Microbial communities of larval rearing systems and wild phyllosoma of the ornate rock lobster, *Panulirus ornatus*, and options for microbial management in aquaculture

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'Microbial communities of larval rearing systems and wild phyllosoma of the ornate rock lobster, *Panulirus ornatus*, and options for microbial management in aquaculture'

The present study was conducted at the Australian Institute of Marine Science (AIMS) from June 2006 – March 2007, under the supervision of Dr. Lone Høj and Dr. Michael R. Hall.

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1. Introduction

1.1 Panulirus ornatus

1.1.1 General characterization

The ornate rock lobster, *Panulirus ornatus* (Crustacea: Decapoda; Figure 1.1), is a tropical species widely distributed throughout the Indo-Pacific region. It belongs to the Palinuridae (spiny lobsters) within the Palinuroidea superfamily, which further comprises Scyllaridae (slipper lobsters) and Synaxidae (coral lobsters), as classified by Bowman and Abele (1982). *Panulirus ornatus* is abundant in the waters off North-East Australia, where numbers have been estimated at 11-17 millions solely in the Torres Strait (CSIRO, 2003), which separates Australia from Papua New Guinea.

The species is characterized by vibrant colorization of the carapace. Like all lobsters, it is a benthic, nocturnal animal only leaving its rocky shelter at night to hunt prey. Usually, *Panulirus ornatus* inhabits a distinct reef complex, though continually moves within this given system (Moore and MacFarlane, 1984). In the third year of age, however, the animal abandons its home territory and migrates hundreds of kilometres to its breeding grounds for mating, walking close to 6 kilometres every day for a period of up to two months (CSIRO, 2003). Within a breeding season, which lasts from November to April, females produce up to three broods. Up to 95% of the lobsters die after the mating event, since the animals are in such poor condition after migrating and mating that there are no reserves left to undergo another moult. *Panulirus ornatus* is the only rock lobster species in which such a massive mortality occurs. Captive animals, however, have lived beyond eight years of age (CSIRO, 2003) and developed into large, robust animals.

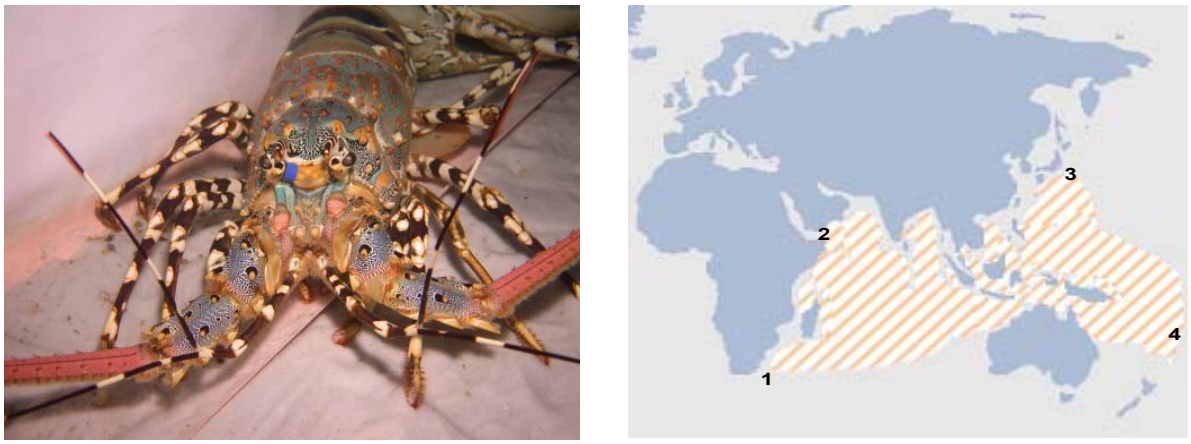


Figure 1.1 Left: Adult *Panulirus ornatus* (Image: AIMS). Right: Distribution of the species, as generated from documented observations (1 – Berry, 1971; 2 – Ben-Tuvia, 1967; 3 & 4 – Pyne, 1974).

1.1.2 Larval phase of Palinuroidea

The larval phase of Palinuroidea is characterized by an extended planktonic period. This period ranges, depending on the species, from six months up to two years (Philips and Sastry, 1980) and takes place in oligotrophic 'blue water' environments.

A female adult releases large amounts of fertilized eggs into the water, where embryos hatch from the eggs and enter the initial *nauplisoma* stage. Minutes to several hours later, larvae moult for the first time into the second phase of development, which represents the largest proportion of the larval life. Larvae are then characterized by a dorsal-ventrally flattened morphology, a translucent body and well developed appendages. They are scientifically named as phyllosomata, or more commonly *phyllosoma* from the greek *phyllos* – meaning *leaf* and *soma* – meaning *body*. This particular larval type (Figure 1.2) is unique characteristic of the living representatives of Palinuroidea (Baisre, 1994), but investigations of fossil phyllosoma have discovered that this type of larvae has diversified little over the past 250 million years (Williamson, 1985).

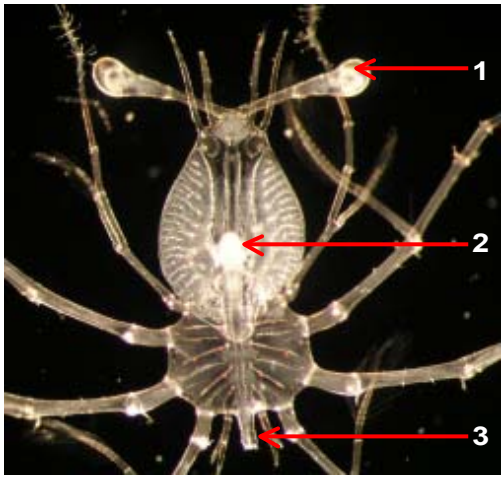


Figure 1.2 Phyllosoma larva. **1:** Eyestalk; **2:** Mouthparts; **3:** Anus (Image: AIMS)

The leaf-like appearance does not change fundamentally during the phyllosoma phase. Larvae usually moult through 11 morphological stages (Lesser, 1978) and 17 or more instars (Booth and Phillips, 1994). A number of instars above 17 indicates supernumerary moults without any morphological changes. The larval phase is overall characterized as diedysic, meaning that short intermoult periods are followed by longer premoult periods.

Phyllosoma are part of the pelagic zooplankton and drifting on prevailing currents in offshore waters. *Panulirus ornatus* larvae have been monitored to drift for up to 2000 kilometres between North-Eastern Australia and Papua New Guinea before settling in adult habitats

(CSIRO, 2002). Larvae undertake diurnal movements within the water column; coming up near the surface at night before descending to deeper strata during the day.

Towards the end of the larval phase, phyllosoma are transported to shallower coastal waters where they moult into the final planktonic *puerulus* stage. This stage is capable of active swimming. The larval morphology is distinctly changed, with pueruli looking like typical, though still transparent, rock lobsters. Pueruli eventually settle from the plankton on rocky reefs, where they undergo another series of moults and develop to juvenile rock lobsters. From then on, rock lobsters remain benthic animals.

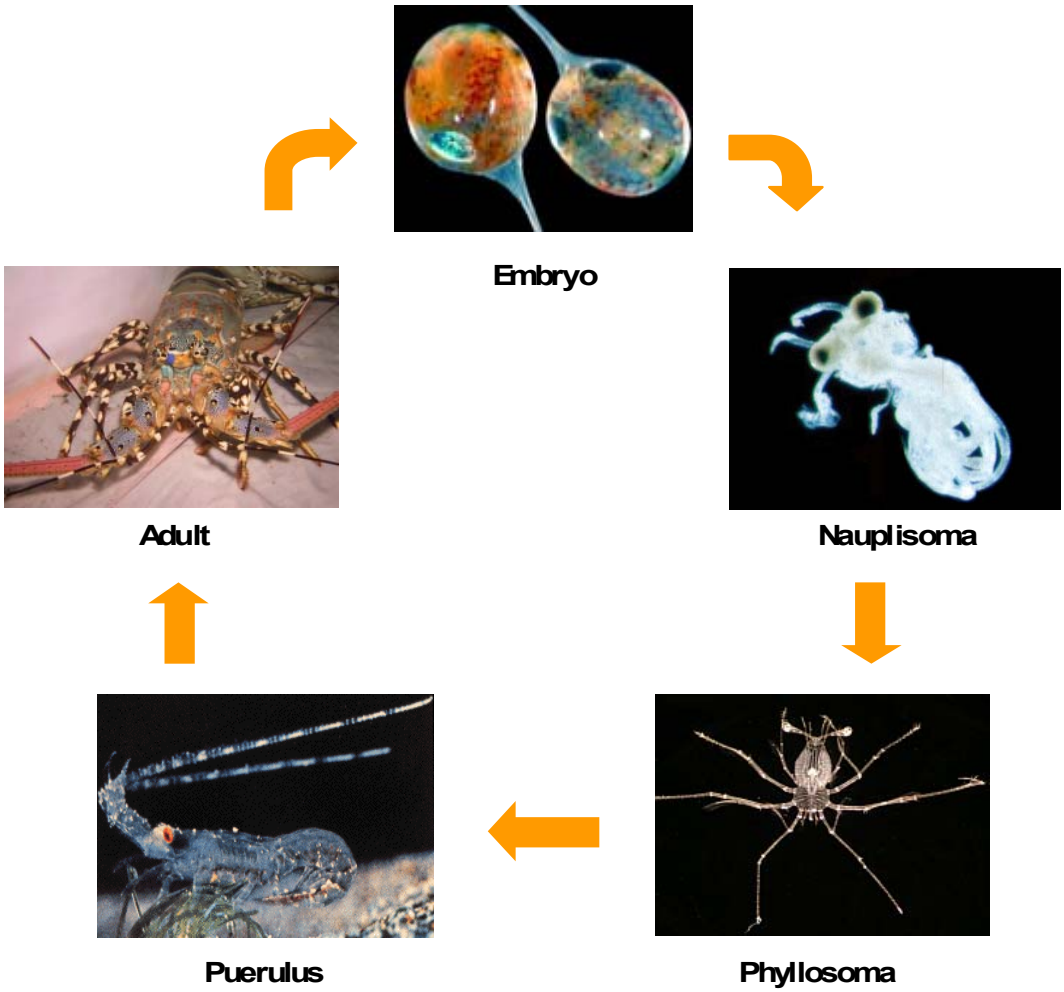


Figure 1.3 Illustration of the life cycle of Palinuroidea lobsters. From top, clockwise: Lobster embryos, *Jasus edwardsii* nauplisoma; *Panulirus ornatus* phyllosoma (Stage 4); *Panulirus argus* puerulus; adult *Panulirus ornatus*. (Images: AIMS; CSIRO; Natural Geographic)

