharp increase in *Vibrio*/TVC percentage in both the larvae and WC with values of 24.7% and 42.6%, respectively.

Clone libraries were undertaken for three samples including 10 DSS not-diseased larvae (L_260909), moribund larvae at 17 DSS (D_031009) and biofilm at 17 DSS (B_031009).

Clone library data are summarised in Table 14 of Chapter 2. All clone libraries had good coverage with at least 84% (Goods formula).

Not-diseased larvae 10 DSS grouped with egg samples of production run 070909 in Unifrac PCA analysis using presence-absence data (Figure 20), which indicates that the egg microbial community of production run 170909 may have been similar to that of 070909. Species composition analysis (Figure 29) indicated that the most predominant phylotypes of 10 DSS larvae samples were also encountered in eggs samples of production run 070909 including *Sphingopyxis litoris*$_{(93\%)}$, *Martelella mediterranea*$_{(97\%)}$, *Sphingomonas paucimobilis*$_{(97\%)}$, and *Ramlibacter tataouinensis*$_{(93\%)}$. In moribund larvae of 17 DSS these species were all still present except for *Sphingopyxis litoris*$_{(93\%)}$, which had been the most predominant species of 10 DSS larvae. Otherwise moribund larvae 17 DSS and not-diseased larvae 10 DSS were quite different, sharing only four phylotypes and the difference was also supported by Unifrac PCA analysis (Figure 20). Other predominant phylotypes of moribund larvae included *Shimia marina*$_{(97\%)}$ and *Arcobacter nitrofigilis*$_{(93\%)}$ both of which were not present in 10 DSS larvae. *Shimia marina*$_{(97\%)}$ was the most predominant phylotype of 17 DSS biofilm. At class level moribund larvae were predominated by *Alphaproteobacteria* (44.7%) and *Betaproteobacteria* (34.2%) as shown in Figure 21.

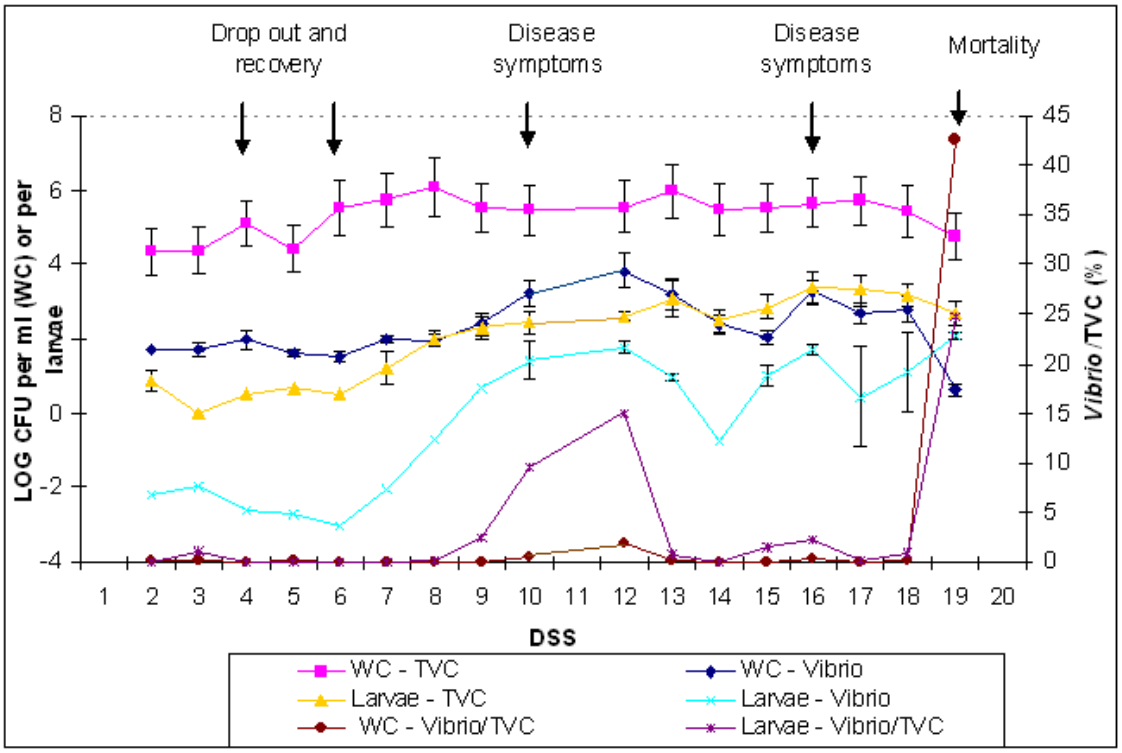


Figure 28 – TVC counts, *Vibrio* counts, and *Vibrio*/TVC percentage for larvae and WC of production run 170909

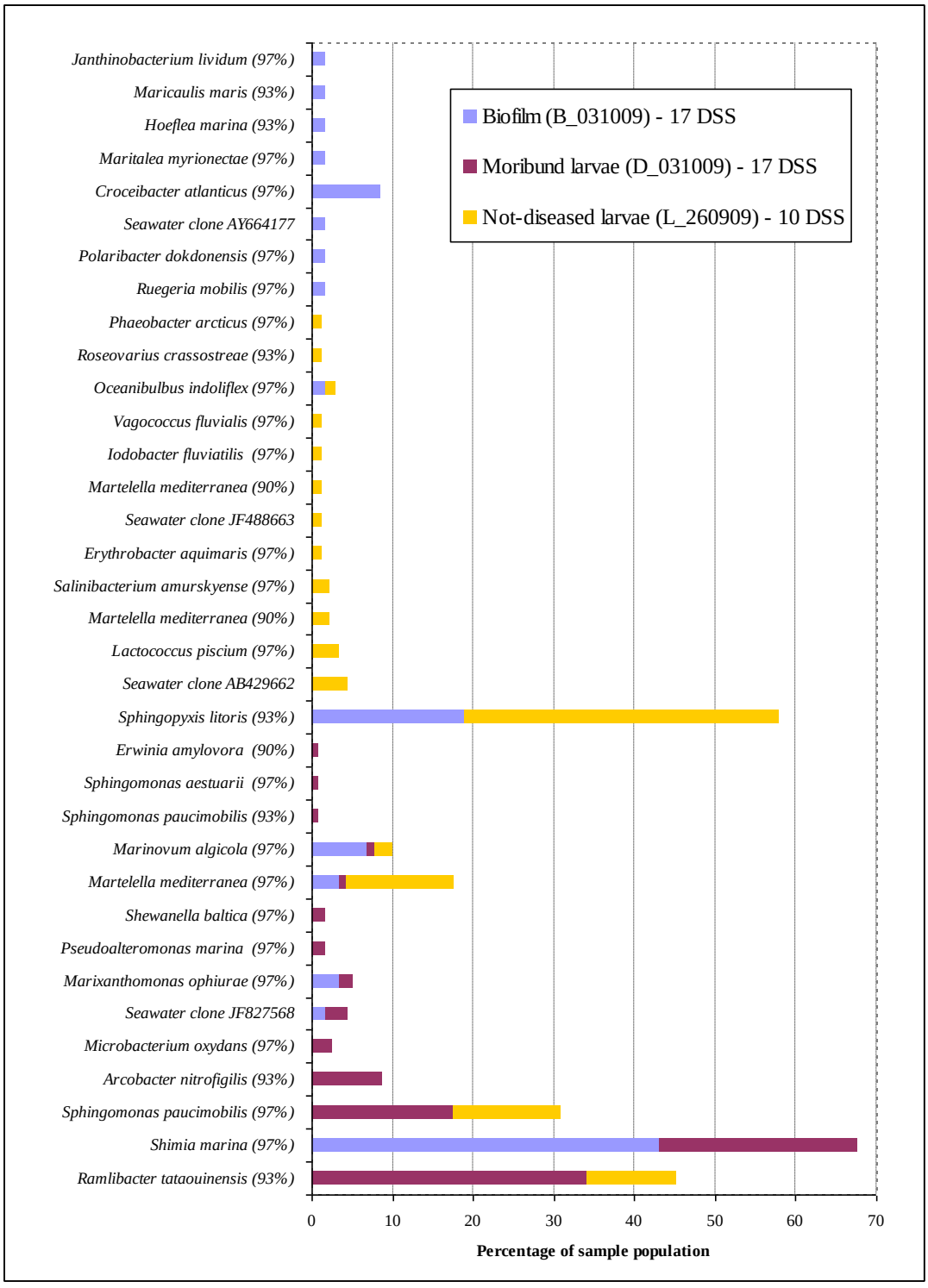


Figure 29 – Summary of clone libraries for Production run 170909

Note: (1) Percentage indicates minimum percentage similarity to type strains

3.4.4 Larvae-bacteria challenge tests

Larvae-bacteria challenge tests were undertaken with bacterial isolates from moribund larvae and a strain of *Vibrio tubiashii*, obtained from Animal Health Laboratories (Isolate ID: 09/2885-1). Isolates included predominant species of moribund larvae isolated on MA and TCBS. Only seven such species were obtained and Table 20 identifies their source and relative predominance in moribund larvae in which they were observed.

Table 20 – Bacteria used in challenge tests and predominance in clone library samples

Isolate	**Isolate found in the following clone libraries:**
Pseudoalteromonas marina	• D_031009 (1.75%)[1] • D_100209 (6.85%)
Vibrio splendidus	None - Moribund larvae 15/12/09
Pseudomonas fluorescens	• D_150909 (1.14%)
V. tubiashii	None - Animal Health Laboratories
S. sciuri	• D_100209 (42.5%)
Nautella italica	• D_150909 (61.4%)
V. pomeroyii	None - Moribund larvae 28/12/08
Sphingopyxis litoris	• D_150909 (2.27%)

Note: (1) numbers in brackets are percentage of total population

Challenge results (Figure 30) indicated that *V. tubiashii* was more pathogenic than other isolates, causing mortality at the lowest concentration of 2 log CFU/ml after 72 h. Cultivation of the WC from the 2 log CFU/ml challenge at 72 h gave counts of 5.19 (+/- 4.89) log CFU/ml; indicating that the *Vibrio* concentration had increased 3 log units since the beginning of the experiment. Isolates that were taken from the hatchery all caused high mortality in challenge tests but only at higher concentrations. *Sphingopyxis litoris* may have been more pathogenic than the other species, causing greater than 50% mortality at levels around 6 log CFU/ml after 72 h. Other species caused higher mortality after 72 h at levels greater than 6 log CFU/ml.

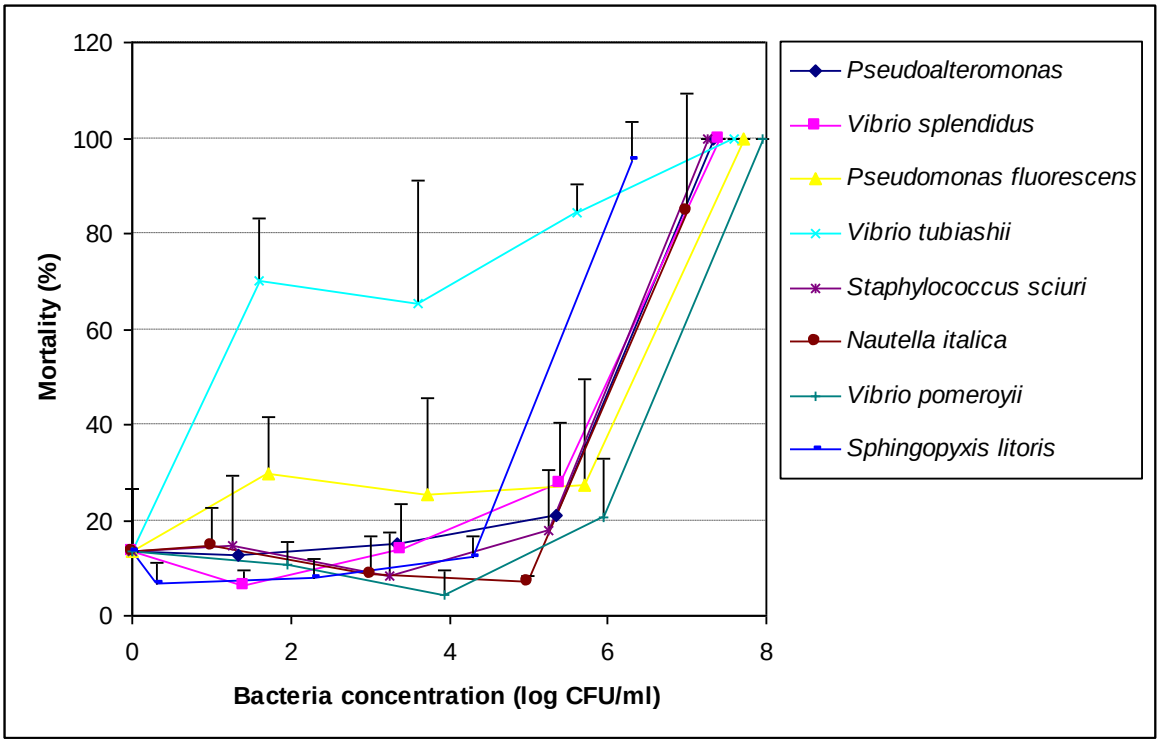


Figure 30 – Larvae-bacteria challenge test results after 72 h

3.5 Discussion

Typical disease symptoms encountered in oyster larvae epizootics in this monitoring study including damaged or detached vela, abnormal swimming behaviour, reduced activity, poor faecal consistency, necrotic soft tissues and mass mortality are consistent with those caused by bacteria and described by Tubiash et al (1965) as bacillary necrosis. However, definitive affirmation that disease was caused by bacteria was not achieved in this study. Viruses from the family *Herpesviridae* have also been known to cause mass mortalities in oyster larvae hatcheries (Hine et al 1992) and diseased larvae samples were not investigated for evidence of virus particles and lesions although evidence from Animal Health Laboratories (Dept Primary Industries, Parks, Water and Environment (DPIPWE)) suggests that virus infection was unlikely. From 1995 to the present (2011) the Animal Health Laboratory has provided testing for annual health surveys of farmed Pacific oysters (including Shellfish Culture) across all marine farming development plan areas (MFDP) in Tasmania for the Tasmanian

Oyster Research Council. Since the program started in 1995 histopathological findings consistent with Oyster Herpesvirus have not been detected in Tasmanian farmed Pacific oysters. In 2011, as part of the National Ostreid Herpesvirus-1 survey, 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. The testing was conducted at the Australian Animal Health Laboratory, CSIRO, Geelong, Victoria (Graeme Knowles, Animal Health Laboratory DPIPWE Tas, pers. comm.). Even so, definitive affirmation of bacillary necrosis would require more detailed histopathology than undertaken here and positive identification of the etiologic agent including isolation and re-infection into healthy larvae according to Koch's postulates (Paillard et al 2004). Nevertheless this study has characterised microbial communities in association with diseased larvae exhibiting symptoms consistent with bacillary necrosis.

3.5.1 Microbial communities before and after emergence of disease symptoms

Microbial communities of diseased oyster larvae and associated WC were shown, using TRFLP analysis, to differ significantly from microbial communities of larvae and WC in which disease symptoms had not yet developed (sections 3.4.1.1 and 3.4.2, for larvae and WC respectively). However, the magnitude of the variation explained by disease status was less than that explained by larvae age (DSS) and, in particular, sampling period (see Chapter 2 for analysis of sampling period and DSS). Furthermore there was no characteristic microbial community associated with disease, and disease status showed significant interaction with sampling period. As such it appears that the change in microbial composition associated with the emergence of disease symptoms may have been different in each sampling period. That is not to say that changes in microbial composition with disease status may not have had common elements across sampling periods, since disease may relate to activities of a single species or microbial community changes occurring below the limits of detection and as such may not have exerted significant influence on the TRFLP fingerprint.

Whilst TRFLP data indicated a difference in microbial communities of larvae with disease status within sampling periods, clone library data from production run 070909 did not support such difference (section 3.4.1.1). Clone libraries of larvae samples from eight DSS, prior to development of disease, and 10 DSS, during a mass mortality event when disease symptoms were clearly evident, were similar based on Unifrac PCA analysis. TRFLP and clone library data may at least be in agreement that the difference between not-diseased and diseased larvae may not have been substantial. Bacterial cultivation data, TVC and *Vibrio* counts, also indicated no significant difference in larvae and WC microbial communities with disease status; although *Vibrio*/TVC percentage was shown to be higher in diseased larvae compared to not-diseased larvae. Moreover, no concomitant changes in underlying drivers of bacterial productivity were observed, including physiochemical properties and nutrient levels in the WC, although the threshold of detection of nutrient level peaks may have been too high.

It is worth noting that Unifrac PCA analysis is based on phylogenetic distance-based metrics, which take little account of differences in closely related species and therefore may not register significant difference among samples dominated by different but closely related species. This may be of consequence since several studies have shown that pathogenicity of bacteria towards mollusc larvae may vary substantially even among strains of the same species (Elston et al 2008). TRFLP may be sensitive to differences in species composition, provided that adequate restriction enzymes are chosen (Camarinha-Silva et al 2011; Waldron et al 2009), although it may be equally ineffective in determining differences among strains. In the present trial TRFLP was a much more powerful tool for detection of community level differences due to inclusion of a greater number of samples.