1.1.3 The aquaculture industry and commercial aspects of rock lobsters

Aquaculture is the cultivation of animals and plants in water, incorporating species from diverse marine and freshwater environments. Aquaculture is also often used for replenishment programs of various aquatic species.
Global production from aquaculture is increasing about 11% per year, making this industry the fastest growing food production sector worldwide (FAO, 2003). By the year 2003, 202 aquatic animals were routinely farmed, of which 131 were finfish, 42 molluscs and 27 crustaceans (FAO, 2003). Crustacean aquaculture at present is dominated by large-scale rearing of prawns, particularly in Asian countries. Seafood is however still the only important food source that is primarily gathered from the wild, despite farmed fish and shellfish account for 39.5% of all seafood consumed by humans (FAO, 2004).

Lobsters represent one of the most valuable seafood commodities. Presently, nearly all production of marine lobsters is based on harvesting from wild populations, whereby Palinuridae lobsters constitute approximately 33% of world harvest (FAO, 2004).
The Australian rock lobster fisheries represent an important marine resource for the domestic and international market. From its natural habitat, commercial fisheries land about 500 tonnes of lobster per year. Whilst the distribution was previously oriented towards the export of frozen tails, animals are now increasingly held in captivity for a shorter period, before being shipped live to mainly Asian markets. The average value for live specimens is around AU$ 40-50 per kg, which equals approximately 50-55 € per kg.
The wild harvest capacity for this high-value seafood is currently at sustainable levels. The ability to meet increasing consumer demand will only be possible by converting the source of supply from conventional fisheries to aquaculture production, enabling both environmentally sustainable and economically advantageous outputs. Although part of the increased demand for lobsters can be met by grow-out of post-settlement juveniles collected from the wild, the primary goal for the industry is development of closed-life cycle breeding and a true farming sector.

Panulirus ornatus has been identified as representing the most promising candidate for larval culture, whereby several characteristics would benefit its introduction to commercial-scale rearing. This species possesses one of the shortest larval phase within the Palinuridae – estimated at a 160 to 180 days (Prasad et al., 1975) – and a higher tolerance to changes in salinity and temperature (Geddes et al., 2000). Juvenile wild-collected specimens have been shown to grow rapidly, with a specific rate of 1.56% per day observed. This is sufficient to permit growth from 3 g to a market size of 1 kg within 18 months (Jones et al., 2001).

1.1.4 Challenges for a rock lobster aquaculture

The introduction of a commercially viable rock lobster aquaculture has proven to be difficult to date. Despite the characteristics, which make *Panulirus ornatus* a promising candidate for larval rearing, several parameters contribute to continuous attrition and high mortalities during the larval rearing attempts.

Extended larval phase. A critical point in the closed life cycle production of aquaculture species is the hatchery phase. The only commercially viable aquaculture sectors to date are those that farm species which have a short larval phase (Lee and Wickins, 1992). Foremost for crustaceans are penaeid prawns with a larval phase of between 15 to 20 days. Such a short larval rearing period enables the maintenance of optimal rearing conditions and facilitates achievable microbial management.
Although the larval phase of *Panulirus ornatus* is one of the shortest of all Palinuridae, it is still of significant duration. This prolonged time that larvae are held in captivity subsequently increases the detrimental effects associated with rearing, and compromises the health of the animals.

Differences to the natural environment. Conditions within a larval rearing system are vastly different to what an animal experiences in the wild. This includes both the water chemistry and quality, and variations in temperature and salinity. Within culture, larvae are exposed to an environment of complex organic load, significantly different from the natural oligotrophic oceanic environment. High stocking densities contribute to increased stress levels for culture animals and interfere with the natural developmental cycle. Sub-optimal larval development is characterized by the occurrence of multi-instars within a particular larval stage, a phenomenon usually not observed with wild phyllosoma.

Nutrition. Identification of the optimal phyllosoma feed has proven to be difficult. Little is known about the natural diets of lobsters and their nutritional requirements. Feeding with insufficient diets leads to nutritional undersupply, which may compromise phyllosoma development as well as an animal's immune response.

Challenging microbial flora. A diverse and challenging microbial flora develops within the rearing system, colonizing both the tank environment and the cultured larvae. This flora differs to a great extent from the community found in the wild. Molecular studies have demonstrated that the bacterial flora of cultured phyllosoma was dominated by potentially pathogenic *Vibrio* and filamentous *Thiothrix* populations (Bourne et al., 2004; Webster et al.,

2006), causing various detrimental effects on larvae including disease and colonization of the phyllosoma exterior surface, respectively. In contrast, it was demonstrated that wild phyllosoma harboured only minor *Vibrio* populations, and the community was found to be dominated by α-*Proteobacteria* (Payne et al., unpublished data).
High bacterial loads and external bacterial colonization compromise the fitness of reared phyllosoma, which facilitates the outbreak of disease caused by opportunistic pathogens such as vibrios (Bourne et al., 2004). Although little is known about the immunodefensive system of lobsters, it can be assumed that early stage larvae have poor protection against these pathogens.

The challenges faced for successful large-scale phyllosoma rearing are complex and include an extended larval phase, immunocompromising stress levels, poor nutrition and high bacterial loads. All of these factors interact and contribute to poor larval survival, since phyllosoma cannot master the complexity of challenges.

This study addresses several aspects of the microbiology of *Panulirus ornatus* larval rearing and the microbial communities associated with both the rearing system and the phyllosoma. The microbiological characteristics of this complex system will be introduced in the following pages.

1.2 Microbiology of the larval rearing system

The two fundamental sectors of the *Panulirus ornatus* aquaculture system – the rearing tank environment and the phyllosoma – have been subdivided into several microbial compartments. These include biofilms, live feeds and the water column for the rearing tank; and externally and internally colonizing microorganisms for the phyllosoma (Bourne et al., 2004). A division of the system into compartments is necessary to obtain an understanding of the respectively associated microbial communities, as well as interactions and dynamics of the entire larval rearing system.

While the communities within each of these compartments possess innate characteristics, they simultaneously display interconnected properties. In this section, microbiological aspects relevant to each of these compartments of the larval rearing system will be presented.

1.2.1 Compartment 1 – *Biofilm*

It has been estimated that the majority of bacteria in natural aquatic ecosystems is organized in biofilms (Donlan and Costerton, 2002). Microbial activity is hence mainly associated with surfaces (Costerton et al., 1995; Davey and O'Toole, 2000; O'Toole et al., 2000), and bacteria accumulate rapidly wherever surfaces are immersed in water and offer favourable physiological conditions (Characklis and Marshall, 1990). Sessile bacterial cells differ profoundly from their planktonic counterparts (Costerton et al., 1978), and it has been proposed that bacterial surface attachment and subsequent biofilm growth exemplifies a survival strategy (Costerton et al., 1995).

Biofilm development and structure

The development of a microbial biofilm is separated into 4 stages (Characklis, 1981), which describe a periodical process of cellular attachment and detachment.

Stage 1. Dissolved and particulate matter is passively transported from the bulk fluid to the surface, which is possibly facilitated by an increased adhesiveness of the surface.

Stage 2. Microbial cells settle from the plankton and reversible contact is followed by firm attachment of cells to the surface. Cell divisions begin, leading to the appearance of small cell clusters.

Stage 3. Microbial reproduction within the growing biofilm results in high cell numbers and development of a rich microbiota. This biofilm build-up is often associated with a bacterial production of organic compounds, which are generally referred to as extracellular polymeric substances (EPS). The production and secretion of complex polysaccharides lead to the constitution of the biofilm being a gelatinous 'slime' matrix.

Stage 4. Single cells, filamentous bacteria, multicellular biofilm and EPS matrix detach from the surface, primarily due to fluid sheer stress.

Re-establishment of biofilm, beginning from stage 1, can be regarded as a fifth phase of development, making biofilm development a cyclic pathway. New bacterial attachment may be facilitated when previous biofilm has not been fully detached and particulate matter is left on the surface.

Active, mature biofilms are frequently represented by structural heterogeneity. Lawrence et al. (1991) however identified a basic scheme consisting of microcolonies of sessile, EPS matrix-enclosed cells with intervening water channels. This allows a convective water flow in between microcolonies, which reduces sheer forces and micro-turbulences and makes the biofilm architecture more stable and resistant against disturbance (Stoodley et al, 1994). Such typical biofilm structures have been observed with single- or multispecies biofilms; featuring spatial aggregates often referred to as towers or mushrooms. A variety of additional elements, such as cellular bridges between towers, can occur (Costerton, 2004).
Growth within biofilms is advantageous for a number of reasons. Surfaces absorb and concentrate scarce nutrients from the water column. The EPS matrix both provides additional protection and enables metabolically cooperative bacteria to form stable multispecies consortia. Complex interactions facilitate nutrient uptake, the degradation of complex substrates and recycling of decomposition products. Thus, proliferation within a biofilm offers significant metabolic advantages, represented by an increased activity of biofilm communities compared to planktonic populations (Kudo et al., 1987).