That the microbial community of larvae may not have differed substantially with the emergence of disease symptoms indicates that diseased larvae may have been affected by a bacterial pathogen that was not numerically predominant or below the level of detection of TRFLP and clone library methodologies. This idea is explored further in discussion on *Vibrio* spp. in section 3.5.4. An alternative hypothesis is that the putative bacterial pathogen operated remotely, from outside the diseased larvae, by mechanisms such as toxin

production. Toxin production is a known mechanism of pathogenesis in some *Vibrio* spp. such as *V. tubiashii*, which produce extracellular metalloproteases that are highly toxic to oyster larvae (Hasegawa et al 2009). Hasegawa et al (2009) found that the cell-free supernatant from *V. tubiashii* caused similar disease symptoms to those caused when larvae were exposed to bacteria cells, which demonstrates that pathogenic bacteria can cause disease symptoms remotely.

A remotely located putative pathogen may have been operative from moribund larvae, or from other compartments of the larvae tank. Due to the ability of surface and particle-associated bacteria to reach densities up to 1,000 to 10,000 times greater than water-borne bacteria in mature biofilms (Bruhn et al 2007) and their propensity for collaborative behaviour such as production of secondary metabolites including quorum factors and toxins (Porsby et al 2008; Yan et al 2002), biofilm structures may be suspected of harbouring toxin-producing pathogenic bacteria. Although it is not clear how such biofilm activities may be affected by the regular, 48 h, disturbance experienced in the larvae tank due to cleaning, sanitation and desiccation.

Other researchers investigating disease pathogenicity have described an intimate association of larvae and pathogenic bacteria. Elston et al (1980) described in detail the alternative points of entry through which different pathogenic *Vibrio* spp. entered the bivalve larvae. The authors described three different pathways including attachment to the larvae shell and growth along the mantle into the visceral cavity, invasion through the digestive system, and invasion of the velum. In another study of *Vibrio* spp. pathogenic to shellfish, Brown et al (1981), suggested that the bacterial pathogen may be located in the WC initially but after colonisation of larvae surfaces multiplies rapidly and becomes more difficult to detect in the water. Prado et al (2005) in a study of epizootics in shellfish hatcheries similarly found that only bacterial isolates from larvae, and not other compartments of the larvae tank, were able to cause disease in bacterial challenges with larvae. These studies indicate that pathogenic bacteria typically colonise and multiply within the larvae, directly. Thus if diseased larvae were affected by toxins produced remotely by pathogenic bacteria, it seems more likely that such pathogens may have been operative from moribund larvae rather than biofilm.

3.5.2 Microbial communities of moribund larvae

Moribund larvae sampled from the tank bottom were different from diseased and not-diseased larvae, based on Unifrac PCA analysis of clone libraries (Figure 20). Bacterial cultivation data, TVC and *Vibrio* counts, also indicated a difference between moribund and not-diseased larvae with densities of bacteria in association with moribund larvae six- to seven-fold higher compared to not-diseased larvae (Table 18).

A total of three clone libraries were constructed for moribund larvae from three different production runs that indicated that there was no characteristic bacterial community associated with moribund larvae common to all production runs. Moribund larvae of production run 070909 were dominated by *Alphaproteobacteria* and also had a high proportion of *Flavobacteria*. Production run 170909 moribund larvae were dominated by *Alphaproteobacteria* and *Betaproteobacteria* whereas production run 280109 moribund larvae were dominated by *Bacilli* and *Gammaproteobacteria* (Figure 21). No culture-independent microbial community studies have been reported for oyster larvae but limited research exists for other aquacultured species. A study of a great scallop (*Pecten maximus*) hatchery using DGGE showed that moribund larvae were dominated by *Pseudoalteromonas* spp. (*Gammaproteobacteria*) (Sandaa et al 2008). In a study of rock lobster larvae (*Panulirus ornatus*) using DGGE, Payne et al (2006) observed that the WC was dominated by *Alphaproteobacteria* and *Gammaproteobacteria* following disease incidence at 10 DSS, and *Vibrio* spp. were detected in clone libraries.

Unifrac PCA analysis indicated that moribund samples could be more similar to WC, algae and biofilm in terms of microbial composition (Figure 20), and clone libraries from production run 070909 provided evidence that advanced stages of disease may be associated with displacement of indigenous microbial species by new predominant species. In particular predominant species present in eggs and larvae from eight and 10 DSS, including *Sphingomonas paucimobilis*$_{(97\%)}$, *Ramlibacter tataouinensis*$_{(93\%)}$ and *Roseisalinus antarcticus*$_{(93\%)}$, were absent in moribund larvae, and other predominant species undetected in the not-diseased larvae were present including *Nautella italica*$_{(97\%)}$ and an unidentified *Flavobacteria*$_{(>10\%)}$. The idea of displacement of the indigenous microbial community with

new predominant species takes some support from other production runs. In production run 280109 2/4 phylotypes from nine DSS larvae and 7/10 phylotypes from 14 DSS larvae, including the predominant phylotype *Shimia marina*$_{(97\%)}$, were no longer present in moribund larvae of seven DSS larvae. Moribund larvae also had predominant species absent in not-diseased larvae including *Marinovum algicola*$_{(97\%)}$ and *Arcobacter nitrofigilis*$_{(93\%)}$. However, seven and nine DSS larvae did not share a single phylotype, which may indicate that the larvae microbial community was not persistent in production run 280109. In production run 170909, 13/17 phylotypes of 10 DSS not-diseased larvae were not detected in moribund larvae including the most predominant phylotype *Sphingopyxis litoris*$_{(93\%)}$. In addition predominant species of moribund larvae including *Shimia marina*$_{(97\%)}$ and *Arcobacter nitrofigilis*$_{(93\%)}$ were not found in not-diseased or diseased larvae.

The predominance of *Sphingomonas paucimobilis*$_{(97\%)}$ and *Ramlibacter tataouinensis*$_{(93\%)}$ in the microbial communities of egg and larvae of production run 070909 is note worthy since they are not normally encountered in the marine environment and hence their eventual displacement in moribund larvae may be related to their poor suitability to the saline conditions. This idea was discussed in Chapter 2 in the context of poorly adapted indigenous species contributing to inherent instability of the microbial community (section 2.5.7), and has additional traction with incorporation of data from production run 070909, in which predominant indigenous species, *Sphingomonas paucimobilis*$_{(97\%)}$ and *Ramlibacter tataouinensis*$_{(93\%)}$, were displaced in moribund larvae, in the present chapter. *Sphingomonas paucimobilis*$_{(97\%)}$ and *Ramlibacter tataouinensis*$_{(93\%)}$ were both also present in larvae samples from production run 170909 but were not displaced in moribund larvae; although production run 170909 did not experience mass mortality, rather a more gradual attrition up to and beyond metamorphosis. That both *Sphingomonas paucimobilis*$_{(97\%)}$ and *Ramlibacter tataouinensis*$_{(93\%)}$ were present in larvae samples from production run 070909 and 170909 suggests a prevalent source. Potential sources may include implements or fresh water used during spawning, or broodstock, which were housed in the same semi-closed facility in Pipeclay lagoon.

3.5.3 Larvae aggregative behaviour: active or passive response?

In disease incidence the first symptoms to emerge may provide clues to the primary cause of disease. In two out of the three production runs monitored intensively (070909 and 170909) and in six out of seven unsuccessful production runs monitored, the first sign of disease was consistent: 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 and no other disease symptoms were observed (henceforth referred to as "swarming"). Larvae swarming was either associated with an increase in TVC counts in the larvae or in the WC or no change at all. In both 070909 and 170909, larvae *Vibrio* counts or *Vibrio*/TVC percentage did not increase at the first incidence of swarming but began to increase daily from the second swarming incident until a *Vibrio* peak occurred in association with a disease incident.

According to the Bicheno hatchery manager, swarming is the first sign of disease in 80% of disease incidents and although recovery sometimes takes place, the likelihood of disease occurring following recovery is elevated. Such behaviour is likely to create conditions conducive to proliferation of opportunistic pathogens within the larvae due to the high densities achieved in swarms and their proximity to the biofilm. 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).

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 larvae hatchery by Sugumar et al (1998), visible aggregation of larvae on the tank bottom was noted as the first sign of disease. The present study is in

agreement that the first sign of disease may be aggregative behaviour; however, the high level of activity of larvae in swarms noted in the present study 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.

Whether by a coordinated active swarming response or by passive processes, it appears that this behavioural response may be of importance in observed swarming in larvae tanks. Anthropogenic contaminants are known to alter larvae behaviour. For example Thompson et al (1997) showed that in 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 mg/L NaOCl (1 ppm)) before activity levels began to increase after one hour and returned to normal after six hours while 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 and His et al (2000) has proposed that swimming behaviour of bivalve larvae could be used in bioassays to detect sub lethal levels of toxicants. Thus swarming could be caused by anthropogenic contamination from within the hatchery or from outside the hatchery. Nearby potential sources of anthropogenic contamination included a boat slip, abalone factory, boat ramp, and forestry plantations (Figure 2). 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). Kairomones released from fish-predators could theoretically inform larvae of increased predation risk and cause swarming behaviour, although there was no source of kairomones within the hatchery vicinity, except perhaps from fish residue discarded from nearby fishing vessels. The highly volatile chemical dimethylsulphide (DMS) is a breakdown product of the algae osmoregulant dimethylsulfoniopropionate (DMSP) and an important "info-chemical" in the plankton environment that has been shown to elicit responses across a wide range of marine species. For example, sea birds are attracted to algal blooms from kilometres away through detection of DMS (Nevitt & Haberman 2003). Copeopods (such as *Temora longicornis*) were shown to be attracted to DMS plumes (Steinke et al 2006). Lyons et al (2007) found DMS and acrylic acid to be feeding deterrents to sea urchins at "natural" concentrations. DMSP has also been shown to elicit behavioural responses in planktivorous reef fishes where it is active as a foraging cue (DeBose et al 2008).

Elevated levels of DMS in the marine environment are associated with the collapse of algal blooms as DMSP is released with zooplankton grazing of micro-algae and DMSP is then degraded to DMS and acrylic acid by bacteria such as members of the *Roseobacter* clade. The grazing-induced production of DMS may be analogous to the production of volatile info-chemicals during herbivore grazing on terrestrial plants that utilize such trace gases to attract carnivores (Nejstgaard et al 2007). Hence the observed swarming response of larvae may be a chemically induced response to increased predation risk associated with algal blooms and mediated by DMS.

Potential for concentration of DMSP, DMS and acrylic acid exists in the commercial hatchery. Large quantities of DMSP-containing micro-algae are fed to high densities of oyster larvae and DMSP is released through grazing. DMSP is then degraded by bacterial species such as those of the *Roseobacter* clade, which were shown in the Chapter 2 to

dominate microbial communities of the larvae tank (section 2.4.6). One of these chemicals may have a role in altering larvae behaviour and may be an idea worth investigating in future research.

3.5.4 *Vibrio* growth spikes: What role do *Vibrio* spp. play?

In the three production runs monitored intensively, a common feature of disease incidents was a growth spike in *Vibrio* counts, which expressed most reliably, across all three production runs, as a peak in *Vibrio*/TVC percentage in the larvae and WC. The nature of the spike was different in each production run. In production run 280109 a *Vibrio* spike was observed coincidental with emergence of disease symptoms and *Vibrio*/TVC percentage reached 100% in the larvae when mass mortality occurred. In production run 070909 a spike in *Vibrio*/TVC percentage was observed the day prior to emergence of disease symptoms with a peak of 35.4% associated with the larvae, although *Vibrio* percentage declined as disease symptoms emerged and was less than 1% when mass mortality occurred. In production run 170909, peaks in the *Vibrio* counts of larvae and *Vibrio*/TVC percentage (9.6%) were coincidental with first emergence of disease symptoms. Emergence of disease symptoms at 16 DSS was accompanied by rising larvae *Vibrio* counts and the *Vibrio*/TVC percentage increased over the period from 16 to 18 DSS to levels of 24.7% for larvae samples and 42.6% in the WC. In general *Vibrio* growth spikes were observed in both the larvae and the WC. Within the Bicheno hatchery disease incidence is always accompanied by *Vibrio* spikes in the WC (hatchery manager. pers. comm.) while bacteria associated with larvae are not usually assessed.

Vibrio growth spikes when associated with the emergence of disease symptoms indicate that *Vibrio* spp. may be the etiologic agent and this conclusion is all the more attractive for the large body of scientific literature implicating *Vibrio* in bivalve hatchery epizootics. However, *Vibrio* only appears as a minor component in one larvae clone library and is absent from the remainder including moribund larvae and other sample types, which indicates that *Vibrio* is over-represented using culture-based methods of enumeration. This raises the question of whether *Vibrio* could cause disease at less than 1% of the population

or alternatively whether *Vibrio* growth spikes may be indicative of changing conditions favourable to *Vibrio* spp.

Total numbers of *Vibrio* bacteria may be more important in pathogenesis than their proportional representation in the total population. Results indicated no significant relationship between *Vibrio* counts in larvae or WC and disease status. However, this result provides no information on how the population of any particular *Vibrio* sp. may have changed with emergence of disease symptoms other than indicating the maximum population size that may have been achieved by a particular *Vibrio* sp. (if it were 100% of the genus *Vibrio*).

Larvae-bacteria challenge tests in other studies have shown that *Vibrio* spp. isolated from moribund larvae were 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 using the challenge protocol proposed by Estes et al (2004). Prado et al (2005) noted significant mortality in challenge tests after 24 h using 4 log CFU/ml of *Vibrio splendidus*. However, neither of these studies evaluated the number of bacteria in the WC when mortality assessments were made. Hence it is not known whether the *Vibrio* sp. reached higher concentrations in the WC before the *Vibrio* sp. infected the larvae. Due to the use of sterile water in challenge tests it seems likely that the introduced bacteria might replicate rapidly in the absence of competing bacteria.

A larvae-bacterial challenge test with a highly pathogenic species of *V. tubiashii* was undertaken in the present study and relatively high mortality of 70% was observed at initial levels of 2 log CFU/ml after 72 h. *Vibrio* counts taken at 72 h indicated that *Vibrio* counts were 5.19 log CFU/ml. This result indicates that much higher levels of pathogenic bacteria may be required to cause disease than the initial level of 2 log CFU/ml, but also highlights the difficulty in using laboratory-based challenge tests to infer larvae-pathogen relationships in a hatchery environment. Elston et al (2008) found that lower concentrations of bacteria were required to cause mortality in laboratory challenge tests compared to production facilities and proposes 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.

Vibrio counts in the WC in the larvae tank were assessed and are compared here against values found in other studies. In production run 280109 disease symptoms emerged when *Vibrio* counts in the WC reached 4.16 log CFU/ml and mass mortality occurred at 5.01 log CFU/ml. However, since it was shown that the predominant phylotype in larvae, *S. sciuri*$_{(97\%)}$ was capable of growth on TCBS, these numbers cannot be considered as representative of the *Vibrio* population and hence *Vibrio* numbers are not known. In production run 070909 *Vibrio* levels in the WC were 4.85 log CFU/ml when disease symptoms first emerged. In production run 170909 *Vibrio* levels in the WC were 3.21 log CFU/ml when disease symptoms first emerged at 10 DSS and reached a maximum of 3.83 log CFU/ml at 12 DSS, although levels may have been higher at 11 DSS, which was not sampled. In hatchery assessments of *Vibrio* populations, Elston et al (2008) found a maximum count of *Vibrio* 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) suggested that 4 log CFU/ml of *Vibrio* in hatchery water caused vibriosis in scallop larvae. Thus it appears that *Vibrio* numbers in the WC in the present study are of a similar magnitude to *Vibrio* numbers encountered in other hatchery studies where *Vibrio* has been implicated as the primary etiologic agent. As such it remains a possibility that disease was caused by *Vibrio* spp. despite representing less than 1% of the total bacterial population as indicated by clone libraries.

An alternative explanation to the *Vibrio* growth spikes is a change in conditions for microbiological growth that suits *Vibrio* spp. *Vibrio* spp. are known to have relatively high growth rates compared to other bacteria and respond quickly to organic pulses (Rehnstam-Holm et al 2010), and therefore it may be that *Vibrio* growth spikes result from increased organic material in the WC as sick larvae release large amounts of organic matter due to vomiting or diarrhoea caused by non-*Vibrio* bacterial species or other unknown causes. Another possible scenario is increased organic matter release due to lysis of micro-algae in the larvae tank, which could possibly be caused by algicidal bacterial species such as *Kordia*

algicidia (which was predominant in algal samples). However, levels of key nutrients were measured in the larvae tank during disease incidents and these remained below minimum detection levels; although minimum detection levels may have been too high and DOC was not measured during disease incidents.

3.5.5 Predominant bacteria of moribund larvae as suspected pathogens

Predominant bacteria associated with moribund larvae may be suspect pathogens of larvae; however, no bacterial species identified in clone libraries of moribund larvae or other compartments of the larvae tank are recognised pathogens of bivalves. Larvae challenge tests with isolates from the hatchery showed that all isolates caused mortality at the highest densities (>6 log CFU/ml) but none were pathogenic at moderate or low densities, like the highly pathogenic strain *V. tubiashii* that caused mortality at 2 log CFU/ml. In larvae-bacterial challenge tests, Garland et al (1983) found that all of the 34 species tested caused mortality at concentrations of 7 log CFU/ml and only considered species as pathogenic when they caused mortality at lower concentrations of 6 log CFU/ml, which included seven species. Note that direct comparisons with bacterial concentrations used by Garland et al need to be made with caution due to differences in larvae-bacteria challenge methodology.