Biofilms and pathogenic bacteria

In medical sciences, biofilms are well recognized as a causative agent for clinical infections, representing a reservoir for pathogenic bacteria (Ehrlich et al., 2004; Hall-Stoodley and Stoodley, 2005). Human pathology has documented biofilm growth on implants, as well as biofilm-mediated infections and their association with diseases such as cystic fibrosis. With the increasing understanding of biofilms in marine systems, it has been discovered that the

identification of biofilms as a reservoir for detrimental bacteria is transferable to aquaculture facilities.

Bacteria growing within the stable microbiota of a biofilm feature an increased tolerance against antibiotics and disinfectants compared to their planktonic counterparts. Establishment of *Vibrio harveyi* within biofilms has been reported to be connected with high resistance to water sanitizers and antibiotics (Karunasagar and Otta, 1996). On average, the killing dose for any given antibiotic was shown to be more than 1,000 times higher for biofilm bacteria than for planktonic bacteria of the same strain (Ehrlich et al., 2004).

The increased resistance, however, cannot always be attributed for poor antibiotic penetration throughout the biofilm due to the presence of extracellular polysaccharides. Previous studies have shown that various antibiotics readily penetrate *Pseudomonas aeruginosa* biofilms, accumulating within hours at concentrations that would be bactericidal for planktonic forms (Yasuda et al., 1993; Suci et al., 1994; Vrany et al., 1997; Shigeta et al., 1997; Walters et al., 2003). Additional protective mechanisms were demonstrated by the identification of bacterial sub-populations in deeper regions of biofilms, which were viable but not dividing and resided in a state of decreased metabolism. Since most antibiotics target dividing bacteria, the reduced metabolic and divisional rates served to protect them from antibiotic-induced eradication (Anwar et al., 1992; Brown et al., 1998). These bacteria were then able to repopulate the biofilm following antibiotic killing of the peripheral bacteria.

Therefore, biofilms can enhance the survival and growth of pathogens by acting as permanent or temporary refuge. Studies of biofilm formation in a 5,000 litre phyllosoma larval rearing tank resulted in the isolation of a *Vibrio harveyi* strain which caused increased phyllosoma mortalities in subsequent small-scale challenge trials (Bourne et al., 2006). Moreover, molecular analysis of the biofilm development demonstrated that a *Vibrio* population, which was not detectable in the early stages of the biofilm, became a predominant population at the time of a phyllosoma mass mortality event (Bourne et al., 2006).

Quorum sensing

Numerous bacterial communities are capable of employing cell-cell communication pathways through the synthesis of, and response to, intercellular signal molecules enabling the community to exchange information in a density-dependent manner. This regulatory principle is referred to as *quorum sensing* (Fuqua et al., 1994).

A diverse range of metabolites have been identified as signal molecules, of which the most intensively studied are represented by the *N*-acyl-homoserine lactones (AHL) in Gram-negative bacteria. The classic example for AHL-mediated quorum sensing is the control of bioluminescence in *Vibrio fischerii* (Nealson et al., 1970).

Previous work described quorum sensing pathways as playing an important role during later stages of biofilm development, but not in the initial adhesion process (Sauer and Camper, 2001). In addition, quorum sensing frequently contributes to pathogenicity; principles that will be introduced in 1.2.4.

1.2.2 Compartment 2 – *Water column*

Flow cytometry and molecular microbial analyses on water samples obtained from *P. ornatus* phyllosoma rearing tanks demonstrated a dynamic microbial community within the water column during a standard larval rearing run (Payne et al., 2006). Distinct changes in the bacterial diversity occurred from day 1 to 2 and from day 4 to day 5 following initial stocking of phyllosoma. The community diversity in subsequent days of the larval rearing trial remained relatively stable (Payne et al., 2006). Despite a stable microbial community structure, a major increase in bacterial load at the beginning of the first larval moult event was recorded, which markedly decreased 2 days later when >50% of the larvae had moulted. A clone library of a water sample taken following a mass larval mortality event reflected high microbial diversity. Sequences retrieved from both clone libraries and denaturing gradient gel electrophoresis were dominated by bacteria being mainly affiliated with γ- and α-*Proteobacteria*, and additional sequences affiliated with a diverse community of β- and ε-*Proteobacteria*, *Bacteroidetes*, *Cytophagales*, *Chlamydiales*, and *Vibrionaceae* (Payne et al., 2006).

1.2.3 Compartment 3 – *Live feed* (*Artemia*)

The small crustacean *Artemia* is commonly used as live feed in aquaculture. It represents an ideal hatchery food, since dried cysts remain viable in cold storage for many years and can be induced to hatch on demand. This allows to accurately plan and control production schedules.

An ongoing cultivation of *Artemia* has been established at the aquaculture facilities of AIMS, which ensures a continuous production of adult nauplii serving as live feed in phyllosoma rearing. Presently, the optimal *Artemia* as a dietary item is considered to be an on-grown, micro-algae enriched animal grown in high density dedicated production tanks. They are fed on a combination of micro-algae, since each of the algal species possesses a different protein and lipid profile. Enrichment of algal nutrients over the *Artemia* step is necessary, since phyllosoma cannot filter feed or directly capture micro-algae. This however has the

beneficial effect that the micro-algal diet influences the total body composition of *Artemia* by the successive enrichment of algal nutrients within the animals, a process referred to as 'bioencapsulation'. When *Artemia* are consumed by phyllosoma, larvae receive the benefit of concentrated algal nutrients.

The role of *Artemia* in pathogen transmission

The intensive production of live feeds in larval hatcheries has the inherent challenge that the routine addition of on-grown feeds to rearing tanks can yield the transmission of disease. The enrichment procedures imply bacterial contamination of on-grown feed. High organic loads, typical for live feed culture, encourage the rapid proliferation and domination of potential pathogenic bacteria at the expense of autochthonous flora (Skjermo et al., 1997). Thus, *Artemia* represent a significant vector for the transfection of pathogens and bacterial contaminants with subsequent aetiology of infectious disease in larval culture (Verdonck et al., 1994; Lopez-Torres and Lizzaraga-Partida, 2001). Previous studies have shown that aquatic larvae progressively establish their intestinal flora from the environment they inhabit. In addition, the bacterial flora of live feeds and the bacterial flora of the larval species to which they are fed have been shown directly correlated (Nicolas et al., 1989; Muroga et al., 1990; Otta et al., 1999).

The transmission of bacterial pathogens to phyllosoma rearing, after contamination and proliferation on *Artemia* exoskeleton and within their intestinal tract during the grow-out, represents an important threat for larval rearing trials. Once introduced into larval rearing tanks, pathogenic populations potentially reside within the protective microenvironment of a biofilm and may subsequently facilitate disease outbreaks.

1.2.4 Compartment 4 – *Phyllosoma*

Diseases of crustaceans

Infectious diseases of crustaceans are separated into four categories; viral, bacterial, fungal and parasitic infections. These microbial entities are capable of causing mass mortalities of culture animals.

Viral infections have been described by Edgerton (1996) for the Australian freshwater crayfish, *Cherax destructor*. Other viruses, such as the white spot syndrome virus or gill-associated virus, have had major impacts on penaeid prawn aquaculture around the world, as reviewed by Flegel (1997). Fungal and parasitic infections are less commonly reported

and typically less serious, except for the highly infectious *crayfish plague* caused by the fungus *Aphanomyces astaci* (Alderman and Polglase, 1986).

The majority of crustacean diseases is represented by bacteriological infections. Principal examples are gaffkemia, shell disease and vibriosis (Sindermann, 1989; Evans and Brock, 1994; Abraham et al., 1996), which are widespread among almost all farmed species.

As outlined in 1.1.3, the outbreak and spread of disease in aquaculture is facilitated by stress that the animals encounter when exposed to sub-optimal hatchery conditions (Evans and Brock, 1994; Aguado and Bashirullah, 1996), resulting in animals being increasingly susceptible to infection (Takahashi et al., 1995). This can induce a massive proliferation of opportunistic pathogenic bacteria, which normally co-exist with the host, but can proliferate rapidly once external factors change.

The somatic protection of larvae is further diminished around moult cycles, resulting in an increased vulnerability until the new exterior surface is developed. Furthermore, the high stocking densities within culture promote external injuries through physical competition between animals. In this context, several bacterial infections were described as secondary following physical impairment that provided a gate for the causative pathogen. External injuries and subsequent infection, for instance, were accounted for severe shell disease in both *Macrobrachium rosenbergii* and *Palaemon serratus* (Cook and Lofton, 1973; Delves-Broughton and Poupard, 1976).

The genus *Vibrio*

The Gram-negative *Vibrionaceae* family, within the genera *Vibrio*, *Aeromonas*, *Photobacterium* and *Plesiomonas*, is widespread in the marine environment. Many new species have been described over the past 20 years, leading to a broad diversification of the taxonomy (Austin et al., 2005).

It is established that *Vibrio* sp. represents the major pathogen for crustacean larvae and juveniles, as well as for various other aquatic organisms (Bachère, 2003; Vandenberghe et al., 2003) by inducing infectious disease, known as vibriosis. Pathogenic *Vibrionaceae* are often characterized by expression of density-dependent bioluminescence. Quorum sensing is regularly employed also to regulate the expression of virulence factors including a range of potent entero- and exotoxins linked to pathogenicity (Camara et al., 2002; Henke and Bassler, 2004), such as the production of heat stable exotoxins by the human pathogen *Vibrio cholerae* (Dalsgaard et al., 1995). The establishment of vibrios within biofilms has been demonstrated (Karunasagar and Otta, 1996; Croxatto et al., 2002), emphasizing the biofilm capability as a potential refuge for pathogens (see above).