A number of predominant phylotypes of moribund larvae belonging to the *Roseobacter* clade were identified including *Marinovum algicola*$_{(97\%)}$, *Nautella italica*$_{(97\%)}$, and *Shimia marina*$_{(97\%)}$. None of these have been implicated in disease of aquatic animals. *Nautella italica* was used in larvae challenge tests in the present study and found to cause high larvae mortality at a concentration greater then 6 log CFU/ml after 72 h. As such *Nautella italica* was not considered highly pathogenic.

Arcobacter spp., which were predominant in moribund larvae of production run 170909 (*Arcobacter nitrofigilis*$_{(93\%)}$) have been known to cause disease in warm blooded animals (McClung et al 1983) but have not been identified as pathogenic towards aquatic invertebrates. *Arcobacter nitrofigilis* is a nitrogen-fixing symbiotic species found in the marine environment and has been isolated from shellfish (Figueras et al 2011). No *Arcobacter* spp. were isolated in the present study.

S. sciuri, which was predominant in production run 280109 (*S. sciuri*$_{(97\%)}$) is widely distributed in nature and is a human pathogen responsible for endocarditis, peritonitis, septic shock, urinary tract infection, pelvic inflammatory disease and wound infections (Chen et al 2007). *Staphylococcus warneri* was found in scallop eggs by Sandaa et al (2003) and was also isolated from diseased Rainbow trout (*Oncorhynchus mykiss*) (Gil et al 2000), where the authors suggested that it may have been an opportunistic pathogen. In the present study *S. sciuri* was found to be pathogenic towards larvae at a concentration of greater than 6 log CFU/ml after 72 h.

Thus whilst it appears that larvae mortality was caused by bacillary necrosis, no specific bacterial pathogen was identified in this study.

3.5.6 Study limitations

Limitations of the monitoring study outlined in the previous chapter in section 2.5.9 are also valid in this chapter. Additional limitations relevant to the material in this chapter are discussed here.

Detailed histological examination, not undertaken in this study, could have provided additional information regarding disease etiology since tissues of abnormal larval bivalve with bacillary necrosis have been well described with histological immunofluorescent and ultrastructure techniques (Paillard et al 2004). Additionally only seven bacterial species isolated were trialled in larvae challenge tests and more success in identifying specific bacterial pathogens may have been achieved with a greater effort in isolating bacterial species from the hatchery.

It was demonstrated in this study that *Vibrio* spp. represented less than 1% of the population and that *Vibrio* spp. may have been sufficient in number to cause disease in observed mortality incidents. For the purpose of investigating the role of *Vibrio* spp. in disease incidence where the *Vibrio* population may be less than 1%, the principal investigative tool used here, TRFLP, may be of limited value. A better microbiological tool to allow study of *Vibrio* populations in the context of the whole microbial community could include next-generation sequencing (Zhanjiang 2011).

An additional limitation of this study is the lack of successful production runs for comparison with diseased runs. Only one of eight production runs monitored was successful and none of the three production runs monitored intensively were successful. Hence production run outcome, in terms of success or failure, was not investigated as it was not possible to separate any observed differences with production run outcome from the effects of other independent variables. Instead this study characterised changes occurring in microbial communities with emergence of disease symptoms.

Investigation into the underlying causes of disease was limited to a set of known independent variables but disease may have been related to unidentified factors. In particular, bacillary necrosis is considered a management disease (Elston 1990), and management factors were not considered in this study. Accounting for the activities of staff in adhering to various protocols such as daily and weekly cleaning schedules, animal husbandry, feeding rates, and equipment maintenance was beyond the scope of this study. Since this study was limited to a single hatchery it was particularly prone to the influence of hatchery practices. Other factors not accounted for included larvae-centric factors such as the immunological and genetic status of the larvae, which according to Sainz-Hernandez (2005) may determine the fate of a batch of larvae under culture. Additionally larvae stress, which is thought to increase susceptibility to disease incidence (Bourne et al 2004), was not evaluated.

3.6 Summary and Conclusion

Evidence was presented, using TRFLP analysis, that microbial communities associated with larvae and WC experienced significant change with the emergence of disease symptoms characteristic of bacillary necrosis. However the change experienced appeared to be unique within each sampling period and there was no predominant characteristic microbial community associated with the emergence of disease symptoms, and no recognised bacterial pathogens were detected using 16S rRNA clone libraries. Larvae-bacteria challenge trials with predominant bacterial species of diseased larvae indicated that predominant bacterial species were not highly pathogenic. Furthermore the change observed in diseased larvae

was less significant than the change experienced with DSS and sampling period, and may not have been substantial. On the other hand, microbial communities of moribund larvae were substantially different than diseased and not-diseased larvae, and highly divergent among different production runs. Evidence indicated that advanced stages of disease, in moribund larvae, were associated with displacement of the predominant species of not-diseased larvae and a shift towards the microbial communities of the WC, biofilm and algae. Poor suitability of predominant species of the larvae indigenous microbial community, *Sphingomonas paucimobilis*$_{(97\%)}$ and *Ramlibacter tataouinensis*$_{(93\%)}$, to conditions of culture might have related to their displacement in moribund larvae.

High mortality disease incidents were accompanied by population peaks in *Vibrio* spp. and despite representing less than 1% of the total population using 16S rRNA clone libraries, the total number of *Vibrio* spp. may have been sufficiently abundant to cause disease. Larvae aggregative behaviour near the tank bottom prior to the development of definitive disease symptoms may indicate a non-microbiological primary cause of disease, or a microbiological etiology that occurred below the detection limits of 16S rRNA gene-based analyses used. Non-microbiological causes may have included toxins or behavioural stimulants of natural or anthropogenic origin.

4 Chapter 4: Environmental stressors and larvae susceptibility to bacterial challenge

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. Two different stressors included exposure of larvae to copper and, in a separate trial, heat, administered prior to bacterial challenge. It was shown that sub-lethal levels of copper could increase larvae susceptibility to bacterial pathogens under some conditions. Larvae behaviour was modified at copper levels as low as 2.5 ppb, which indicated that behaviour could be used as a sensitive biomarker of copper stress and might also be of use in assessment of other types of chemical stress and susceptibility to bacterial disease. The behavioural response to different concentrations of copper 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. Preliminary investigation into heat shock treatment demonstrated increased larvae activity, which may have been correlated with survival, when challenged with bacteria, following heat treatments of 30 or 45 min at 37^{o}C.

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 agents 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 copper 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 copper 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 copper phagocytosis was stimulated but was inhibited at high levels of copper. 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, copper is highly toxic to bivalve larvae and is a common contaminant in the coastal marine environment. Calabrese et al (1977) found that copper 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 copper levels as low as 0.6 ppb. Sources of copper within the hatchery include brass fittings and pump bearings, both of which may "leak" copper ions as corrosion takes place (Jones 2006). The main sources of copper 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 copper 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 the hatchery. 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). Other pollutants may accumulate at the air-surface interface at concentrations 100 to 1,000-fold greater than in the water column (Hardy 1982) including toxic hydrocarbons, heavy metals, lipids and proteins. Since hatchery water inlets are usually located subsurface, water intake is likely to be more affected by sediment re-suspension than surface accumulation.

Contamination from within the hatchery may also cause stress to 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 21) but contribute little to the prediction of the direct consequences for the organism or population in question (Depledge & Fossi 1994).

Table 21 – 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 The heat shock response

The cellular response to stress is often complex and may involve adaptive mechanisms designed to protect the cell from further assault, and one such example is the heat shock response. The heat shock phenomenon was first observed as a response to heat stress involving 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). The heat shock response has since been found to be an almost ubiquitous phenomena occurring across almost all species of prokaryotes, archaea, and eukaryotes (Hochachka & Somero 2002). The induction of HSP has also been demonstrated in oyster larvae (*Ostrea conchaphila* in Brown et al (2004), *C. virginica* in Ueda and Boettcher (2009)); and Ueda and Boettcher (2009) suggested that increased susceptibility of larvae to diverse environmental stress may be associated with low expression of HSP. However, there does not appear to be any research investigating the effect of heat shock on subsequent resistance to bacterial pathogens in bivalve molluscs.

4.2.5 Research objectives

In this chapter attention was turned towards the host and environment. Larvae susceptibility to bacterial attack was assessed following exposure to two different types of stress: copper as a chemical stress and heat as a physiological stress; to meet hypotheses B and C, defined in Table 1 and associated research objectives (No. 3 to 5). 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 copper-based anti-fouling paints are used regularly. Heat shock was investigated to establish whether the recognised protective effects induced by heat shock can serve to decrease larvae susceptibility to bacterial disease. Investigation into copper stress includes larvae behaviour as a potential biomarker for application in the hatchery environment. According to Oliver and Fisher (1999) little is known about the relationship between biomarkers and susceptibility to disease, and Depledges and Fossi (1994) advocate research to relate stress-induced changes in biomarkers to survival, which can then be related to exposure to a known amount of the pollutant.

4.3 Methods

Trials involved exposure of larvae to stress followed by bacterial challenge. Two types of stress were investigated including (1) exposure of larvae to copper sulphate and (2) exposure of larvae to heat stress. In both cases the larvae stress treatment and bacterial challenge were performed sequentially with the stress prior to the challenge. Separation of the two may not have accurately represented likely culture conditions but was done to avoid potential interaction between the stress or unknown component of the culture water and pathogenic mechanism. For example if copper exposure and bacterial challenge were combined it would not be clear whether the presence of copper increased bacterial pathogenicity towards larvae or instead exerted some influence on larvae such that susceptibility was enhanced. With separation of the two, interaction between stress and bacterial challenge was taken as due to enhanced susceptibility of the larvae caused by the

stress treatment; although, other factors such as the effect of copper treatment on the resident microbial community of the larvae cannot not be wholly discounted.

4.3.1 Copper sulphate exposure followed by bacterial challenge

Six DSS larvae 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 as indicated in the treatment plan below in Table 22. 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.

Table 22 – $CuSO_4$ treatment plan prior to bacterial challenge

	$CuSO_4$ conc.	**ppb**	**0 (control)**	**10**	**30**	**60**	**100**	**500**	**1000**	**3000**
		log ppb	**0[1]**	**1.0**	**1.5**	**1.8**	**2.0**	**2.7**	**3.0**	**3.5**
Exposure time (h)	**Cu conc.**	**ppb**	**0**	**2.5**	**7.6**	**15**	**25**	**127**	**254**	**763**
24			3	3	3	3	3	3	3	3
48			3	3	3	3	3	3	3	3

(1) Log values are included because they are used in figures. Note that log of zero is mathematical nonsense but zero is used as an approximation

$CuSO_4$ exposure was conducted in 250 ml Erlenmeyer beakers at a density of 100 larvae per ml, in 200 ml autoclave sterilised artificial seawater (SSW). 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 heavy metals as indicated in Table 23. Full chemical analysis is available online from http://www.redseafish.com/ (01/01/2012).

Table 23 – Heavy metal content in natural "Red sea" water and Red Sea Salts

Element	Natural "Red sea" water (ppb)	Red sea salts (ppb) at 33 g/ L
Copper	0.2	trace
Mercury	0.005	trace
Nickel	0.7	0.3
Silver	0.02	trace
Zinc	0.5	9

$CuSO_4$ stock solution was diluted in a small volume of sulphuric acid and treatments adjusted to a pH range of 8.1 to 8.2 using 5M sodium hydroxide. 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 separate challenge tests with three different bacterial species (*Vibrio pomeroyii*, *Staphylococcus sciuri* and *Pseudomonas fluorescens)*, or a no-bacteria control, according to the plan given in Table 24. All challenge tests were done in triplicate using all copper treatment replicates.

Table 24 – Bacterial-larvae challenge plan following $CuSO_4$ treatment

	Control (no bacteria)	*V. pomeroyii* (log CFU/ml)			*S. sciuri* (log CFU/ml)			*P. fluorescens* (log CFU/ml)		
		3	**4**	**5**	**5**	**6**	**7**	**5**	**6**	**7**
24 h	3	3	3	3	3	3	3	3	3	3
48 h	3	3	3	3	3	3	3	3	3	3

Larvae were challenged with bacteria in 12-well tissue culture plates according to the methodology detailed in section 2.3.5, adapted from a protocol proposed by Estes et al (2004).

Statistical analyses were conducted separately for each combination of $CuSO_4$ exposure time and bacterial species, which included a total of six analyses (statistical model design given in Appendix 14: HH to MM). All statistical analyses were conducted using SAS

GLM (SAS software (version 9.2) general linear model (GLM)) as described in section 2.3.4.2. Statistical models included modelling 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 mobility

In a separate trial run in conjunction with $CuSO_4$ exposure and bacterial challenge, larvae mobility 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 different concentrations of $CuSO_4$ for a range of exposure periods from 0.5 h to 54 h, according to the plan in Table 25. 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 mobility 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).

Table 25 – Copper sulphate exposure plan for assessment of larvae mobility

	$CuSO_4$ concentration								
	ppb	**0**	**10**	**30**	**60**	**100**	**500**	**1000**	**3000**
Exposure time (h)	**log ppb**	**0**	**1**	**1.5**	**1.8**	**2**	**2.7**	**3**	**3.5**
0.5		3	3	3	3	3	3	3	3
13		3	3	3	3	3	3	3	3
18		3	3	3	3	3	3	3	3
28		3	3	3	3	3	3	3	3
54		3	3	3	3	3	3	3	3

The protocol for assessment of larvae mobility 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 the exposure times indicated in Table 25, 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 15. All treatments were filmed beginning with the lowest $CuSO_4$ concentration and moving through to the highest concentration. Filming took approximately one hour and the times indicated in Table 25 represented the time that filming commenced. After filming, the movies were downloaded to a PC computer and each replicate was assessed for % SL and % CL. % CL was evaluated over the full two min and larvae were counted as they crossed a line drawn in the middle of the cuvette prior to the commencement of the experiment, whether ascending or descending. The % SL was evaluated from a still image of the last frame of the two min. Full counts of larvae in each cuvette were made at the conclusion of the trial so that % SL and % CL could be calculated accurately.

Two separate statistical analyses were conducted including one analysis for % SL (Statistical model NN) and another for % CL (Statistical model OO). Both analyses were conducted using SAS GLM (SAS software (version 9.2) general linear model (GLM)), as described in section 2.3.4.2, and included modelling of main effects of $CuSO_4$ level and exposure time, as well as the interaction between these two factors.

4.3.3 Heat shock treatment followed by bacterial challenge

After collection from the Bicheno hatchery, larvae were held at 10^{o}C for three to four hours before experimentation began. Prior to heat shock larvae were held at 25^{o}C for one hour. Larvae were exposed to a heat shock prior to challenge against *V. pomeroyii* isolated from a diseased larvae sample. The heat shock treatment involved exposure of larvae to 37^{o}C for 10, 30, 45 or 60 min followed by six hours recovery at 25^{o}C before being challenged against *V. pomeroyii* at concentrations of 1 log, 3 log and 5 log CFU/ml, according to the plan given

in Table 26. Heat shock treatment was done in triplicate and each treatment replicate was challenged by each level of *V. pomeroyii* to achieve triplicate replication for bacterial challenge.

Table 26 – Heat shock treatment and bacterial challenge plan

***V. pomeroyii* challenge concentration**	**Heat shock duration (min)**				
	0	**10**	**30**	**45**	**60**
0 (control)	3	3	3	3	3
1 log CFU/ml	3	3	3	3	3
3 log CFU/ml	3	3	3	3	3
5 log CFU/ml	3	3	3	3	3

A total of 15 tubes were exposed to heat shock treatment (including the no-heat shock control) and each tube contained 1,000-1,500 larvae in 10 ml SSW, prepared as in the previous section except without filtration to 0.45 µm. Tubes were immersed into a 37^{o}C water bath for durations indicated in Table 26 before being placed into a 25^{o}C water bath for recovery. After a six h recovery period larvae were challenged against *V. pomeroyii* by dividing each treatment replicate into four new 15 ml falcon tubes (a total of 60 tubes) to make up 10 ml with bacterial concentrations as indicated in Table 26 and larvae densities of 20 -30 larvae per ml. The bacterial challenge protocol employed was adapted from Estes et al (2004), in which 15 ml tubes were recommended for assessment of swimming behaviour. The bacterial culture was prepared according to the procedure detailed in section 2.3.5 (Chapter 2). At 12 h after initiation of the bacterial challenge, the top 3 ml of the tube was removed using a 1 ml pipette and larvae placed into a petri-dish for counting and then discarded. At 24 h the heat shock experiment was terminated early because all larvae, including the untreated control, were dead or inactive. The total number of larvae in each challenge test was established. Clearly the experimental conditions were not optimal and it was subsequently determined that the likely cause of death was some component in the SSW that could be removed by filtration to 0.45 µm, presumably in association with undissolved particles. Because of time restraints, the experiment was not repeated.