Vibriosis represents one of the most serious diseases in aquaculture worldwide, affecting the cultivation of various animals from crustaceans to fish (King and Flick, 2001) and often causing mass mortalities. For instance, the pathogens *Vibrio alginolyticus* and *Vibrio harveyi* were demonstrated to affect larval cultures of the prawn species *Penaeus monodon* and *Penaeus japonicus* by their production of proteinaceous exotoxins (Chen et al., 1999; Harris and Owens, 1999). These two bacterial species were also identified as the causative agents of disease within *Panulirus homarus* rearing after being isolated from both exoskeleton and haemolymph lesions (Abraham et al., 1996). Further work described stages of luminous vibriosis in reared phyllosoma of the New Zealand spiny lobster *Jasus verreauxi* (Diggles, 2000) and bacterial enteritis in cultures of the Southern rock lobster *Jasus edwardsii* (Handlinger et al., 1999).

For studies on the ornate rock lobster, Bourne et al. (2004) identified the presence of *Vibrio parahaemolyticus* and *Vibrio harveyi* present prior to and during mass mortality events of phyllosoma. *Vibrio* exotoxin production may have further accounted for deteriorated hepatopancreas tissue observed within phyllosoma sections (Webster et al., 2006).

Bacterial colonization of phyllosoma

Bacterial colonization of phyllosoma is of particular concern in the early larval stages, as this is the time when phyllosoma are exceptionally vulnerable to bacterial colonization and pathogen infection. Previous work at AIMS consistently observed high larval mortalities within the first (P1-P2) and second developmental stage (P3-P4). Their individual duration is usually 9 days and 12 days, respectively, and occur from days 1-9 and 16-28 of the total larval age.

a. External colonization

A significant microbial challenge confronting the phyllosoma within the larval rearing environment is external bacterial colonization by filamentous bacteria, in this context sometimes also referred to as biofouling. Handlinger et al. (1999) reported massive external colonization for *Jasus verrauxii* phyllosoma with *Leucothrix*-like filamentous bacteria, which additionally provided a protective habitat for the proliferation of a complex microbiota consisting of sessile protozoa, rod-shaped bacteria, ciliates and occasionally fungi. The bacterial filaments were preferentially associated with nutrient-rich regions, such as the mouthparts and the anus of larvae. By entangling mouthparts, filamentous bacteria hamper the larval ability to feed and compete for nutrients. The poor nutritional supply of the larvae is enhanced, which further compromises the health status of the animal.

Heavy epibiont growth also contributes to difficulties in moult shedding and reduces respiratory effectiveness, when demand for oxygen increases during a moulting event (Crear and Forteath, 1998; Diggles, 1999; Handlinger et al., 1999). This phenomenon has been reported for penaeid prawns infected with the filamentous bacterium *Leucothrix mucor* (Lightner, 1983), and likely explains mass mortalities around moult periods. However, moult shedding is likely able to effectively eliminate the colonization of the larva when the exterior surface is removed.

Previous studies of *P. ornatus* phyllosoma detected a *Thiothrix* affiliated sequence retrieved from DNA extracted directly from larvae (Bourne et al., 2004). The association of *Thiothrix* with larvae was confirmed by using a rRNA targeted *Thiothrix* probe and fluorescence *in situ* hybridization. *Thiothrix* is preferably found on biological, 'living' surfaces, such as algal epibiont, strongly attaching with holdfasts (Brigmon et al., 1994; Polz et al., 1994). It is an obligate chemoautotroph, dependent on higher sulfide concentrations, and utilizes reduced sulfur compounds as an electron source leading to the formation of sulfate (Munn, 2004). *Leucothrix* sp., which was also detected within *P. ornatus* larval rearing systems, possesses similar filament morphology features, but filaments are arranged in characteristic rosettes. *Leucothrix* sp. was described as the chemoheterotrophic counterpart of *Thiothrix* (Harold and Stanier, 1955) and is not capable of metabolizing sulfuric compounds.

b. Internal colonization

The term internal colonization refers to the proliferation of bacteria within the intestinal tract of phyllosoma, typically the hepatopancreas. When pathogenic bacteria colonize this tract, the proceeding infection is commonly represented by tissue lesions and distinct numbers of bacterial cells that can be visualized by histology, Gram staining or fluorescence *in situ* hybridization.

Using hybridization techniques, the significant invasion of the phyllosoma digestive tract by *Vibrio* sp. was demonstrated and associated with mass mortalities among cultured larvae (Webster et al., 2006). Nevertheless, it is important to emphasize that internal bacterial proliferation does not necessarily yield disease, since most organisms harbour a specific microflora which can contribute to beneficial metabolic effects. This principle may also apply to wild, disease-free phyllosoma. Clone libraries of bacterial communities associated with wild Palinuridae and Scyllaridae phyllosoma, collected in the Coral Sea and Great Barrier Reef waters, revealed that the majority of the bacterial flora of wild larvae was represented by α-*Proteobacteria*, mainly comprising genera related to *Sulfitobacter* and *Roseobacter*. Although in minor proportions, various members of the *Cytophaga-Flavobacterium* group of the CFB phylum were also found in association with wild larvae (Payne et al., unpublished

data). The majority of these bacteria is believed to be internally associated, and it is possible that larvae benefit from their colonization. *Vibrionaceae* were also demonstrated to colonize wild larvae, although their association was lower than with the cultured phyllosoma. Nevertheless, the status of vibrios as belonging to the normal intestine microflora of many marine species (Vandenberghe et al., 1998; Conejero and Hedreya, 2003), including lobsters (Rosemark and Fisher, 1988), was highlighted. It is hence likely that opportunistic *Vibrio* species are only rendered pathogenic when sub-optimal conditions within a system occur (see 1.1.4).

1.3 Methods of microbial management

1.3.1 Ozonation of seawater

The use of ozone as a powerful oxidizing agent and disinfectant in aquatic systems is well established (Summerfelt and Hochheimer, 1997). Once ozone enters into water, it becomes highly unstable and rapidly decomposes through a complex series of reactions. The weak chemical bonds within the three-atom allotrope of oxygen (O_3) facilitate a single atom to break away, which readily reacts with most molecules leading to their oxidation (Lawson, 1995). Ozone further decomposes into highly reactive radicals (Sugita et al, 1992; Lawson, 1995), of which the hydroxyl radical ($HO^{\bullet}$) is the most important species. In addition, ozonation commonly leads to the production of brominated by-products.

Ozonation addresses both the elimination of microorganisms and enhancement of water quality by removal of dissolved organic and inorganic waste, the increase of nitrification rates and the control of suspended solids (Paller and Lewis 1988; Lawson, 1995). It effectively destroys bacteria, fungi, algae and protozoa by disrupting cell membrane function and interfering with nuclear chemistry within the cells (Lawson, 1995). Previous work achieved 99.99% reductions in viable counts of four pathogenic strains within 3 min of exposure to ozone (Liltved et al., 1995). A similar study by Sugita et al. (1992) demonstrated 99% inactivation of *Enterococcus seriolicida*, *Pasteurella piscicida* and *Vibrio anguillarum* within 1 min at various ozone concentrations. Thus, subsequent disinfection of the culture water and improvement of overall system performance are achieved. The effects of ozonation do not only exceed those of basic water filtration, but have been also shown to outreach other non-specific chemical treatments such as chlorination (Caravelli et al., 2006).

Problems of usage

Since both the water quality and target organisms determine the required ozone concentration and contact time for successful decontamination of a system, these parameters have to be specifically evaluated for each aquaculture facility (Lawson, 1995; Summerfelt and Hochheimer, 1997). This is of particular concern, since microbial reductions are essentially limited by the ability to maintain a specific ozone concentration within a system (Summerfelt and Hochheimer, 1997). This may prove problematic, since the average half life of ozone is about 1 hour and it may be depleted before microorganisms can be eradicated (Liltved and Landfald, 1995; Summerfelt and Hochheimer, 1997). Furthermore, the above mentioned reactivity with bromide incorporates the possibility of bromate formation, a compound regarded as carcinogenic.

Ozonation and biofilms

Despite its decontaminating capability, ozone reacting with natural organic matter increases the content of available organic carbon (Janssens et al., 1984). The resulting organic compounds are easily biodegradable, and may promote microbial growth and biofilm formation instead of controlling it (Glaze, 1987).

Ozonation was introduced in the aquaculture facilities at AIMS to control the build-up of biofilm in larval rearing tanks, and also as an attempt to limit the proliferation of filamentous bacteria both within biofilms and externally on phyllosoma. The potential value of biofilm control was highlighted by observations of significant biofilm development and sloughing within phyllosoma rearing tanks (Bourne et al., 2004) (see above). This advised to test potential biofilm-controlling protocols, including ozonation.

1.3.2 Disinfection of live feed

A principal goal of live feed production states the reduction of the associated bacterial load. The reduction of this load would result in improved microbial management in the target larval culture, since contaminated live feed that is added to the culture potentially allows pathogenic organisms to enter the rearing system.

A variety of methods have been investigated to control the bacterial contamination of live feed. Perez-Benavente and Gatesoupe (1998) reported significantly improved turbot larvae survival by implementation of antibiotics reducing the bacterial load in both rotifers and *Artemia* prior to feeding. However, in the pursuit of environmental awareness and consumer health, the use of chemical disinfectants and antibiotics is becoming increasingly unsuitable.