Preparation of the bacterial cells used in the challenge is described in section 2.3.5. A single statistical analysis was conducted for the heat shock trial and included modelling of main effects of heat shock duration and *V. pomeroyii* concentration, as well as the interaction between these two factors (Statistical model PP).

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 was investigated using separate statistical analyses for each bacterial species (three species) and exposure length (24 h or 48 h) combination, a total of six statistical analyses. All analyses are represented in Figure 31 (A-F), where percentage mortality was plotted against $CuSO_4$ treatment levels. The interaction between $CuSO_4$ and bacterial challenge was the focus of this study, although the main effects of both variables were highly significant. In brief, $CuSO_4$ level and bacteria level had a positive correlation between mortality and both factors. 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, although 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 31 (B), Pr = 0.0018); 24 h exposure followed by *P. fluorescens* challenge (see Figure 31 (E), Pr < 0.0001); 48 h exposure followed by *S. sciuri* challenge (see Figure 31 (D), Pr = 0.0279); and 24 h treatment followed by *S. sciuri* challenge (see Figure 31 (C), Pr = 0.0279), although the latter two were of marginal significance.

The strongest interactions between CuSO4 and bacterial challenge were seen in B and E (Figure 31) 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 with a decrease in mortality at 100 ppb, while the no-bacteria control showed no increase in mortality until 60-100 ppb. In 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 C and D (Figure 31) reflected similar trends in a less consistent way. Similarly to the 6 and 7 log CFU/ml challenge of E, 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. 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 mobility

Larvae mobility in response to different $CuSO_4$ concentrations, assessed as % SL and % CL, was plotted in Figure 32 (A and B for % SL and % CL, respectively). Statistical analysis indicated that larvae mobility was significantly affected by $CuSO_4$ exposure (% SL: $F_{7,119}$ = 7.33, 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 mobility 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 (Statistical model NN); % CL: Pr < 0.0001 (Statistical model OO)), 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 A and B of Figure 32 by the different shaped curves of different time series. Apart from the assessments at 0.5 h in A, and 0.5 h and 13 h in B, 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 A and B. 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, as shown in both A and B of Figure 32.

Sensitivity of larvae to 10 ppb $CuSO_4$ was further evidenced by inclusion of LSD analysis of larvae mobility as reported in Table 27. 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% vs 14.89%) while the % CL was not significantly different, although from B of Figure 32 it seems that the reduction in activity at 10 ppb was only evident with longer exposure.

Table 27 – LSD grouping of larvae mobility 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 (Statistical model NN)				LSD = 3.83. Pr < 0.0001 (Statistical model OO)			

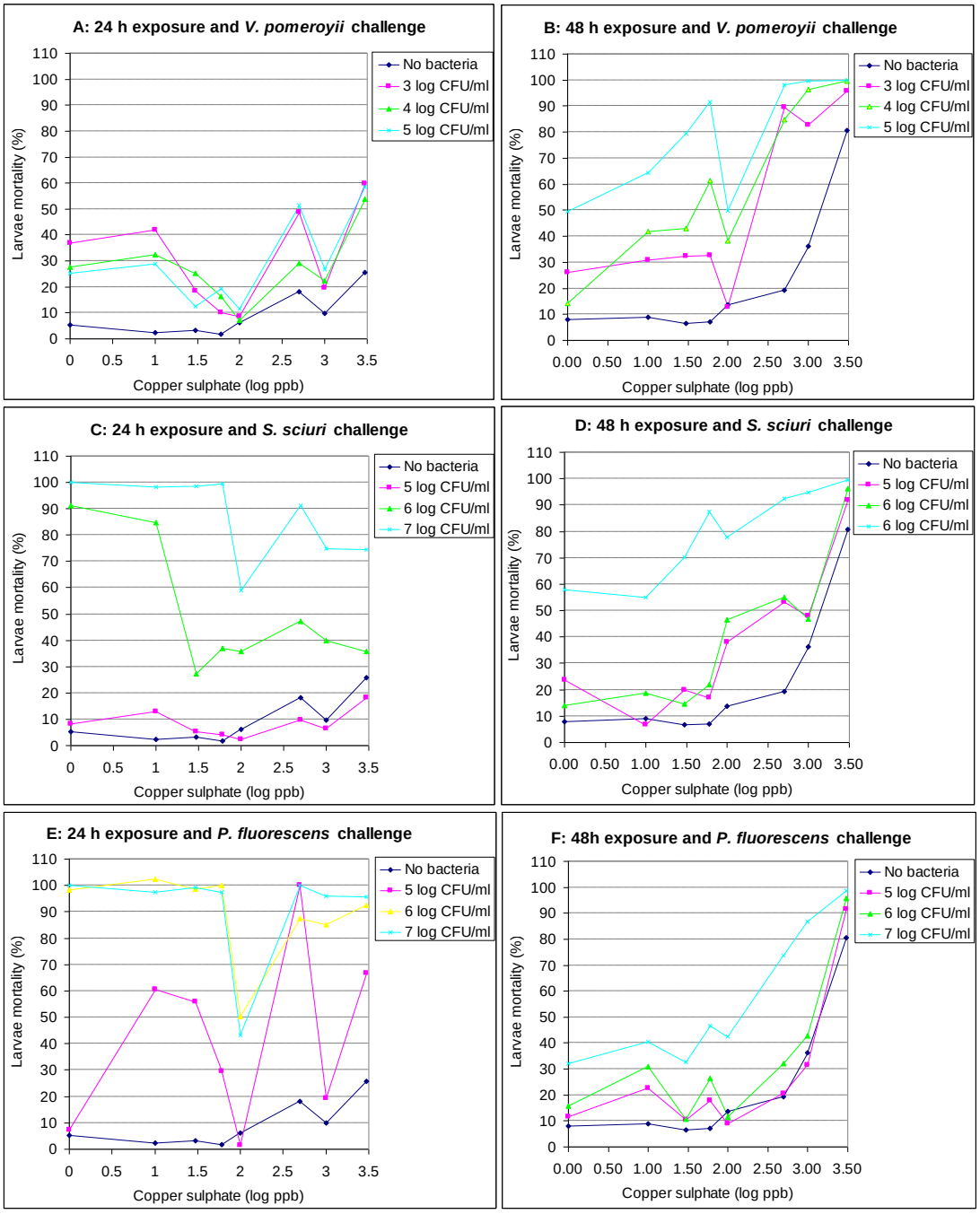


Figure 31 – Larvae mortality in response to 24 or 48 h $CuSO_4$ exposure followed by 24 h exposure to bacteria challenge

Interaction between $CuSO_4$ concentration and bacteria challenge: A: Pr = 0.5238 (Statistical model HH). B: Pr = 0.0018 (Statistical model II). C: Pr = 0.0296 (Statistical model JJ). D: Pr = 0.0279 (Statistical model KK). E: Pr < 0.0001 (Statistical model LL). F: Pr = 0.117 (Statistical model MM).

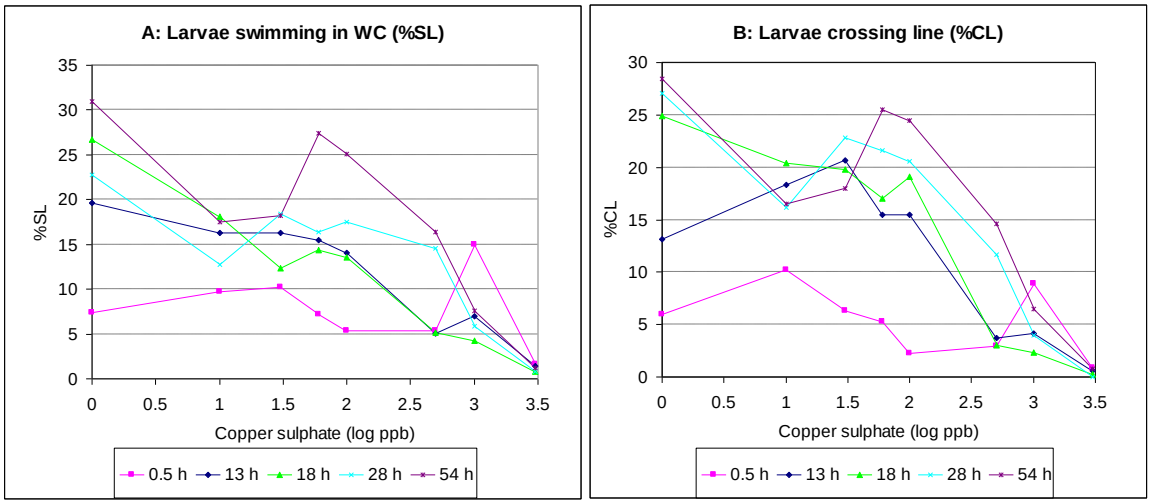


Figure 32 – Larvae swimming behavioural response to different periods of $CuSO_4$ exposure

Interaction between $CuSO_4$ concentration and exposure time: A: Pr < 0.0001 (Statistical model NN). B: Pr < 0.0001 (Statistical model OO)

4.4.3 Heat shock treatment followed by bacterial challenge

The larvae response to heat shock and subsequent bacterial challenge is represented graphically in Figure 33, which indicates that heat shock of 30 and 45 min may have increased larvae mobility in the absence of bacteria and with 1 log and 3 log CFU/ml *V. pomeroyii* challenge. Statistical analysis, in Statistical model PP, indicated that the effect of heat shock was highly significant (Pr < 0.0001) and that the interaction with bacterial level was also significant (Pr = 0.0105).

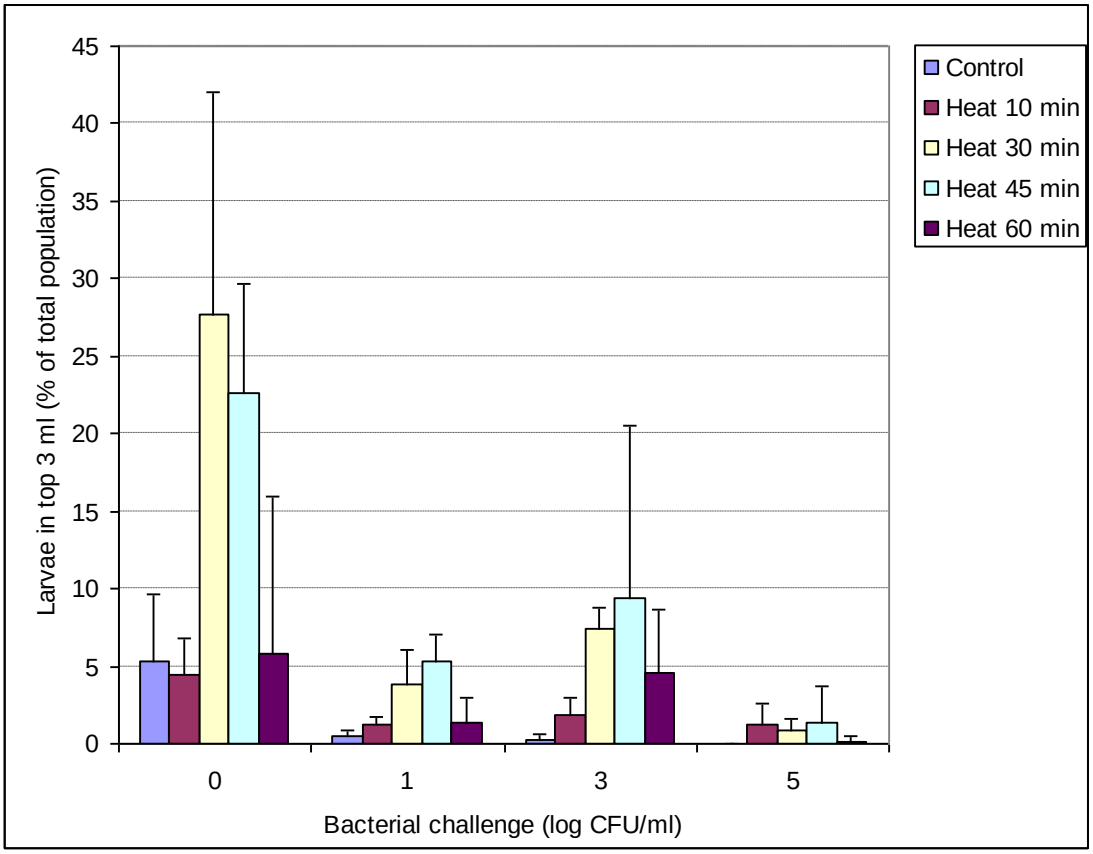


Figure 33 – Effect of heat shock on larvae activity under bacterial challenge

Heat shock duration: Pr < 0.0001; Bacterial level: Pr < 0.0001; Heat shock duration and bacterial level interaction: Pr = 0.0105 (Statistical model PP)

4.5 Discussion

4.5.1 Copper stress, disease susceptibility and larvae behaviour

Results indicated that Cu stress may have increased susceptibility to disease at sub-lethal levels, although in an inconsistent manner, at levels as low as 10 ppb $CuSO_4$. In those stress – bacteria combinations in which the interaction between $CuSO_4$ level and bacterial level was highly significant (B and E in Figure 31), 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 (C and D of

Figure 31), some evidence of increased mortality at sub lethal $CuSO_4$ levels and decreased mortality at the lethality threshold was evident in an inconsistent way. The two stress-bacteria combinations, A and F of Figure 31, showed no significant interaction between $CuSO_4$ stress and bacteria levels.

It is not clear why the interaction between Cu stress and bacterial challenge levels 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 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 potentially increase larvae susceptibility to disease in the hatchery environment. A small increase in disease incidence may be amplified in the conditions of the hatchery where a reduction in swimming activity caused by disease results in dense aggregations of larvae at the tank bottom which may favor contagious spread of the disease.

Separation of the Cu stress treatment and bacterial challenge may have decreased the sensitivity of the test by allowing time for larvae to recover before bacteria could take advantage of the compromised larvae. Separation of the two was undertaken to avoid potential interaction between the stress or unknown component of the culture water and pathogenic mechanism, as explained in methods section 4.3. The trial may have been enhanced by undertaking the Cu stress and bacterial challenge both separately and in combination.

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 as described in the introduction (section 4.2). 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 and 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. Confirmation of this effect through quantification of HSPs was not undertaken, although this may have enhanced the study.

Assessment of larvae motility using 4.5 ml cuvettes according to the protocol employed by Prael et al (2001) provided further evidence that larvae were sensitive to sub-lethal levels of Cu as low as 10 ppb $CuSO_4$, which equates to 2.5 ppb Cu ions, and 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 which application the biomarker would need to be simply and rapidly determined, and not require sophisticated or expensive equipment. In feasibility terms monitoring of larvae swimming 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, and so would 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 2.5 ppb Cu in the present study, although lower levels were not tested, while other studies have indicated a range of Cu concentrations for embryogenesis (EC50 = 50% normal development) as indicated in Table 28 adapted from His et al (1999). The average EC50 for Cu was 22 ppb, although values ranged from 2.72 through to 130 ppb

(Table 28). Thus it appears that swimming behaviour may be at least as sensitive as embryogenesis.

Table 28 – Concentrations of Cu causing abnormal embryogenesis in *C. gigas*

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

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

Research into the behaviour of oyster larvae 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) notes that larvae behaviour in experimental vessels changes with larvae age and therefore urges caution in interpretation of behaviour experiments. The differential behavioural responses (% CL and % SL) to different concentrations of Cu at different time periods of assessment noted in the present study (Figure 32) 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 a response at the level of the whole organism.

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 increase near the toxicity threshold (when testing environmental water quality, a concentration range can be achieved through dilution with SSW); (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); and (3) assessment of the number of larvae in suspension as well as a measure of activity, are different behaviour attributes and should both be included in assessment.

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 21, but characterisation of the stress response should not be limited to these and in particular inclusion of immunological defence activity (mechanisms identified in Table 3, Chapter 1) 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. Research into stress-induced induction of the disease process could additionally enhance understanding of pathogenicity in known pathogenic *Vibrio* spp.

4.5.2 Heat treatment and disease susceptibility

Heat shock treatment had a marked effect on larvae behavior at 12 h in the no-bacteria control and low to moderate levels of bacterial challenge with *V. pomeroyii*. The 30 min heat shock treatment had a greater proportion of the population contained within the top 3 ml than the no-heat shock control in the no-bacteria control, 1 and 3 log CFU/ml challenge with differences of seven, eight and 24 fold, respectively. Although only minimal assessment of the trial was made due to non-survival of larvae including the control, the experiment provided an indication that heat shock treatment may have improved larvae performance against the stressful culture conditions, evident from complete mortality at 24 h, and bacterial challenge.

Increased larvae numbers in the top 3 ml of the experimental tubes under heat shock treatment could be explained by increased survival due to induction of the heat shock response. As discussed in the introduction (section 4.2.4), the heat shock response is a protective affect against a broad range of stresses induced by a short duration of heat shock and other sub lethal stress, and mediated by production of membrane bound HSP. HSP levels were not determined in the present trial due to the poor survival of the larvae under bacterial challenge, as the trial was then considered only preliminary. Although it was intended that the heat shock trial be repeated, insufficient time prevented this being done.

The induction of HSP and increased thermotolerance has previously been demonstrated in oyster larvae. Brown et al (2004) showed that veliger larvae of Californian native oyster (*Ostreola conchaphila*) had elevated levels of HSP (in the size range of 69 k Da) following exposure to 34^{o}C for one hour. Elevated HSP levels were detected six h after treatment and were a further four fold higher after 12 h. Elevated HSP levels were associated with increased survival to the previously lethal temperature of 39^{o}C, although survival was higher at six h than 12 h. Ueda and Boettcher (2009) also demonstrated increased levels of HSP (69 k Da) in oyster larvae (*C. virginica)* after heat shock of 10^{o}C above normal culture temperature for one hour.

Whilst the induction of HSP in oyster larvae has been demonstrated it has not yet been determined how long the effect might last and whether a protective effect against a broad

range of stresses could be achieved, as has been observed in other heat shock studies with diverse species (Hochachka & Somero 2002). It would be of particular interest to determine whether heat shock treatment could induce a protective effect against bacterial pathogens. Such an effect was demonstrated by Sung et al (Sung et al 2008) in experiments with *Artemia franciscana*. One-day old *Artemia* treated with the optimal heat shock of 37^{o}C for 30 min followed by six hour recovery, increased in expression of HSP (70 k Da) and when subsequently challenged with pathogenic *Vibrio* spp. (*V. campbellii* and *V. proteolyticus*) a 100% increase in survival was observed after a 24 h in bacterial challenge. The authors argue that the increase in HSP may have activated the innate immune system of *Artemia*. Indeed, induction of a strong immune response by stress regulated HSPs has been demonstrated in vertebrates including activation of an inflammatory response and T-cells (Pockley 2003; Roberts et al 2010); although in invertebrates too little research on short-term stress and immune response has been performed to establish a clear association between HSP production and activation of the innate immune response.

Use of heat shock as a treatment for commercial applications has been considered previously for other aquacultural species. In research on heat shock of eight-day old fresh water prawn larvae (*Macrobrachium rosenbergii*), Rahman et al (2004) demonstrated increased thermotolerance and tolerance to hypersalinity, which lasted for four days, and the authors recommended that the treatment could be used to improve the performance of larvae during transport and / or initial inoculation into grow out ponds. Other researchers have demonstrated induction of a heat shock response without the need for heat shock treatment. Sung et al (2008) fed brine shrimp with *E. coli* which had been previously heat-shocked to induce production of HSPs. These exogenous HSPs were shown to increase brine shrimp gut levels of HSP and enhance resistance to challenge with *Vibrio* species. The authors suggested that the treatment may be a viable application in management of Vibriosis. Numerous other examples of exogenous HSP stimulation are provided in a review on the potential role of HSPs in aquaculture by Roberts et al (2010).

The results obtained in the present trial, supported by other scientific literature, provide an indication that heat shock treatment might induce cross-protection of larvae against stress and bacterial pathogens. Further research into heat shock treatment may enhance

understanding of larvae immune function and lead to development of a commercially applicable treatment to enhance survival under stressful conditions. Incorporation of a 30 min water bath at elevated temperature between larvae tank water changes might be feasible in a commercial oyster hatchery although research and development potentially might lead to a treatment to induce HSPs without heat shock. Initial research should include optimization of the heat shock procedure, determination of HSP levels in larvae, demonstration of protective effect under a range of different challenge conditions, and determination of the duration of the protective effect.

5 Synopsis and Recommendations

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, microbiological tools were selected to study whole microbial communities (16S rRNA gene clone libraries and TRFLP analysis) in the larvae tank and tank inputs under commercial conditions. In Chapter 3, using data from seven diseased production runs, it was shown that microbial communities associated with larvae and WC experienced significant change with the emergence of disease symptoms characteristic of bacillary necrosis. However the change was unique within each sampling period and there was no predominant characteristic microbial community associated with the emergence of disease symptoms; as such Hypothesis A was negated. The precise cause of disease was not determined in this study although some possible causes were considered.

No known bacterial pathogens were detected using 16S rRNA clone libraries and larvae challenge tests, while limited in the number of bacterial species isolated and tested, indicated that predominant species were not highly pathogenic. It was speculated that disease incidence may have related to displacement of predominant species of the larvae. This idea arose since predominant bacterial species of not-diseased larvae were not detected in moribund larvae, which had microbial communities more closely related to communities of algae, biofilm and WC. The displacement of non-typical marine species, *Sphingomonas paucimobilis*$_{(97\%)}$ and *Ramlibacter tataouinensis*$_{(93\%)}$, in production run 070909 indicated that displacement and disease might be more likely when larvae indigenous species are poorly adapted to the conditions of culture. However, the displacement of predominant bacterial species observed in moribund larvae was not observed in diseased larvae, as such changes occurring in microbial communities of moribund larvae may not relate to the primary cause of disease.

Through daily monitoring of microbial abundance in three production runs it was shown that *Vibrio* spp. experienced population peaks, as a percentage of the total population, in

association with emergence of disease symptoms, despite not being detected in clone libraries of diseased and moribund larvae and other compartments of the larvae tank. Total *Vibrio* counts at population peaks were approximately 4 log CFU/ml in the WC, which has been shown by other researchers to be sufficient to cause bacillary necrosis. These results indicated that *Vibrio* spp. might have been involved in pathogenesis despite being present at less than 1% of the total microbial population. However, since *Vibrio* population peaks were not associated with the first disease symptoms their role in disease incidence is not clear. It may be that *Vibrio* peaks are associated with secondary infections or a response to increased organic matter due to larvae disease.

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 and peaks in *Vibrio* spp. population, which may indicate 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. Such behaviour could be explained by either exposure to info-chemicals in the marine environment signalling increased predation risk or sub-lethal levels of toxicants. In either case aggregative behaviour may favour opportunistic bacterial pathogens through creating locally high densities of larvae in contact with tank-bottom debris.

In Chapter 2, it was shown that the larvae indigenous microbial community was formed within hours of fertilisation and 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. The indigenous microbial community of the larvae is influenced by the initial egg microbial community from the broodstock, the rearing water and other sources of contamination such as implements used during fertilisation, fresh water used for cleaning, the hatchery floor from splashing, human hands and residual biofilms. 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. Seasonal

variability in sea water microbial communities may have caused variability in the larvae and WC communities among sampling periods.

As Hypothesis A was negated, and larvae aggregative behaviour indicated the possibility of a non-microbiological primary cause of disease, it was speculated that disease incidence may have been caused by exposure to some toxin that predisposed larvae to bacterial attack. Because nearby sources of copper were identified (boatslip in Figure 2) and because copper can persist in sediment it was hypothesised that exposure to sub-lethal levels of copper may affect larvae susceptibility to bacterial disease (Hypothesis B; Table 1). In Chapter 4 it was shown that exposure to sub-lethal levels of copper could increase susceptibility of larvae to bacterial attack under certain conditions, in support of the hypothesis. 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. It was further shown that behavioural changes occurred upon exposure to sub-lethal levels of copper as low as 2.5 ppb, which indicates that exposure to copper, or perhaps some other toxin, might explain larvae aggregative behaviour occurring prior to the emergence of definitive disease symptoms as observed during the monitoring study. The results also indicated that larvae behaviour might be a more sensitive biomarker of copper contamination than embryogenesis and might be as sensitive as growth. It follows that larvae behaviour could be further developed as a sensitive and simple to use biomarker for detection of chemical contamination or behavioural stimulants, for use in water quality monitoring. The results indicated that behavioural assessments should be made using a broad range of concentrations across a 24 to 48 h time period.

Finally, in a more explorative approach, based on the recognised heat shock phenomena and research in which heat shock was shown to improve performance of *Artemia* sp. against bacterial pathogens, it was hypothesised that heat shock treatment may affect larvae susceptibility to bacterial disease (Hypothesis C; Table 1). This hypothesis was addressed in Chapter 4, as an investigation into stress responses. Only a preliminary investigation was undertaken but the results showed that heat shock may improve larvae performance under bacterial challenge.

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. Broodstock should be maintained in fresh flowing seawater to minimise the chance of contamination from this source. Broodstock may be a source of undesirable bacteria to the larvae indigenous microbial community.
3. Daily monitoring of *Vibrio* levels expressed as a percentage of the total culturable population (*Vibrio*/TVC) may be more useful than *Vibrio* levels alone (Figure 26). Absence of a *Vibrio*/TVC growth spike during incidence of disease or aggregative behaviour might indicate a non-microbiological cause. *Vibrio*/TVC ratio in larvae and WC samples appears to correlate with incidence of bacillary necrosis.
4. Aggregative behaviour in the absence of any other disease symptoms or *Vibrio*/TVC peaks might be caused by a non-microbiological cause such as chemical stressor or behavioural stimulant in the water.
5. Intensive culture conditions (IC tank) and batch feeding may favour *Vibrio* spp.; although it is uncertain how this may impact upon incidence of disease.

Recommendations for research:

6. Characterise larvae biochemical response to bacterial attack to further elicit the disease etiology and develop tools for monitoring. In particular identify larvae immune response biochemical markers.

7. Characterise larvae biochemical response to the combination of diverse stressors and bacterial attack to identify stress biomarkers that correlate with increased susceptibility to disease incidence.
8. Investigate larvae behaviour in tanks to understand the underlying cause of larvae aggregative behaviour. As a starting point it should be determined whether reduced larvae activity and tank flow dynamics could account for aggregative behaviour. Subsequent investigations may include larvae response to a range of stressors and marine info-chemicals such as DMS.
9. Develop larvae behaviour as a useful, simply applied biomarker for detection of toxins or behavioural stimulants in hatchery water to be used by hatchery staff.
10. Investigate whether different indigenous microbial communities of larvae, manipulated through exposure to different microbial communities during fertilisation, may affect subsequent performance in larvae-bacteria challenge tests.
11. Further investigate heat shock as a treatment to decrease larvae susceptibility to stress and bacterial pathogens.
12. Undertake further hatchery monitoring using Next Generation Sequencing (NGS) including both successful and unsuccessful production runs to:
    a. Determine whether disease incidence is characterised by the presence or absence of particular bacterial groups in the larvae tank whether predominant or relatively minor.
    b. Identify differences between successful and unsuccessful production runs.
13. Further hatchery monitoring using NGS may be undertaken using a functional approach rather than species recognition approach. This would involve using NGS to identify bacterial groups according to particular functional genes relating to microbial lifestyle. Such an approach may inform our understanding of the types of selective pressures driving microbial transformations underlying disease incidence.

6 Appendix

Appendix 1 – Production run dataset: TRFLP-1 analysis

Sampling period	Production run	Sample type	Tank type	2-5 DSS	6-10 DSS		>10 DSS		Eggs
				Disease-free	Disease - free	Diseased	Disease - free	Diseased	
Summer 2009	16/01/09	Larvae	IC				3	1	
			Batch	1	1				
		WC	IC				3		
			Batch	1	1				
	21/01/09	Larvae	IC		1				
			Batch			1	2		
		WC	IC						
			Batch	1		2	2		
	28/0109	Larvae	IC		2		2	3	
			Batch	1	1				
		WC	IC		1		1	3	
			Batch		1			3	
Spring 2009	07/09/09	Larvae	IC		3	3			3
			Batch	3					
		WC	IC		3	3			
			Batch	3					
	17/09/09	Larvae	IC						3
			Batch	3	3	3	3	3	
		WC	IC						
			Batch	3	3	3	3	3	
Summer 2010	27/11/09	Larvae	IC						3
			Batch			3	3		
		WC	IC						
			Batch			3	3		
	01/12/09	Larvae	IC						3
			Batch	3	3		3		
		WC	IC						
			Batch	3	3		3		
	18/01/10	Larvae	IC						
			Batch	3	3			3	
		WC	IC						
			Batch	3	3				

Appendix 2– Production run dataset: bacterial cultivation

				Larvae		WC		CTW		Algae	
Sampling Period	Tank type	DSS	Disease status[1]	TVC	*Vibrio*	TVC	*Vibrio*	TVC	*Vibrio*	TVC	*Vibrio*
Summer 2009	Batch tank	2-5	+	0	0	0	0	0	0	0	0
			-	5	4	3	3	2	2	2	2
		6-10	+	1	1	1	0	0	0	1	1
			-	7	6	6	6	2	2	4	4
		>10	+	3	3	3	3	0	0	3	3
			-	2	2	3	3	1	0	1	2
	IC tank	2-5	+	0	0	0	0	0	0	0	0
			-	0	0	0	0	0	0	0	0
		6-10	+	0	0	0	0	0	0	0	0
			-	4	3	2	2	4	3	2	3
		>10	+	5	5	7	7	5	5	4	4
			-	8	5	3	2	4	1	2	2
Spring 2009	Batch tank	2-5	+	0	0	0	0	0	0	0	0
			-	10	9	10	10	8	8	2	2
		6-10	+	1	1	1	1	1	1	0	0
			-	4	4	6	6	6	6	6	6
		>10	+	5	5	5	4	3	3	5	5
			-	5	5	5	5	2	2	4	4
	IC tank	2-5	+	0	0	0	0	0	0	0	0
			-	1	1	1	1	0	0	1	1
		6-10	+	3	3	3	3	4	4	1	1
			-	7	6	7	7	6	6	7	7
		>10	+	1	1	1	1	0	0	0	0
			-	0	0	0	0	0	0	0	0
Summer 2010	Batch tank	2-5	+	0	0	0	0	0	0	0	0
			-	2	2	2	2	0	0	2	2
		6-10	+	1	1	1	1	0	0	1	1
			-	2	2	2	2	0	0	2	2
		>10	+	2	2	1	1	0	0	2	2
			-	2	2	2	2	0	0	2	2
	IC tank	2-5	+	0	0	0	0	0	0	0	0
			-	0	0	0	0	0	0	0	0
		6-10	+	0	0	0	0	0	0	0	0
			-	0	0	0	0	0	0	0	0
		>10	+	0	0	0	0	0	0	0	0
			-	0	0	0	0	0	0	0	0

(1) "+" = diseased; "-" = not-diseased

Appendix 3 – Sample type data set: TRFLP-2 analysis

Date	Algae	Biofilm	CTW	Eggs	Larvae	WC	Grand Total
07/09/09				3			3
09/09/09	3	3	2		2	3	13
15/09/09	3	3	3		3	3	15
17/09/09				2			2
19/09/09		2			3	3	8
20/09/09			3				3
24/09/09	3	3	3		3	3	15
20/01/10	3	3	2		1	3	12
26/01/10	3	3	3		3	3	15
Grand Total	15	17	16	5	15	18	86

Appendix 4 – Forty-eight hour monitoring: Dependent variables measured in the WC

	Algae addition[1]									Microbial composition (TFFLP-3)
TSW (h)	Bag feed	Calcitrans	TVC	*Vibrio*	Phosphate	Nitrite	Nitrate	Ammonia	DOC	
0.00			X	X	X	X	X	X	X	X
2.50	30	40	X	X						
5.80			X	X						
6.50	30		X	X						
12.50			X	X	X	X	X	X	X	X
13.00	80		X	X	X	X	X	X	X	X
23.75			X	X	X	X	X	X	X	X
27.50	40	20	X	X					X	
32.00			X	X					X	
32.50	40		X	X	X	X	X	X	X	X
36.10			X	X						
36.80	40	20	X	X						
48.25			X	X	X	X	X	X	X	X

(1) Algae additions made 0.5 h prior to indicated TSW

Appendix 5 – Phylotypes identified in the hatchery

ID	Species (97%) or Genbank nearest match (Accession No.)	Genus (93%)	Family (90%)	Order	Class	Phylum
A120	*Microbacterium oxydans*	*Microbacterium*	*Microbacteriaceae*	*Actinomycetales*	*Actinobacteria*	*Actinobacteria*
A152	*Propionibacterium acnes*	*Propionibacterium*	*Propionibacteriaceae*	*Actinomycetales*	*Actinobacteria*	*Actinobacteria*
A162		*Salinicola*	*Micrococcineae*	*Actinomycetales*	*Actinobacteria*	*Actinobacteria*
A58	*Demequina aestuarii*	*Demequina*	*Cellulomonadaceae*	*Actinomycetales*	*Actinobacteria*	*Actinobacteria*
A9	*Salinibacterium amurskyense*	*Salinibacterium*	*Micrococcineae*	*Actinomycetales*	*Actinobacteria*	*Actinobacteria*
SA5		*Salinicola*	*Micrococcineae*	*Actinomycetales*	*Actinobacteria*	*Actinobacteria*
A114	Seawater clone JF488663				*Actinobacteria*	*Actinobacteria*
A159	Soil clone JN409139				*Actinobacteria*	*Actinobacteria*
A102			*Cryomorphaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A104		*Winogradskyella*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A14	*Polaribacter dokdonensis*	*Polaribacter*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A141	*Marixanthomonas ophiurae*	*Marixanthomonas*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A151	*Gramella echinicola*	*Gramella*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A155	*Chryseobacterium gleum*	*Chryseobacterium*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A17	*Croceibacter atlanticus*	*Croceibacter*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A179		*Mesoflavibacter*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A27	*Dokdonia donghaensis*	*Dokdonia*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A31		*Arenibacter*	*Flavobacteriacea*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A34		*Tenacibaculum*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A46	*Muricauda ruestringensis*	*Muricauda*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A47	*Kordia algicida*	*Kordia*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A6		*Brumimicrobium*	*Cryomorphaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A64		*Mesoflavibacter*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A71			*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A82		*Tenacibaculum*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*