This has been emphasized by the emergence of antibiotic resistant bacteria, which represent a significant threat for both human and animal health (Brown, 1998).
Representing a more natural approach, the biocontrol potential of various algae has been recognized. The secretion of different antimicrobial compounds has been shown, such as of polyphenols or tannins (Glombitza, 1979; Reichelt and Borowitzka, 1984). Algal therapeutics was used by Olsen et al. (2000) to achieve a 75% reduction of bacteria and increase of relative bacterial diversity in *Artemia* following an four-hour enrichment on *Tetraselmis* sp. Further work revealed the inhibition of several pathogens by cell extracts of the same algal species (Austin et al., 1992) and sufficient decontamination after *Chaetoceros mülleri* enrichment (Tolomei et al., 2006). However, it was anticipated that contaminant bacteria resident on external surfaces would not be removed by an algal therapeutic. With respect to the decontamination by ozonation, *Artemia* appear resilient to ozone, and bacterial levels in *Artemia* culture water were reduced by 99.9% within minutes of exposure to 4 ppm ozone (Tolomei, unpublished data).

1.4 Probiotic bacteria

1.4.1 General characterization

For more than 50 years, probiotic bacterial strains have been shown to improve intestinal microbial balance and reduce the presence of pathogens within the gastro-intestinal tract of culture species (Fuller, 1995). Gatesoupe (1999) separated aquatic probiotics into three categories, (i) bioremediators that break down waste or pollutants in the water and are not transient or resident in the gastrointestinal tract of animals, (ii) biocontrol agents that are antagonistic to pathogens, but as well are not transient or resident in the gastrointestinal tract, and (iii) 'true' probiotics that are both antagonistic to pathogens and transient or resident in the gastrointestinal tract of animals. Thereby, members belonging to this last category could be seen as the most promising candidates, and several bacterial strains have been suggested as being 'true' probiotics as per this definition (see below). Experience from agriculture and human medicine however suggests that the direct probiotic action involves competitive exclusion, i.e. the probiotic controls proliferation of potential pathogens by antibiosis, competition for nutrients and/or space, or the modification of microbial metabolism (Irianto et al., 2000). In addition, probiotics may act indirectly by nutritional improvement in terms of the production of vitamins, detoxification of compounds in the diet, the breakdown of indigestible components in the diet or supplementation with exogenous enzymes (Irianto et al., 2000).

Overall, an effective probiotic treatment would exclude pathogens from the aquaculture system and stimulate immune responses, probably including broad-spectrum and non-specific disease protection (Rengpipat et al., 2000). Application of probiotics could be performed in a variety of ways, ranging from incorporation of the probiotic in a nutritionally complete, microencapsulated diet (Itami and Takahashi, 1991), addition to the rearing water of live feed (Gatesoupe, 2002) or culture animals (Kennedy et al., 1998) to direct injection of probiotics into the culture organism (Sridhar et al., 2006). It was commonly suggested to include probiotics from the earliest days of rearing (Ringo and Vadstein, 1998; Gatesoupe, 1999; Rengpipat et al., 2000).

1.4.2 Recognized probiotics

Bacillus spp.

Several members of the Gram-positive genus *Bacillus* produce a range of natural antimicrobial compounds and are further enabled to deploy several other strategies to reduce the abundance of competing strains. If *Bacillus* spp. are present in higher numbers, these bacteria have shown to readily displace other bacteria (Moriarty, 1998). *Bacillus* strains are able to circumvent antibiotic resistance of bacteria that grow within biofilms by secretion of enzymes that degrade these protective structures. The produced antibiotic compounds are able to penetrate the biofilm layers and attack Gram-negative bacteria. Growth of potential pathogenic bacteria like *Vibrio* sp. can thus be adversely affected. Furthermore, *Bacillus* spp. can inhibit other bacteria by competition for nutrients or surface space, hindering their ability to multiply and exchange genetic information. This impairs, for instance, the transfer of antibiotic resistance genes and further enhances the probiotic effect.

Roseobacter spp.

The genus *Roseobacter* and species affiliated with this group, the *Roseobacter* clade, belong to the α-*Proteobacteria* and represent important members of the marine microbiota. *Roseobacter* spp. may account for as much as 40% of the prokaryotic DNA from the ocean and is believed to play an important role in the global sulfur cycle and climate (Moran et al., 2003). The metabolism incorporates the oxidation of the greenhouse gas carbon monoxide, and production of the climate-relevant gas dimethylsulfide (DMS) through the degradation of algal osmolytes (Wagner-Döbler and Biebl, 2006). This also accounts for the bacterium's typical association with algae and dinoflagellates (Gonzalez et al., 2000; Eilers et al., 2001; Miller and Belas, 2004). *Roseobacter* spp. has been identified as an excellent biofilm-forming bacterium (Bruhn et al., 2005) and to be among the first and dominant colonizer of surfaces

(Dang and Lovell, 2000). Several species are inhibitory towards other bacteria and were shown to produce antimicrobial compounds. It was hypothesized that this ability, associated with the capability of biofilm formation, may give members of the clade a selective advantage and helps to explain their dominance within the marine algal microbiota (Bruhn et al., 2006). As a specific example of inhibition, which also points to the involvement in the sulfur metabolism, Brinkhoff et al. (2004) demonstrated the *Roseobacter* sp. production of the sulfur-containing compound tropodithietic acid, a precursor of the antibacterial compound thiotropocin.

Previous work identified *Roseobacter* spp. as an abundant member of the bacterial flora associated with wild phyllosoma (Payne et al., unpublished data). Several *Roseobacter* strains have been also isolated from *Panulirus ornatus* larval rearing tanks, confirming the ability of the genus to establish within the bacteria-rich phyllosoma aquaculture environment (Lone Høj, personal communication).

***Vibrio* sp.**

Despite their general status as pathogenic bacteria, *Vibrio* species have not only been suggested as being part of the natural gut microflora of crustaceans and various other animals, but found as featuring probiotic capabilities. Austin et al. (1995) described probiotic effects of *Vibrio alginolyticus* – pathogenic for many marine organisms – by inhibiting growth of *Vibrio ordalii*, *Vibrio anguillarum* and *Aeromonas salmonidica*; thereby reducing mortalities within cultures of the Atlantic salmon (*Salmo salar*). A strain of *Vibrio alginolyticus* (C009), isolated from *Artemia* live feed in the AIMS aquaculture facilities, similarly demonstrated the potential ability to inhibit growth of pathogenic vibrios (Lone Høj, personal communication).

1.5 Goals of this study

The division of the phyllosoma larval rearing system into compartments is essential to improve the understanding of complex microbial interactions leading to phyllosoma mortality, and develop effective strategies of microbial management (Skjermo and Vadstein, 1999). Microbial management and effective disease control in the rock lobster phyllosoma rearing facility is essential, since poor larval survival in previous cultivation trials was indirectly or directly related to increased microbial proliferation.

The colonization of the hepatopancreas of dying larvae by potentially pathogenic vibrios has been demonstrated (Webster et al. 2006), as well as external colonization by filamentous bacteria (Bourne et al., 2004; Payne et al., 2007). Proliferation of potentially pathogenic vibrios within biofilms forming on the surfaces of large-scale 5,000 litre larval rearing tanks

has also been demonstrated (Bourne et al., 2006). In addition, *Artemia* live feed has been suggested to multiply bacterial loads within larval rearing tanks by co-cultivation of (pathogenic) bacteria.

In this study, a polyphasic approach was employed to investigate three of the four microbial compartments associated with the *Panulirus ornatus* larval rearing system. Additional microbial investigations of wild phyllosoma aimed to identify the naturally associated bacterial communities including potential probiotic bacterial strains.

Compartment 1 – Biofilm

Ozonation of incoming seawater was recently implemented in the larval rearing facility at the Australian Institute of Marine Science. This study therefore investigated the effect of seawater ozonation in the presence of biological loading – represented by *Artemia* – on the development and composition of biofilm in larval rearing tanks using microscopical and molecular analyses. Since the biofilm is a potential reservoir for phyllosoma-colonizing filamentous bacteria and a refuge for opportunistic pathogens, control of biofilm build-up in the rearing tanks is important. It was hypothesized that ozonation results in the decontamination of the culture water and therefore inhibition of biofilm formation. By investigating the tank biofilm, conclusions could also be drawn on the effect of ozonation on the microbial load within the water column (compartment 2).

Compartment 3 – Live feed (*Artemia*)

It is believed that *Artemia* live feed pose a vector for entry of pathogenic bacteria into larval rearing systems through the subsequent bacterial colonization of their exterior surface during the enrichment. Thus, methods for improving the microbial status of *Artemia* were evaluated, including the assessment of antibiotic treatment protocols and the use of a commercial probiotic (*Sanolife*) for controlling their load with potential pathogenic *Vibrionaceae*. It is essential to develop reliable decontamination methods for *Artemia*, to reduce compromising effects on the phyllosoma health based on the co-cultivation of pathogens via the *Artemia*.

Compartment 4 – Phyllosoma

The degree of bacterial colonization, both externally and internally, is believed to be enhanced in rearing systems. Microscopical studies of bacterial colonization of both cultured and wild larvae were performed to complement previous studies. The microbial flora associated with reared phyllosoma was compared to the microflora of healthy wild phyllosoma to observe the differences in both bacterial diversity and colonization. Such direct comparisons can aid in the understanding of beneficial bacterial associations and improve

microbial management of the closed rearing system. Potential probiotic strains, particularly targeting strains from the *Roseobacter* clade, were isolated from wild, disease-free larvae and screened for activity against a pathogenic bacterium, with candidates offering one management option for improved rearing protocols.