ID	Species (97%) or Genbank nearest match (Accession No.)	Genus (93%)	Family (90%)	Order	Class	Phylum
A88	*Algibacter mikhailovii*	*Algibacter*	*Flavobacteriaceae*	*Flavobacteriales*	*Flavobacteria*	*Bacteroidetes*
A107	Seawater clone FN433392				*Flavobacteria*	*Bacteroidetes*
A118	Seawater clone GQ274082				*Flavobacteria*	*Bacteroidetes*
A15	Seawater clone JF827568				*Flavobacteria*	*Bacteroidetes*
A166	Uncultured Bacteroidetes AY580686				*Flavobacteria*	*Bacteroidetes*
A170	Seawater clone FR648180				*Flavobacteria*	*Bacteroidetes*
A172	Soil clone JN122782				*Flavobacteria*	*Bacteroidetes*
A22	Seawater clone JN639288				*Flavobacteria*	*Bacteroidetes*
A43	Seawater clone GC385400				*Flavobacteria*	*Bacteroidetes*
A55	Uncultured Bacteroidetes AY225660				*Flavobacteria*	*Bacteroidetes*
A65	Seawater clone FJ202704				*Flavobacteria*	*Bacteroidetes*
A72	Seawater clone GQ325429				*Flavobacteria*	*Bacteroidetes*
A96	Seawater clone HQ163249				*Flavobacteria*	*Bacteroidetes*
SB14	Seawater clone JF683452				*Flavobacteria*	*Bacteroidetes*
A50	*Cytophaga marinoflava*	*Cytophaga*	*Cytophagaceae*	*Sphingobacteriales*	*Sphingobacteria*	*Bacteroidetes*
A183	Seawater clone HQ166790				*Bacteroidetes*	*Bacteroidetes*
A89	Seawater clone HM437477				*Bacteroidetes*	*Bacteroidetes*
A200		*Prochlorococcus*	*Synechococcaceae*	*Synechococcales*	*Cyanobacteria*	*Cyanobacteria*
A109	*Listeria monocytogenes*	*Listeria*	*Listeriaceae*	*Bacillales*	*Bacilli*	*Firmicutes*
A149	*S. sciuri*	*Staphylococcus*	*Staphylococcaceae*	*Bacillales*	*Bacilli*	*Firmicutes*
A29	*Brochothrix campestris*	*Brochothrix*	*Listeriaceae*	*Bacillales*	*Bacilli*	*Firmicutes*
A123	*Lactobacillus divergens*	*Lactobacillus*	*Lactobacillaceae*	*Lactobacillales*	*Bacilli*	*Firmicutes*
A129	*Lactococcus piscium*	*Lactococcus*	*Streptococcaceae*	*Lactobacillales*	*Bacilli*	*Firmicutes*
A135	*Vagococcus fluvialis*	*Vagococcus*	*Enterococcaceae*	*Lactobacillales*	*Bacilli*	*Firmicutes*
A97	*Lactobacillus maltaromicus*	*Lactobacillus*	*Lactobacillaceae*	*Lactobacillales*	*Bacilli*	*Firmicutes*

ID	Species (97%) or Genbank nearest match (Accession No.)	Genus (93%)	Family (90%)	Order	Class	Phylum
A127	Seawater clone AB429662				*Planctomycetacia*	*Planctomycetes*
A79	Seawater clone GU327810				*Planctomycetacia*	*Planctomycetes*
A125			*Aurantimonadaceae*	*Rhizobiales*	*Alphaproteobacteria*	*Proteobacteria*
A130			*Aurantimonadaceae*	*Rhizobiales*	*Alphaproteobacteria*	*Proteobacteria*
A168	*Martelella mediterranea*	*Martelella*	*Aurantimonadaceae*	*Rhizobiales*	*Alphaproteobacteria*	*Proteobacteria*
A20		*Hoeflea*	*Phyllobacteriaceae*	*Rhizobiales*	*Alphaproteobacteria*	*Proteobacteria*
A25	*Hoeflea marina*	*Hoeflea*	*Phyllobacteriaceae*	*Rhizobiales*	*Alphaproteobacteria*	*Proteobacteria*
A10	*Ruegeria mobilis*	*Ruegeria*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A103		*Roseisalinus*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A12	*Shimia marina*	*Shimia*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A122	*Seohicola saemankumensis*	*Seohicola*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A124	*Thalassobacter oligotrophus*	*Thalassobacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A158		*Thalassobacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A174	*Maritalea myrionectae*	*Maritalea*	*Hyphomicrobiaceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A177		*Paracoccus*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A181	*Roseobacter denitrificans*	*Roseobacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A26	*Oceanibulbus indoliflex*	*Oceanibulbus*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A32			*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A36	*Tropicibacter naphthalenivorans*	*Tropicibacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A48	*Antarctobacter heliothermus*	*Antarctobacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A49		*Roseovarius*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A56	*Roseovarius nubinhibens*	*Roseovarius*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A68		*Roseovarius*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A69		*Sulfitobacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A70		*Sulfitobacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A73	*Nautella italica*	*Nautella*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*

ID	Species (97%) or Genbank nearest match (Accession No.)	Genus (93%)	Family (90%)	Order	Class	Phylum
A76		*Marivita*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A85		*Thalassobacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A99	*Phaeobacter arcticus*	*Phaeobacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
SA2	*Marivita cryptomonadis*	*Marivita*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
SB32	*Sulfitobacter donghicola*	*Sulfitobacter*	*Rhodobacteraceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
SB5		*Marinicella*	*Hyphomonadaceae*	*Rhodobacterales*	*Alphaproteobacteria*	*Proteobacteria*
A2	*Thalassospira lucentensis*	*Thalassospira*	*Rhodospirillaceae*	*Rhodospirillales*	*Alphaproteobacteria*	*Proteobacteria*
A1	*Erythrobacter aquimaris*	*Erythrobacter*	*Erythrobacteraceae*	*Sphingomonadales*	*Alphaproteobacteria*	*Proteobacteria*
A139		*Sphingomonas*	*Shingomonadaceae*	*Sphingomonadales*	*Alphaproteobacteria*	*Proteobacteria*
A144	*Sphingomonas paucimobilis*	*Sphingomonas*	*Shingomonadaceae*	*Sphingomonadales*	*Alphaproteobacteria*	*Proteobacteria*
A145	*Sphingomonas aestuarii*	*Sphingomonas*	*Shingomonadaceae*	*Sphingomonadales*	*Alphaproteobacteria*	*Proteobacteria*
A184	*Citromicrobium bathoceanense*	*Citromicrobium*	*Shingomonadaceae*	*Sphingomonadales*	*Alphaproteobacteria*	*Proteobacteria*
A30			*Shingomonadaceae*	*Sphingomonadales*	*Alphaproteobacteria*	*Proteobacteria*
A39			*Shingomonadaceae*	*Sphingomonadales*	*Alphaproteobacteria*	*Proteobacteria*
A5		*Sphingopyxis*	*Shingomonadaceae*	*Sphingomonadales*	*Alphaproteobacteria*	*Proteobacteria*
SB51			*Erythrobacteraceae*	*Sphingomonadales*	*Alphaproteobacteria*	*Proteobacteria*
A16	Seawater clone AY664177				*Alphaproteobacteria*	*Proteobacteria*
A74	Seawater clone GQ350388				*Alphaproteobacteria*	*Proteobacteria*
A75					*Alphaproteobacteria*	*Proteobacteria*
A100		*Ramlibacter*	*Comamonadaceae*	*Burkholderiales*	*Betaproteobacteria*	*Proteobacteria*
A59	*Janthinobacterium lividum*	*Janthinobacterium*	*Oxalobacteraceae*	*Burkholderiales*	*Betaproteobacteria*	*Proteobacteria*
SA53	*Ralstonia pickettii*	*Ralstonia*	Burkholderiaceae	Burkholderiales	*Betaproteobacteria*	Proteobacteria
A126	*Iodobacter fluviatilis*	*Iodobacter*	Neisseriaceae	Neisseriales	*Betaproteobacteria*	Proteobacteria
A167					*Deltaproteobacteria*	Proteobacteria
A138		*Arcobacter*	Campylobacteraceae	Campylobacterales	Epsilonproteobacteria	Proteobacteria
A91		*Arcobacter*	Campylobacteraceae	Campylobacterales	Epsilonproteobacteria	Proteobacteria

ID	Species (97%) or Genbank nearest match (Accession No.)	Genus (93%)	Family (90%)	Order	Class	Phylum
A160	*Pseudoalteromonas marina*	*Pseudoalteromonas*	Pseudoalteromonadaceae	Alteromonadales	*Gammaproteobacteria*	Proteobacteria
A21		*Marinovum*	Alteromonadaceae	Alteromonadales	*Gammaproteobacteria*	Proteobacteria
A3	*Marinovum algicola*	*Marinovum*	Alteromonadaceae	Alteromonadales	*Gammaproteobacteria*	Proteobacteria
A4			Alteromonadaceae	Alteromonadales	*Gammaproteobacteria*	Proteobacteria
A51	*Alteromonas macleodii*	*Alteromonas*	Alteromonadaceae	Alteromonadales	*Gammaproteobacteria*	Proteobacteria
A60	*Shewanella baltica*	*Shewanella*	Shewanellaceae	Alteromonadales	*Gammaproteobacteria*	Proteobacteria
A148			Ectothiorhodospiraceae	Chromatiales	*Gammaproteobacteria*	Proteobacteria
A121	*Serratia proteamaculans*	*Serratia*	Enterobacteriaceae	Enterobacteriales	*Gammaproteobacteria*	Proteobacteria
SA20	*Alcanivorax borkumensis*	*Alcanivorax*	Alcanivoracaceae	Oceanospirillales	*Gammaproteobacteria*	Proteobacteria
A81	*P. fluorescens*	*Pseudomonas*	Pseudomonadaceae	Pseudomonadales	*Gammaproteobacteria*	Proteobacteria
A164	*Vibrio crassostreae*	*Vibrio*	Vibrionaceae	Vibrionales	*Gammaproteobacteria*	Proteobacteria
A154		*Stenotrophomonas*	Xanthomonadaceae	Xanthomonadales	*Gammaproteobacteria*	Proteobacteria
A163					*Gammaproteobacteria*	Proteobacteria
A37					*Gammaproteobacteria*	Proteobacteria
A80					*Gammaproteobacteria*	Proteobacteria
A86					*Gammaproteobacteria*	Proteobacteria
A95					*Gammaproteobacteria*	Proteobacteria
A90					*Verrucomicrobiae*	Verrucomicrobia

Appendix 6 - Nearest species match to the 10 most predominant phylotypes for each sample type

Algae (3)[2]									
Phylotype code	Nearest species match (type strain)	Percent similarity to type strain	Algae	Biofilm	CTW	Larvae	WC	Eggs	No.of sample types phylotype present[3]
A47	*Kordia algicida*	97.0	**16.0**[1]	2.1	0.0	0.0	0.0	0.0	2
A5	*Sphingopyxis* sp.	93.0	**12.3**	7.0	0.0	16.0	10.2	6.2	4
A1	*Erythrobacter aquimaris*	97.0	**10.2**	0.0	4.1	0.3	9.7	0.0	4
A168	*Martelella mediterranea*	97.0	**8.6**	4.9	2.7	6.3	12.2	1.5	5
A48	*Antarctobacter heliothermus*	97.0	**8.6**	0.4	0.0	0.0	0.0	0.0	2
A6	*Brumimicrobium* sp.	93.0	**7.0**	2.5	0.0	0.0	1.0	0.0	3
A99	*Phaeobacter arcticus*	97.0	**5.3**	3.7	0.0	1.4	0.0	3.1	3
A26	*Oceanibulbus indoliflex*	97.0	**4.8**	4.1	1.4	0.9	6.6	0.0	5
A3	*Marinovum algicola*	97.0	**2.7**	4.1	0.0	1.7	12.8	0.0	4
A184	*Citromicrobium bathoceanense*	97.0	**2.1**	2.9	0.0	0.0	5.6	1.5	3
	Total non blank		**10**	9	3	6	7	4	Ubiquitous[4] = 5
Biofilm (3)									
Phylotype code	Nearest species match (type strain)	Percent similarity	Algae	Biofilm	CTW	Larvae	WC	Eggs	No.of sample types phylotype present
A73	*Nautella italica*	97.0	0.0	**14.4**	16.2	1.1	9.2	0.0	4
A12	*Shimia marina*	97.0	0.0	**11.9**	0.0	14.6	0.0	0.0	2
A59	*Janthinobacterium lividum*	97.0	0.0	**8.2**	0.0	0.6	0.0	0.0	2
A5	*Sphingopyxis*	93.0	12.3	**7.0**	0.0	16.0	10.2	6.2	4
A56	*Roseovarius nubinhibens*	97.0	1.1	**5.3**	0.0	0.6	1.5	0.0	4
A168	*Martelella mediterranea*	97.0	8.6	**4.9**	2.7	6.3	12.2	1.5	5
A26	*Oceanibulbus indoliflex*	97.0	4.8	**4.1**	1.4	0.9	6.6	0.0	5
A3	*Marinovum algicola*	97.0	2.7	**4.1**	0.0	1.7	12.8	0.0	4
A99	*Phaeobacter arcticus*	97.0	5.3	**3.7**	0.0	1.4	0.0	3.1	3
A118	Seawater clone GQ274082	97.0	0.0	**3.7**	0.0	0.0	0.5	0.0	2
	Total non blank		6	**10**	3	9	7	3	Ubiquitous = 6
CTW (1)									

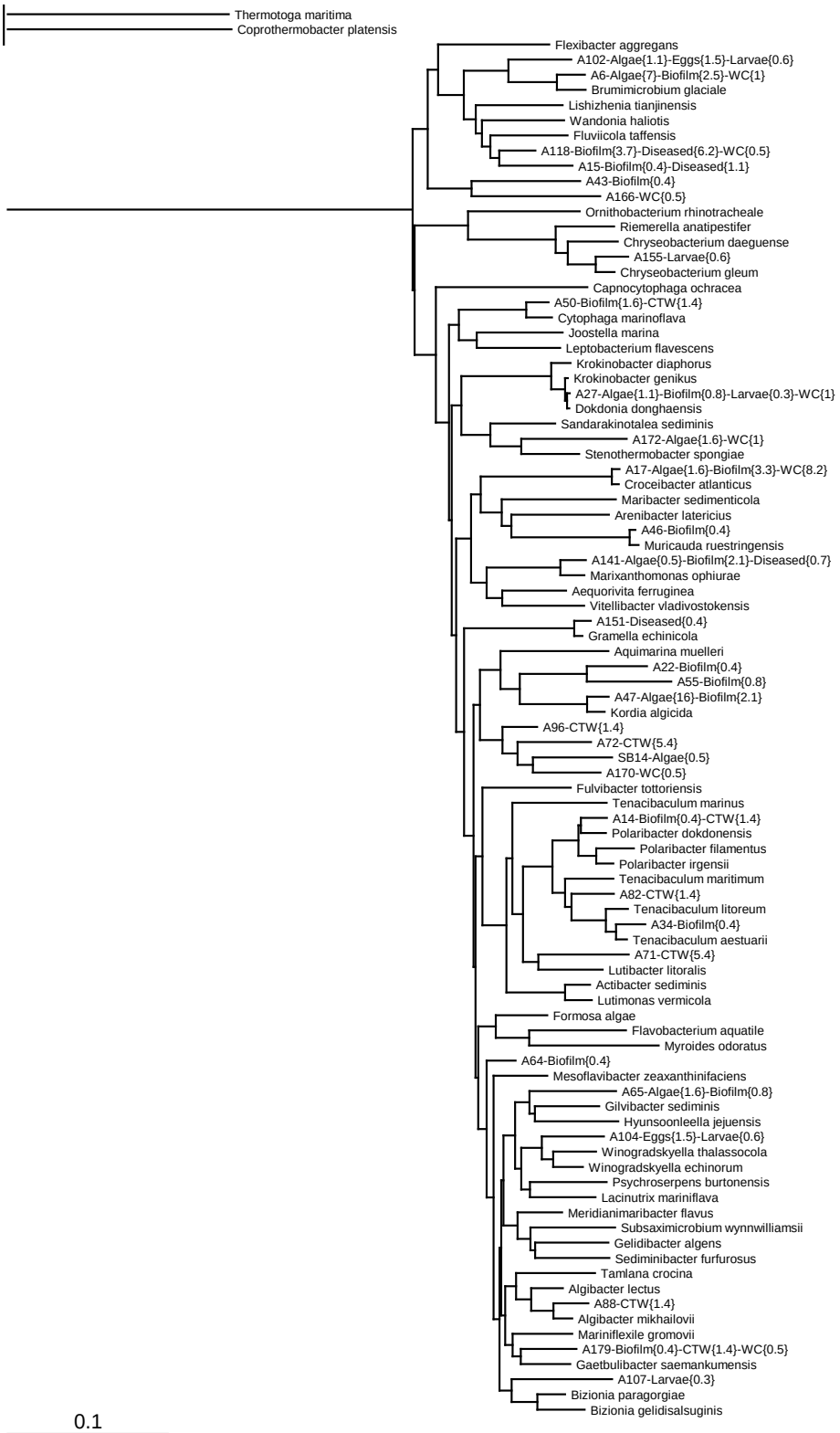
Phylotype code	Nearest species match (type strain)	Percent similarity	Algae	Biofilm	CTW	Larvae	WC	Eggs	No.of sample types phylotype present
A73	*Nautella italica*	97.0	0.0	14.4	**16.2**	1.1	9.2	0.0	4
A76	*Marivita*	93.0	0.0	0.0	**12.2**	0.0	0.0	0.0	1
A68	*Roseovarius*	93.0	0.5	0.0	**6.8**	0.3	0.0	0.0	3
A200	*Prochlorococcus*	93.0	0.0	0.0	**5.4**	0.3	0.5	0.0	3
A70	*Sulfitobacter*	93.0	0.0	0.0	**5.4**	0.0	0.0	0.0	1
A71	Flavobacteriaceae	90.0	0.0	0.0	**5.4**	0.0	0.0	0.0	1
A72	Seawater clone GQ350429	97.0	0.0	0.0	**5.4**	0.0	0.0	0.0	1
A1	*Erythrobacter aquimaris*	97.0	10.2	0.0	**4.1**	0.3	9.7	0.0	4
A69	*Sulfitobacter*	93.0	0.0	0.0	**4.1**	0.0	0.5	0.0	2
A74	Seawater clone GQ350388	97.0	0.0	0.0	**4.1**	0.0	0.0	0.0	1
	Total non blank		2	1	**10**	4	4	0	Ubiquitous = 2
Larvae (4)									
Phylotype code	Nearest species match (type strain)	Percent similarity	Algae	Biofilm	CTW	Larvae	WC	Eggs	No.of sample types phylotype present
A5	*Sphingopyxis*	93.0	12.3	7.0	0.0	**16.0**	10.2	6.2	4
A12	*Shimia marina*	93.0	0.0	11.9	0.0	**14.6**	0.0	0.0	2
A144	*Sphingomonas paucimobilis*	93.0	0.0	0.0	1.4	**11.7**	0.0	30.8	2
A100	*Ramlibacter*	93.0	0.0	0.0	0.0	**10.0**	0.0	41.5	1
A160	*Pseudoalteromonas marina*	93.0	0.0	0.4	0.0	**9.7**	0.0	0.0	2
A168	*Martelella mediterranea*	93.0	8.6	4.9	2.7	**6.3**	12.2	1.5	5
A97	*Carnobacterium maltaromicum*	93.0	0.0	0.0	0.0	**3.7**	0.5	1.5	2
A103	*Roseisalinus*	93.0	1.1	0.0	0.0	**2.6**	0.0	9.2	2
A3	*Marinovum algicola*	93.0	2.7	4.1	0.0	**1.7**	12.8	0.0	4
A114	Seawater clone JF488663	97.0	0.5	0.0	0.0	**1.7**	0.0	0.0	2
	Total non blank		5	5	2	**10**	4	6	Ubiquitous = 3
WC (2)									
Phylotype code	Nearest species match (type strain)	Percent similarity	Algae	Biofilm	CTW	Larvae	WC	Eggs	No.of sample types phylotype present
A3	*Marinovum algicola*	97.0	2.7	4.1	0.0	1.7	**12.8**	0.0	4
A168	*Martelella mediterranea*	97.0	8.6	4.9	2.7	6.3	**12.2**	1.5	5
A5	*Sphingopyxis* sp.	93.0	12.3	7.0	0.0	16.0	**10.2**	6.2	4
A1	*Erythrobacter*	97.0	10.2	0.0	4.1	0.3	**9.7**	0.0	4

	aquimaris								
A73	*Nautella italica*	97.0	0.0	14.4	16.2	1.1	**9.2**	0.0	4
A17	*Croceibacter atlanticus*	97.0	1.6	3.3	0.0	0.0	**8.2**	0.0	3
A177	*Paracoccus*	93.0	0.5	0.0	0.0	0.3	**7.7**	0.0	3
A26	*Oceanibulbus indoliflex*	97.0	4.8	4.1	1.4	0.9	**6.6**	0.0	5
A184	*Citromicrobium bathoceanense*	97.0	2.1	2.9	0.0	0.0	**5.6**	1.5	3
A9	*Salinibacterium amurskyense*	97.0	0.5	0.4	0.0	1.4	**3.1**	0.0	4
	Total non blank		9	8	4	8	**10**	3	Ubiquitous = 7
Eggs (1)									
Phylotype code	Nearest species match (type strain)	Percent similarity	Algae	Biofilm	CTW	Larvae	WC	Eggs	No.of sample types phylotype present
A100	*Ramlibacter*	93.0	0.0	0.0	0.0	10.0	0.0	**41.5**	1
A144	*Sphingomonas paucimobilis*	97.0	0.0	0.0	1.4	11.7	0.0	**30.8**	2
A103	*Roseisalinus*	93.0	1.1	0.0	0.0	2.6	0.0	**9.2**	2
A5	*Sphingopyxis*	93.0	12.3	7.0	0.0	16.0	10.2	**6.2**	4
A99	*Phaeobacter arcticus*	97.0	5.3	3.7	0.0	1.4	0.0	**3.1**	3
A168	*Martelella mediterranea*	97.0	8.6	4.9	2.7	6.3	12.2	**1.5**	5
A120	*Microbacterium oxydans*	97.0	0.0	0.0	0.0	1.4	0.5	**1.5**	2
A184	*Citromicrobium bathoceanense*	97.0	2.1	2.9	0.0	0.0	5.6	**1.5**	3
A102	*Cryomorphaceae*	90.0	1.1	0.0	0.0	0.6	0.0	**1.5**	2
A104	*Winogradskyella*	93.0	0.0	0.0	0.0	0.6	0.0	**1.5**	1
	Total non blank		6	4	2	9	4	**10**	Ubiquitous = 2

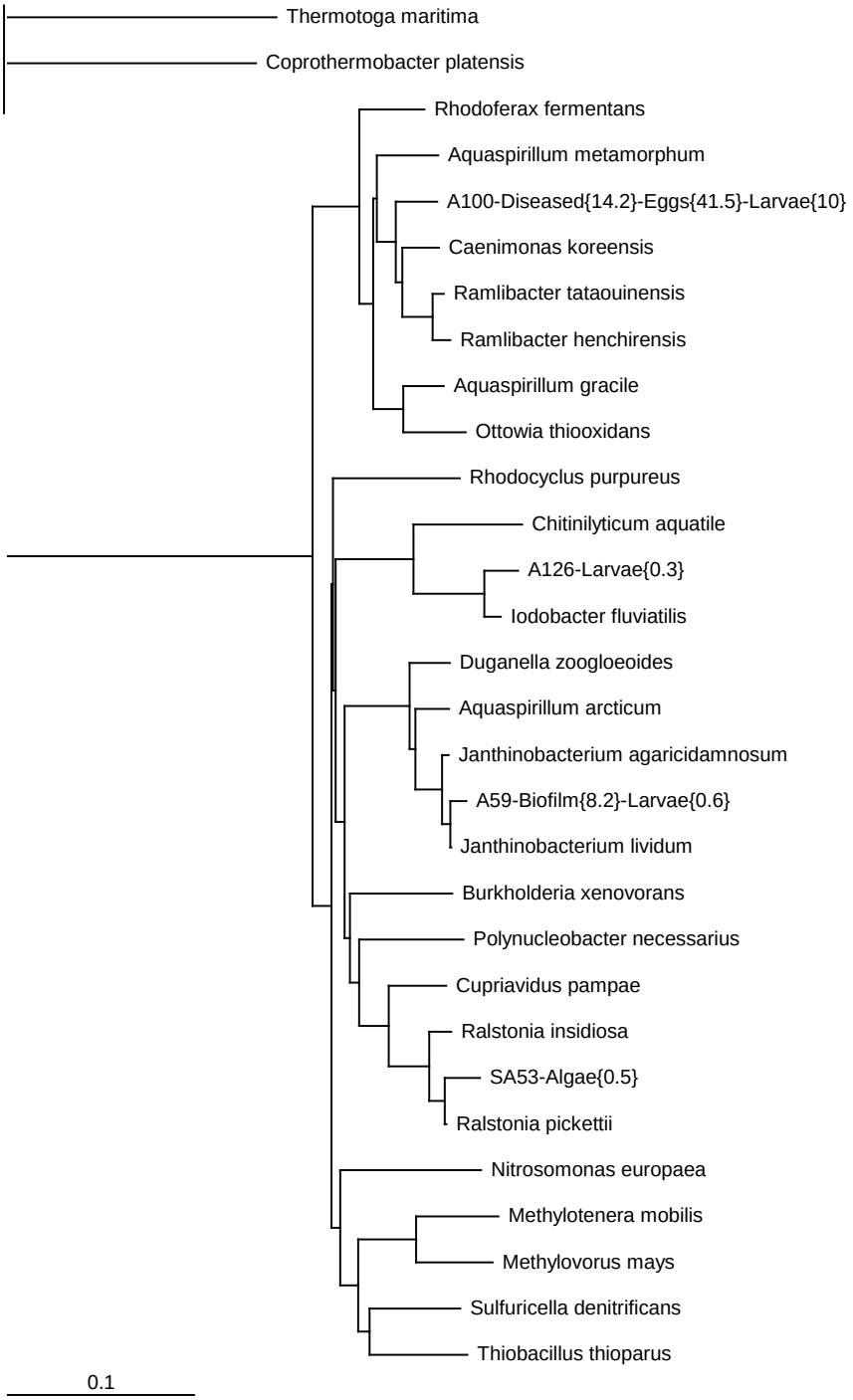
Notes: (1) All figures are percentage of total population for the indicated sample type in column headers. (2) Numbers in brackets indicate the number of clone libraries used in data. (3) "No. of sample types phylotype present" does not include eggs. (4) Ubiquitous = No. of phylotypes that are present in at least 4/5 of the algae, WC, CTW, biofilm and larvae

Appendix 7 – Alpha proteobacteria phylogenetic tree

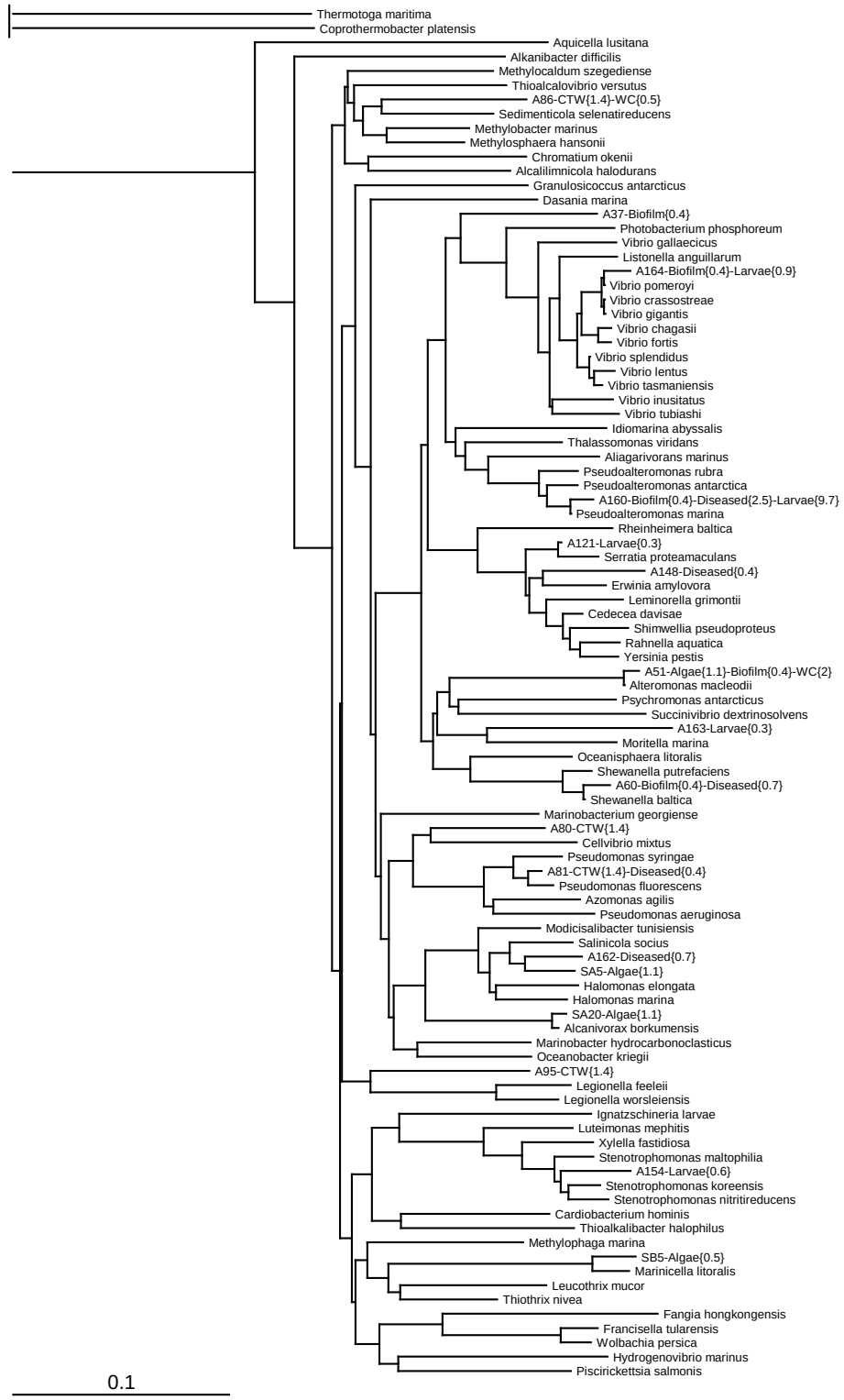
Appendix 8 – *Flavobacteria* phylogenetic tree



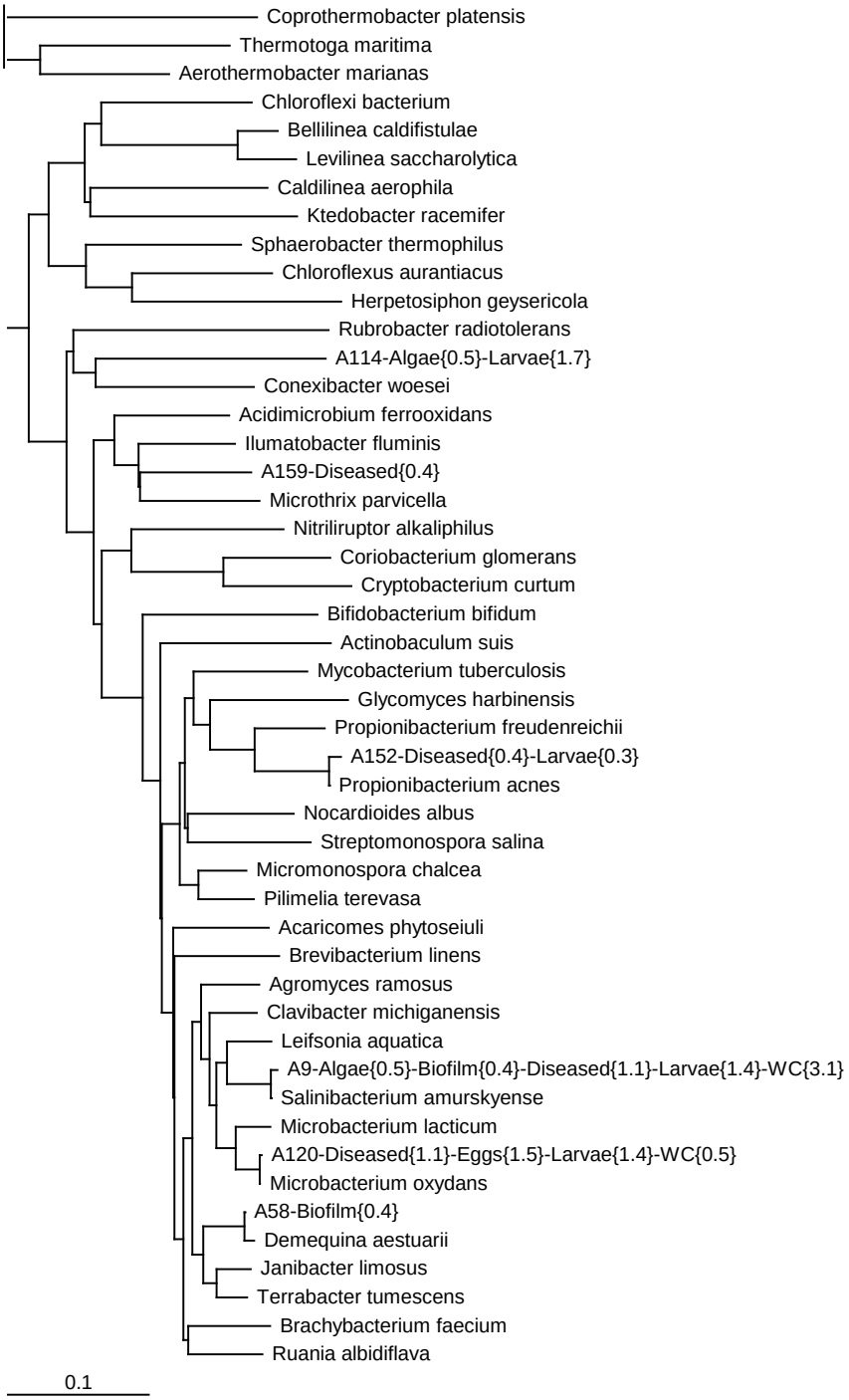
Appendix 9 – Beta proteobacteria phylogenetic tree