In summary, this study was structured in following experiments:

1. Effect of seawater ozonation on development and composition of biofilm within larval rearing tanks
2. *Vibrio* load of *Artemia* and the effect of antimicrobial treatments
3. Microbial colonization of phyllosoma
4. Isolation and identification of potential probiotic strains from wild phyllosoma

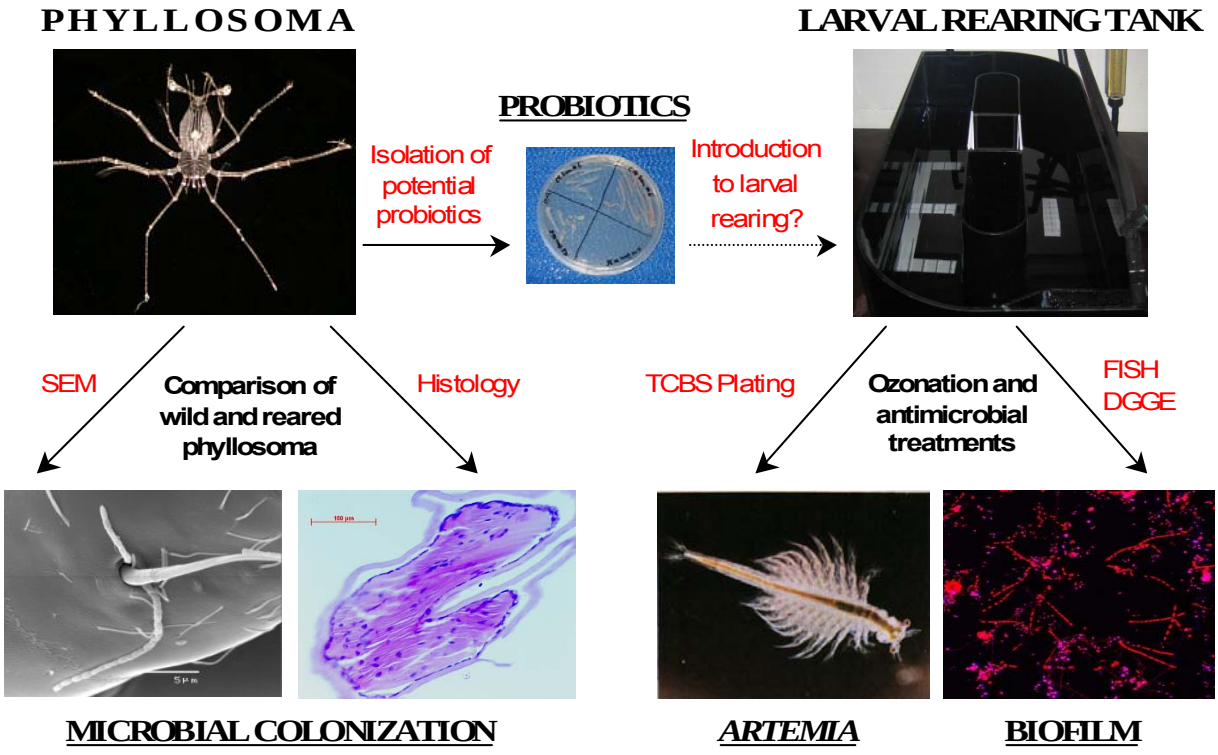


Figure 1.4 Overview of the topics addressed in this study. In the head line, 'Phyllosoma' and 'Larval rearing tank' define the fundamental sectors of aquaculture rearing of *Panulirus ornatus*. The analyzed compartments of the microbial community – biofilm, *Artemia* and the phyllosoma-associated external and internal microbial colonization – are printed bold and underlined. The designation 'Probiotics' refers to the isolatation of potential probiotic strains from wild phyllosoma, aimed at a possible implementation of those strains in the rearing process. In red: Reference to the applied methods, as outlined in Chapter 3.

2. Materials

2.1 Laboratory devices

Agarose gel electrophoresis unit		Bio-Rad, Hercules, USA
Bead Beater		BioSpec, Bartlesville, USA
Cameras	Radiance 2000	Bio-Rad, Hercules, USA
	AxioCam MRc 5	Zeiss, Jena, Germany
Centrifuges	5415 D	Eppendorf, Hamburg, Germany
	Microfuge 22R	Beckman Coulter, Fullerton, USA
	Savant SpeedVac	GMI, Ramsey, USA
DGGE System	DCode	Bio-Rad, Hercules, USA
Histology devices	Shandon Hypocentre XP	Thermo Electron, Waltham, USA
	Shandon Histocentre 2	
Hybridization oven	Xtron Hi 2002	Bartels, Heidelberg West, Australia
Fluorimager	FluorS Multimager	Bio-Rad, Hercules, USA
Microscopes	CLSM (Eclipse E600)	Nikon, Tokyo, Japan
	Light microscope (Axioskop 2)	Zeiss, Jena, Germany
	SEM (JSM-5410LV)	Jeol, Tokio, Japan
Microtome		Leitz, Wetzlar, Germany
Motor Pellet Pestle		Kimble / Kontes, Vineland, USA
Thermal PCR cycler	2720	Applied Biosystems, Foster City, USA

2.2 Consumables

96-well filter plates	Pall, East Hill, USA
Antibiotic assay disks	Whatman, Maidstone, UK
Carbon tabs (IA023)	ProSciTech, Thuringowa, Australia
Pin-type SEM mounts (ø 25 mm)	ProSciTech, Thuringowa, Australia
Serological pipettes (5 & 10 ml)	Sarstedt, Nürnbrecht, Germany
Petri dishes (ø 9 cm)	Sarstedt, Nürnbrecht, Germany
Polypropylene reaction tubes (0.6 ml, 1.5 ml, 2 ml)	Porex QSP, Fairburn, USA
Polypropylene screw-cap tubes (15 & 50 ml)	Sarstedt, Nürnbrecht, Germany
Teflon-coated 8-well microscope slides	ProSciTech, Thuringowa, Australia

2.3 Reagents

10x PCR Buffer	QIAgen, Hilden, Germany
Acrylamide (30%; 37.5:1)	Bio-Rad, Hercules, USA
Antibiotics (Erythromycin, oxolinic acid, oxytetracycline, streptomycin, tetracycline)	Sigma, St. Louis, USA

Artemia AAA Cysts	INVE, Phichit, Thailand
Agarose (LE SeaKem)	Cambrex, Rockland, USA
Ammonium chloride (NH_4Cl)	APS Chemicals, Seven Hills, Australia
Ammonium persulfate (APS)	Bio-Rad, Hercules, USA
Bacteriological Agar	Oxoid, Hampshire, UK
Biomedia Gel / Mount	ProSciTech, Thuringowa, Australia
Bovine serum albumin (BSA)	BioLabs, Frankfurt/Main, Germany
Casamino acids	Becton Dickinson, Sparks, USA
D(+)-Glucose	Sigma, St. Louis, USA
dNTP Mix	QIAgen, Hilden, Germany
Dixylol-phthalate (DPX) Histology Mount	ProSciTech, Thuringowa, Australia
Ethanol, 96%	APS Chemicals, Seven Hills, Australia
Ethidium bromide	MO BIO Laboratories, Carlsbad, USA
Ethylenediamine-tetraacetate disodium salt (Na_2EDTA)	Sigma, St. Louis, USA
Formamide	Sigma, St. Louis, USA
GeneRule 100 bp Ladder	Fermentas, Burlington, CA
Glycerol	Sigma, St. Louis, USA
Glutaraldehyde	ProSciTech, Thuringowa, AU
Hexamethyldisilazane (HMDS)	Sigma, St. Louis, USA
Marine Agar 2216	Becton Dickinson, Sparks, USA
Marine Broth 2216	Becton Dickinson, Sparks, USA
Magnesium chloride ($MgCl_2$, PCR grade)	QIAgen, Hilden, Germany
Paraffin	Sigma, St. Louis, USA
Paraformaldehyde	ProSciTech, Thuringowa, Australia
Repel-Silane	Amersham, Buckinghamshire, UK
Sanolife Mic®	INVE, Phichit, Thailand
Sodium chloride (NaCl)	APS Chemicals, Seven Hills, Australia
Sodium dodecyl sulfate (SDS)	Amresco, Solon, USA
Sodium hydroxide (NaOH)	APS Chemicals, Seven Hills, Australia
Sucrose	Sigma, St. Louis, USA
Taq & Hot Star *Taq* polymerase	Sigma, St. Louis, USA
Thiosulfate-citrate-bile-sucrose (TCBS) Agar	Oxoid, Hampshire, UK
Tetramethylethylendiamine (TEMED)	Bio-Rad, Hercules, USA
Tris Base-HCl	Amresco, Solon, USA
Xylene	Sigma, St. Louis, USA

2.4 Commercial kits

QIAquick Gel Extraction Kit	QIAgen, Hilden, Germany
QIAquick PCR Purification Kit	QIAgen, Hilden, Germany
Wizard Genomic DNA Purification Kit	Promega, Madison, USA

3. Methods

3.1 Biofilm experiments

3.1.1 Experimental setup

Larval rearing tanks

Six *raceway* phyllosoma rearing tanks (770 x 330 x 200 mm; designed by the Aquaculture facilities of the Australian Institute of Marine Science, Figure 3.1) were manually cleansed, and disinfected by filling with 0.1 ppm chlorinated freshwater and left overnight. Tanks were emptied, rinsed with freshwater and connected to a water supply and drain system. The water flow was started with a constant flow-through rate of 1.0 to 1.2 l min^{-1} and the drain system adjusted to maintain a water mass of 16 litres per tank. Three tanks were supplied with filtered, ozone-treated seawater (herein referred to as *ozonated water*) and three tanks with filtered, non-ozonated seawater. The procedures of water filtration and ozonation are outlined in 7.1.