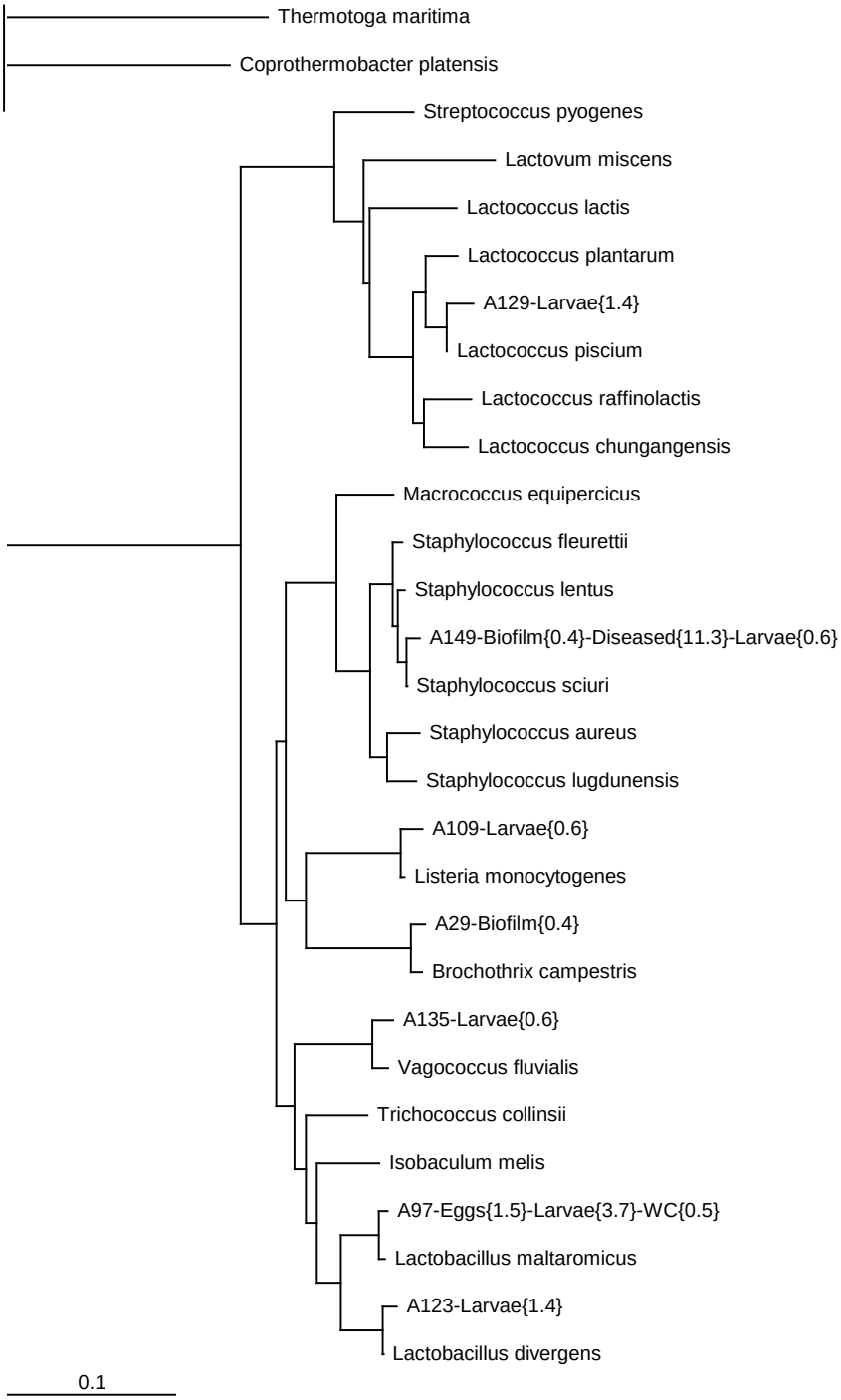
Appendix 10 – Gamma proteobacteria



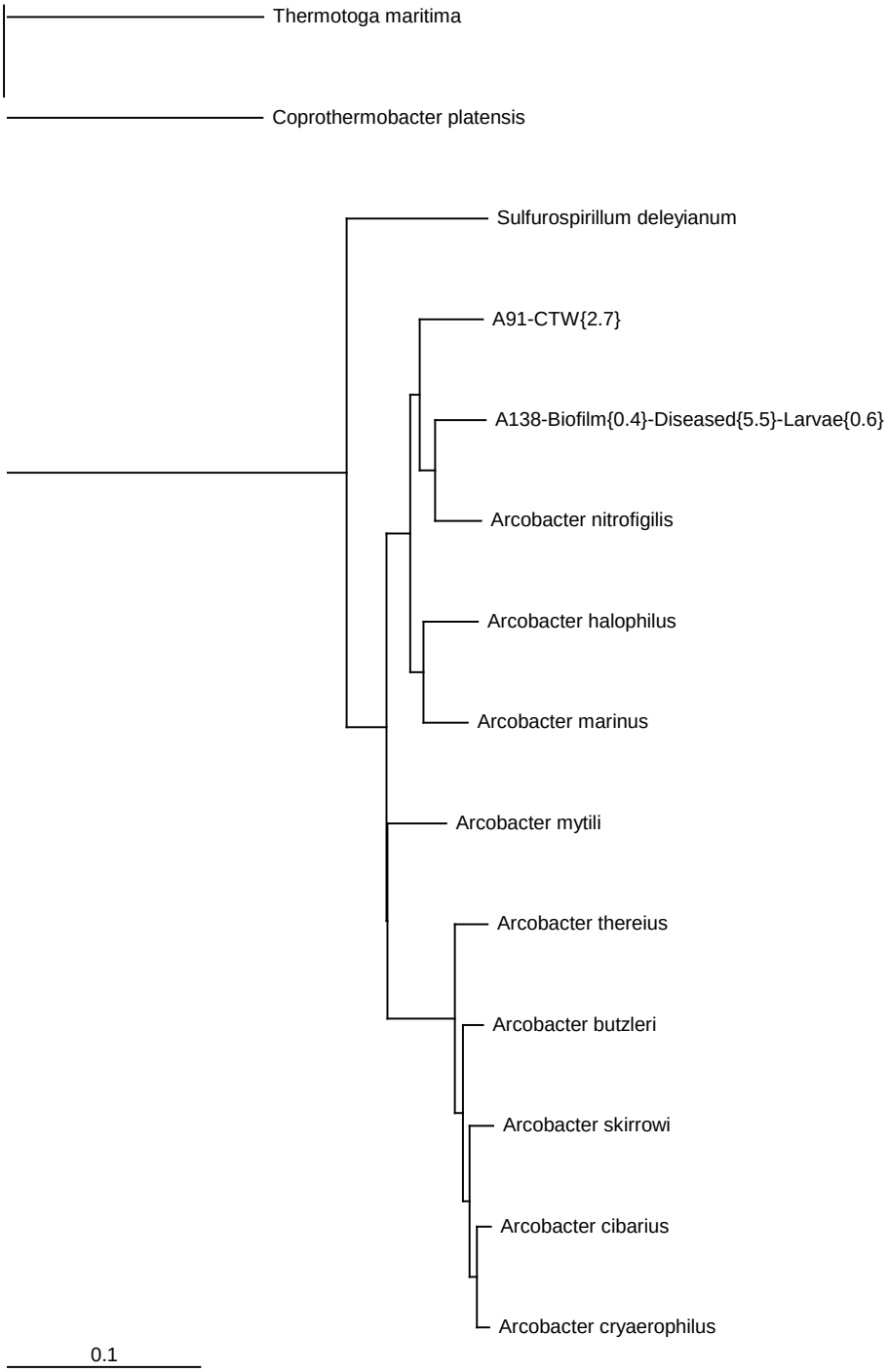
Appendix 11 – Actinobacteria phylogenetic tree



Appendix 12 – Bacilli phylogenetic tree



Appendix 13 – Epsilon proteobacteria phylogenetic tree



Appendix 14 – Statistical model design

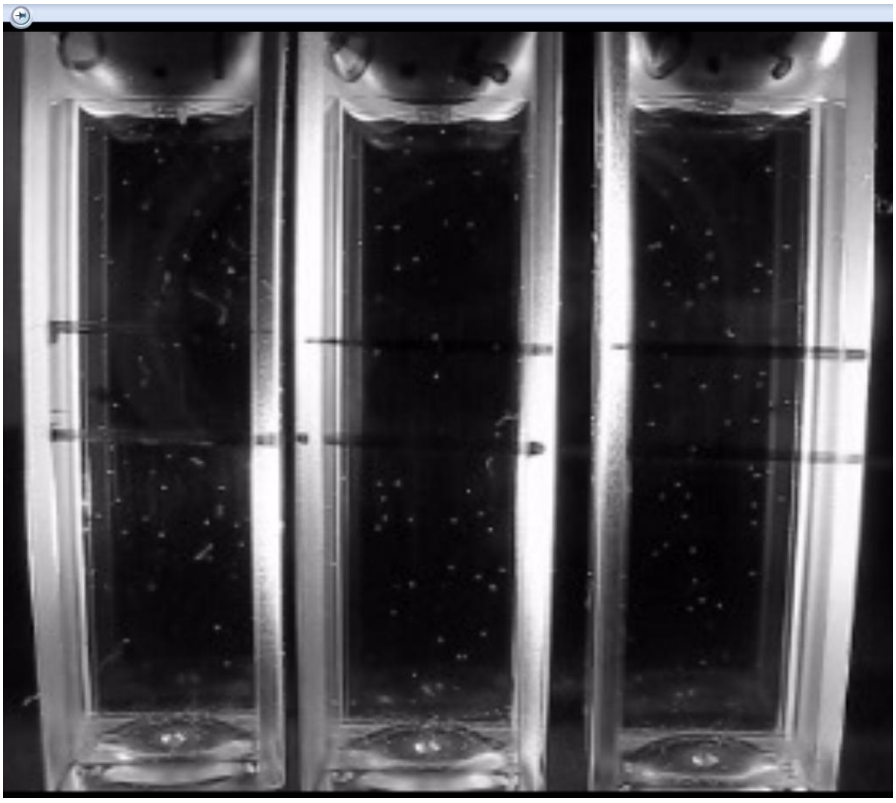
Statistical model	Procedure	Monitoring program / Trial	Dataset	Data excluded	Independent variable(s) included in model	Dependent variable	No. of samples
A. Statistical model A	SAS regression	Production run	Bacterial enunmeration	None	DSS	Larvae TVC counts	81
B. Statistical model B	SAS GLM	Production run	Bacterial enunmeration	None	DSS, Disease status	Larvae *Vibrio* counts	73
C. Statistical model C	SAS GLM	Production run	Bacterial enunmeration	None	DSS, Disease status, Tank type	Larvae *Vibrio/TVC*	76
D. Statistical model D	SAS GLM	Production run	Bacterial enunmeration	None	Sampling period, DSS, Tank type	WC TVC counts	75
E. Statistical model E	SAS GLM	Production run	Bacterial enunmeration	None	DSS, Disease status, Tank type	WC *Vibrio* counts	72
F. Statistical model F	SAS GLM	Production run	Bacterial enunmeration	None	DSS, Disease status, Tank type	WC *Vibrio* /TVC	72
G. Statistical model G	SAS GLM	Production run	Bacterial enunmeration	IC tank data	TSW, DSS	Larvae TVC counts	51
H. Statistical model H	SAS GLM	Production run	Bacterial enunmeration	IC tank data	TSW, DSS	Larvae *Vibrio* counts	48
I. Statistical model I	SAS GLM	Production run	Bacterial enunmeration	IC tank data	TSW, Sampling period, DSS	WC TVC counts	51
J. Statistical model J	SAS GLM	Production run	Bacterial enunmeration	IC tank data	TSW, DSS	WC *Vibrio* counts	49
K. Statistical model K	SAS GLM	Production run	Bacterial enunmeration	None	Sample type, Tank type, DSS	*Vibrio*/TVC counts	188
L. Statistical model L	SAS GLM	Production run	Bacterial enunmeration	None	Sample type	Log total TVC counts	115
M. Statistical model M	SAS GLM	Production run	Bacterial enunmeration	None	Sample type	Log total *Vibrio* counts	90
N. Statistical model N	SAS GLM	Production run	Physiochemical	None	Tank type	pH	60

Statistical model	Procedure	Monitoring program / Trial	Dataset	Data excluded	Independent variable(s) included in model	Dependent variable	No. of samples
			data				
O. Statistical model O	SAS GLM	Production run	Physiochemical data	None	Tank type	Temperature	52
P. Statistical model P	SAS REG	Production run	Bacterial enunmeration	None	None	Larvae TVC, *Vibrio* and *Vibrio*/TVC. WC TVC, *Vibrio* and *Vibrio*/TVC	
Q. Statistical model Q	PERMANOVA	Sample type	TRFLP-2	None	Sample type	MC[1]	86
R. Statistical model R	PERMANOVA	Sample type	TRFLP-2	Abundance data	Sample type	MC	86
S. Statistical model S	PERMANOVA	Production run	TRFLP-1: Larvae	Summer 2010; 2-5 DSS	Tank type, Sampling period, DSS	MC	35
T. Statistical model T	PERMANOVA	Production run	TRFLP-1: Larvae	None	Disease status, Sampling period, DSS	MC	67
U. Statistical model U	PERMANOVA	Production run	TRFLP-1: Larvae	None	Sampling period, DSS	MC	67
V. Statistical model V	PERMANOVA	Production run	TRFLP-1: Larvae	IC tank	Disease status, Sampling period, DSS	MC	49
W. Statistical model W	PERMANOVA	Production run	TRFLP-1: Larvae	IC tank	Sampling period, DSS	MC	49
X. Statistical model X	PERMANOVA	Production run	TRFLP-1: WC	Summer 2010; 2-5 DSS	Tank type , Sampling period, DSS	MC	34
Y. Statistical model Y	PERMANOVA	Production run	TRFLP-1: WC	None	Disease status, Sampling period, DSS	MC	63
Z. Statistical model Z	PERMANOVA	Production run	TRFLP-1: WC	None	Sampling period, DSS	MC	63
AA. Statistical model AA	PERMANOVA	Production run	TRFLP-1: WC	IC tank	Disease status, Sampling period, DSS	MC	49
BB. Statistical model BB	PERMANOVA	Production run	TRFLP-1: WC	IC tank	Sampling period, DSS	MC	49
CC. Statistical model CC	PERMANOVA	Production run	TRFLP-1: Sample type	None	Sample type	MC	142

Statistical model	Procedure	Monitoring program / Trial	Dataset	Data excluded	Independent variable(s) included in model	Dependent variable	No. of samples
DD. Statistical model DD	PERMANOVA	Production run	TRFLP-1: Sample type	None	Sample type	MC	142
EE. Statistical model EE	SAS GLM	Production run	TRFLP-1	None	Sample type	Diversity index H'	135
FF. Statistical model FF	SAS GLM	Production run	TRFLP-1	None	Sample type	Diversity index d	135
GG. Statistical model GG	SAS GLM	Production run	TRFLP-1	None	Sample type	Diversity index J'	135
HH. Statistical model HH	SAS GLM	$CuSO_4$ + bacteria	24 h $CuSO_4$ + *V. pomeroyii*	None	$CuSO_4$ level, Bacteria level	% mortality	90
II. Statistical model II	SAS GLM	$CuSO_4$ + bacteria	48 h $CuSO_4$ + V. pomeroyii	None	$CuSO_4$ level, Bacteria level	% mortality	96
JJ. Statistical model JJ	SAS GLM	$CuSO_4$ + bacteria	24 h $CuSO_4$ + *P. fluorescens*	None	$CuSO_4$ level, Bacteria level	% mortality	90
KK. Statistical model KK	SAS GLM	$CuSO_4$ + bacteria	48 h $CuSO_4$ + *P. fluorescens*	None	$CuSO_4$ level, Bacteria level	% mortality	96
LL. Statistical model LL	SAS GLM	$CuSO_4$ + bacteria	24 h $CuSO_4$ + *S. sciuri*	None	$CuSO_4$ level, Bacteria level	% mortality	90
MM. Statistical model MM	SAS GLM	$CuSO_4$ + bacteria	48 h $CuSO_4$ + *S. sciuri*	None	$CuSO_4$ level, Bacteria level	% mortality	96
NN. Statistical model NN	SAS GLM	$CuSO_4$ + behaviour	$CuSO_4$ + behaviour	None	$CuSO_4$	% SL	120
OO. Statistical model OO	SAS GLM	$CuSO_4$ + behaviour	$CuSO_4$ + behaviour	None	$CuSO_4$	% CL	120
PP. Statistical model PP	SAS GLM	Heat shock	Heat shock	None	Heat duration, Bacteria level	Larvae in top 3 ml	60

(1) MC = Microbial community

Appendix 15 – Larvae behaviour observations in 4.5 ml cuvettes



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