Teflon-coated 8-well microscope slides for fluorescent *in situ* hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE) were attached upright to acrylic racks by affixing to silicone (Figure 3.1). Glass disks for scanning electron microscopy (SEM) were attached similarly to smaller acrylic racks. Racks were disinfected by immersion in 0.1 ppm chlorinated freshwater for 1 h. Each one slide and one disk rack was applied to the bottom of each tank with the surface of slides and disks parallel to the wall, respectively (Figure 3.1).

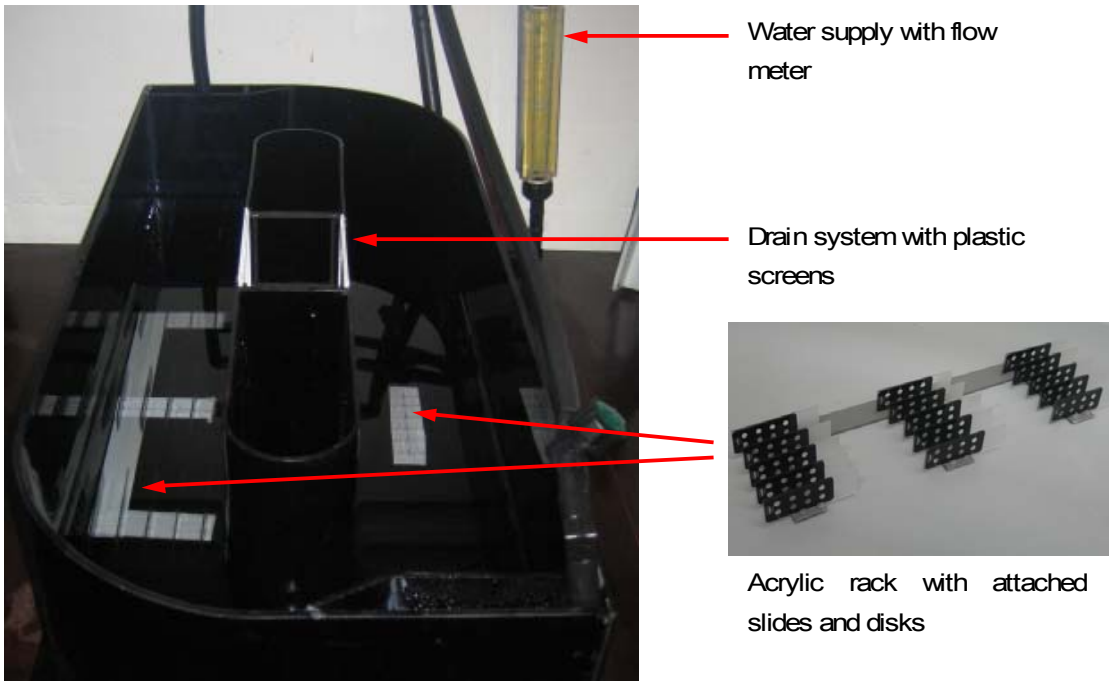


Figure 3.1 Setup of larval rearing tanks

Artemia

5 g of *Artemia* cysts were hydrated for 1 h in freshwater with continuous aeration. Decapsulation of cysts was achieved by vigorous agitation in an Erlenmeyer flask containing 2 ml NaOH (20%), 18 ml NaOCl and 50 ml freshwater. The preparation was rinsed with freshwater. *Artemia* were grown for 24 h with continuous aeration in 30 l filtered seawater at a constant temperature of 28°C.

The *Artemia* culture was harvested the next day by filtration through a screen. To completely remove the egg shells remaining from cyst decapsulation, *Artemia* were washed twice with air-enriched water so that the shells accumulated on the water level where they could easily be siphoned off. Washed specimens were concentrated in 2 l of seawater and transferred to a 800 litre tank. Artemia were on-grown in mass culture for 7 days while fed on the four microalgae species *Chaetoceros* sp. (Bacillariophyceae), *Tetraselmis* sp. (Prasinophyceae), *Isochrysis* sp. (Haptophyceae) and *Pavlova* sp. (Prymnesiophyceae), which were separately cultivated and supplied to *Artemia* enrichment tanks.

After 7 days, 10 l of the mass culture were harvested, from which a 200 ml aliquot was transferred into a beaker. 10 ppm formalin was added and the aliquot left for 3 h. The aliquot was transferred into 10 l of filtered algae culture and 400 ppm formalin was added. *Artemia* were filtered through a screen, briefly rinsed with freshwater and transferred into a gently aerated beaker containing 1800 ml of ozonated seawater. 10 ppm streptomycin, 5 ml carotenoid solution and 0.25 g ascorbate were added and culture left for 1 h.

Artemia were added to each tank to a concentration of approximately 1 nauplius ml^{-1}. Previously disinfected (in 0.1 ppm chlorinated freshwater) plastic screens (Figure 3.1) were inserted to the tanks to prevent *Artemia* being flushed away through the drain system. The *Artemia* culture was exchanged every day by removal of residual and addition of freshly harvested 7-day on-grown *Artemia* as described above. Per substitution, screens were cleaned, disinfected by 1 h immersion in 0.1 ppm chlorinated freshwater and reinserted; as well as dead *Artemia* and as much particulate matter as possible was siphoned off each tank's bottom.

Sample collection and fixation

FISH. Glass slides with biofilm formation for FISH analysis were collected 1 h (Day 0) and 24 h (Day 1) after first addition of *Artemia*. Subsequently, sampling was continued every second day (Day 3, 5, 7, 9, 11, 14, 16, 18, 20) and weekly (Day 28, 36, 44).

For each tank at each sampling time point, a microscope slide was detached from the silicone with a sterile scalpel blade and immediately transferred to a microscope slide holder containing freshly prepared 4% paraformaldehyde suspended in phosphate buffered saline

(PBS). Biofilm was fixed by incubation at 4°C for 5-6 hours. The fixative was then replaced by 1:1 (v/v) ethanol:PBS and sample stored at –20°C until further processing.

SEM. Glass disks with biofilm formation for SEM analysis were collected on the same days as samples for FISH analysis were collected (see above). For each tank at each sampling time point, a glass disk was detached from the silicone with a sterile scalpel blade and sterile forceps and transferred to a 15 ml polypropylene screw-cap tube containing freshly prepared 3% glutaraldehyde suspended in artificial seawater (ASW). Biofilm was fixed by incubation for 5-6 hours at 4°C and sample stored at 4°C until further processing.

DGGE. Glass slides with biofilm formation for DGGE analysis were collected on days 21 and 44 after first addition of *Artemia*. For each tank at each sampling time point, 2 microscope slides were detached from the silicone with a sterile scalpel blade and immediately frozen at –80°C until further processing.
After draining the tank water on day 44, additional samples were obtained from each tank wall by taking biofilm swabs of still moist biofilm using a sterile cotton tab. Each swab was immediately transferred to a 2 ml microcentrifuge tube containing 1 ml of ASW and vigorously moved to transfer as much biomass as possible from the cotton tab to the water. Biomass was pelleted by centrifugation at 16000 x g for 5 min (repeatedly if required). The supernatant was removed and pellets immediately frozen at –80°C until further processing.

3.1.2 Fluorescence *in situ* hybridization (FISH)

Embedding of biofilm

Fluorescent probing of biofilm samples was tested with a modified FISH protocol according to the method outlined in Daims et al. (2006), which describes a preservation of spatial biofilm structures by embedding biofilm within a protective matrix prior to FISH processing. The associated image analysis software *daime* then enables three-dimensional (3D) visualization of biofilm based on X-Y series obtained using a confocal laser scanning microscope (referred to as *3D-FISH*).

Agarose. Teflon-coated 8-well microscope slides were removed from a raceway larval rearing tank containing non-ozonated water after 16 days of biofilm build-up. The attached biofilm on the slides was fixed as described above before being dried for 15 min at 46°C. Samples were embedded by dipping several slides into 0.5% and several others into 1% hand-warm agarose, and the slides then placed horizontally on ice to harden the agarose.

Embedded biofilm was subsequently dehydrated through a 50% / 75% / 85% / 96% ethanol series for 10 min each and slides dried for 15 min at 46°C.
It was found that 0.5% agarose yielded a thin film on the surface, whereas application of 1% agarose resulted in a thicker layer on the slides that was suspected to affect microscopical analysis and/or hybridization. Therefore, hybridization was performed on 0.5% agarose treated slides only with the oligonucleotide probes according to Table 3.1 and using a standard FISH protocol (see below).

Polyacrylamide. Teflon-coated 8-well microscope slides were removed from a raceway larval rearing tank containing non-ozonated water after 16 days of biofilm build-up. The attached biofilm was fixed as described above. Each slide was dipped into PBS to remove any residual ethanol that was found to inhibit the polymerization of acrylamide (Daims et al., 2006) and dried for 15 min at 46°C. To each microscope well, 4 µl of freshly prepared 10% polyacrylamide (PAA) solution (10% (w/v) acrylamide, 0.1% (w/v) APS, 1% (v/v) TEMED) was added. A 24x60 mm cover slip, pretreated with Repel-Silane according to the manufacturer's instructions, was applied to each slide in order to minimize oxygen exposure. Polymerization was carried out for at least 20 min to ensure complete gel polymerization before the cover slip was removed. Embedded biofilm was subsequently dehydrated through a 50% / 75% / 85% / 96% ethanol series for 10 min each and slides left air-dry until the ethanol evaporated. Hybridization was performed with the oligonucleotide probes according to Table 3.1 and using a standard FISH protocol (see below).

Hybridization of biofilm

All oligonucleotide probes (Table 3.1) were purchased from Sigma (St. Louis, USA) and labelled with either the indocarbocyanine fluorochrome Cy3 (EUB338) or Cy5 (all group specific probes). Negative control reactions were performed on all replicate samples with the Cy3 labelled antisense probe Non-EUB338.
In situ hybridizations were carried out in 50 ml polypropylene screw-cap tubes each containing a tissue paper moistened with Milli-Q water (chamber). For each hybridization reaction, 8 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl pH 8.0, 0.01% SDS) containing the appropriate formamide concentration (Table 3.1) was mixed with 1 µl (50 ng μl^{-1}) of oligonucleotide probe. Both the hybridization chambers and hybridization solutions were preheated to 46°C in a hybridization oven. Each processed microscope slide was placed on a 46°C heating block and 9 µl of the appropriate hybridization mix was applied to each well. The slide was immediately inserted into the chamber and hybridization performed for 2 hours at 46°C. For each processed slide, 50 ml of 20% wash buffer (0.215 M NaCl, 20 mM Tris-HCl pH 8.0, 5 mM Na_2EDTA, 0.01% SDS) was prepared in 50 ml polypropylene

screw-cap tubes and preheated to 48°C. After hybridization, each slide was rinsed with a small amount of wash buffer, then immersed in the wash buffer for 20 min at 48°C. Each slide was rinsed with Milli-Q and air-dried on filter paper covered with aluminium foil. Each sample was mounted with 2 drops of BioMedia Gel, covered with a 24x60 mm coverslip and stored at –20°C until microscopical analysis undertaken within two days of processing. Fluorescence signals were recorded using a confocal laser scanning microscope (CLSM) at the Advanced Analytical Centre (James Cook University, Townsville). Both randomly selected 2D pictures and 3D X-Y image stacks in 0.5 µm sections were generated.

Table 3.1 Oligonucleotide FISH probes used in this study. BET42a, GAM42a, PLA886 and G123T were used in combination with equimolar amounts of their respective unlabelled competitor probes.

Probe	Specificity	Target site[a]	Sequence from 5'–3'	FA[b]	Reference
EUB338[c]	*Bacteria*	16S, 338–355	GCTGCCTCCCGTAGGAGT	20 – 40[d]	Amann et al., 1990
Non-EUB338	Complementary to EUB338	---	ACTCCTACGGGAGGCAGC	35	Wallner et al., 1993
ALF1b	α-*Proteobacteria*	16S, 19–35	CGTTCGYTCTGAGCCAG[e]	20	Manz et al., 1992
BET42a	β-*Proteobacteria*	23S, 1027–1043	GCCTTCCCACTTCGTTT	35	Manz et al., 1992
GAM42a	γ-*Proteobacteria*	23S, 1027–1043	GCCTTCCCACATCGTTT	35	Manz et al., 1992
CF319a	*Cytophaga-Flavobacterium* cluster of CFB	16S, 319–336	TGGTCCGTGTCTCAGTAC	35	Manz et al., 1992
G V	*Vibrionaceae*	16S, 822–841	AGGCCACAACCTCCAAGTAG	30	Giuliano et al., 1999
PLA886	*Planctomycetes*	16S, 886–904	GCCTTGCGACCATACTCCC	35	Neef et al., 1998
PLA46	*Planctomycetes*	16S, 46–63	GACTTGCATGCCTAATCC	35	Neef et al., 1998
G123T	*Thiothrix* spp.	16S, 697–714	CCTTCCGATCTCTACGCA	40	Kanagawa et al., 2000

[a] *Escherichia coli* numbering of rRNA position

[b] Percent formamide in hybridization buffer

[c] 1:1:1 mix of probes EUB338 (Amann et al., 1990), EUB338-II and EUB338-III (Daims et al., 1999)

[d] Percentage of formamide was dependent on the stringency of the respective group specific probe

[e] Y – variable pyrimidine base

3.1.3 Scanning electron microscopy (SEM)

Fixed biofilm samples on glass disks were dehydrated according to the method outlined in Table 3.2. Disks were gently blotted on filter paper and dried overnight inside a laminar flood hood. Disks were mounted on pin-type SEM mounts using adhesive carbon tabs. Samples were sputter coated with gold at 25 mA for 150 sec in a Balzars MFD 020 sputter coating unit and analyzed using a scanning electron microscope at 10 kV at the Advanced Analytical Centre (James Cook University, Townsville).

Table 3.2 Dehydration protocol for SEM preparation

Solution	Incubation
50 – 60 – 70 – 80 – 90% ethanol	10 min each
96% ethanol	3 x 10 min
96% ethanol:100% HMDS[a] 1:1 (v/v)	10 min
100% HMDS	3 x 10 min

[a] HMDS: Hexamethyldisilazane

3.1.4 Denaturing gradient gel electrophoresis (DGGE)

DNA extraction

The back surface of microscope slides was scraped with a sterile scalpel blade to remove attached biofilm. Biomass was transferred with a sterile toothpick to a 2 ml microcentrifuge tube containing 440 µl lysis buffer (40 mM Na_2EDTA, 50 mM Tris-HCl, 0.75 M sucrose, pH 8.3) and acid-washed glass beads. Lysis buffer and glass beads were directly added to pelleted biofilm from biofilm swabs already in 2 ml microcentrifuge tubes (see above).

To achieve cell lysis, bead beating was performed for 30 sec, 1 mg ml^{-1} lysozyme added and sample incubated for 1 h at 37°C. 1% SDS and 0.2 mg ml^{-1} of Proteinase K were added and sample incubated for a further 1 h. To inactivate DNases and RNases, the sample was boiled for 1 min and cooled to room temperature. Proteins were precipitated by phenol-chloroform-isoamyl alcohol (25:24:1, pH 8.0) extraction and centrifugation for 10 min at 16000 x g. The aqueous layer was transferred to a fresh 1.5 ml polypropylene reaction tube and the extraction repeated as described above. An equal volume of chloroform-isoamyl alcohol (24:1) was added, centrifuged for 10 min at 16000 x g and the upper aqueous layer transferred to a fresh 2.0 ml polypropylene reaction tube.

Precipitation of cellular DNA was achieved by addition of 2.5 volumes of 96% ethanol and 0.1 volumes of 3 M sodium acetate and incubation at –20°C for at least 2 hours. DNA was pelleted by centrifugation for 30 min at 16000 x g and 4°C. The pellets were washed twice

with 70% ethanol by repeated centrifugation for 10 min at 16000 x g and 4°C. The supernatant was removed and DNA pellets rehydrated in 20 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 4°C overnight.

Purification of DNA extracts

The quality and quantity of the extracted DNA was analyzed by agarose gel electrophoresis. For each DNA extract, 5 µl was mixed with 6x gel loading buffer and separated on a 0.8% agarose gel in TAE buffer (40 mM Tris-HCl, 20 mM sodium acetate, 1 mM Na_2EDTA) stained with ethidium bromide (0.5 µg ml^{-1} agarose). A 100 bp ladder was used as a size standard for DNA fragments. Under low intensity UV light, gel parts displaying fragments >2 kb were excised with a sterile scalpel blade and transferred to pre-weighed 1.5 ml polypropylene reaction tubes. Gel slices were weighed, with the weight corresponding to 1 volume in the following steps.

Purification was performed using the QIAquick Gel Extraction Kit (Qiagen). For each gel slice, 3 volumes of buffer QG were added to 1 volume of gel. The tube was incubated for 10 min at 50°C and repeatedly vortexed to completely dissolve the agarose. A yellow colour of the extract confirmed an appropriate pH. One gel volume of isopropanol was added and the sample mixed. A QIAquick column was placed in a 1.5 ml collection tube, the sample applied to the column and centrifuged for 1 min at 16000 x g. The flow-through was discarded and column placed back into the same collection tube. 500 µl of buffer QG was applied to the column, the centrifugation repeated and the flow-through discarded. 750 µl of buffer PE was applied to the column, the sample left for 5 min and then centrifuged for 1 min at 16000 x g. The flow-through was discarded, the sample centrifuged for 1 min at 16000 x g and the column placed in a fresh 1.5 ml polypropylene reaction tube. DNA was eluted by application of 30 µl of buffer EB and centrifugation for 1 min at 16000 x g.

Polymerase Chain Reaction (PCR)

PCR amplification of (purified) 16S rDNA gene fragments was performed with primers 1055F (5'-ATGGCTGTCGTCAGCT-3') and 1392R-GC (5'-GCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCACGGGCGGTGTGTAC-3') (Ferris et al., 1996). The underlined region of primer 1392R-GC defines the primer sequence, which is attached to a 40-bp rich GC clamp at the 5' end (Muyzer et al., 1996). These primers amplify a 323-bp section of the 16S rDNA gene of members of the domain *Bacteria*, including the highly variable V9 region (Gray et al., 1984; Ferris et al., 1996).

The PCR reactions were carried out in 0.2 ml tubes with a final volume of 50 µl. Each reaction mixture contained 1x PCR buffer, additional 1.5 mM $MgCl_2$, 100 µM each dNTP, 0.5 pmol each primer, 1.25 U *Taq* polymerase and 5 µl template in sterile Milli-Q water.
The amplification was performed using a touchdown profile. An initial denaturation at 94°C for 3 min was followed by 10 cycles at 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min. Primer annealing temperature of the reaction was decreased 1°C per cycle.
This was followed by 20 additional cycles of 94°C for 1 min, 43°C for 1 min and 72°C for 1 min, with a final elongation at 72°C for 10 min. PCR products were separated by electrophoresis on a 0.8% agarose gel as described above and photographed under UV transillumination.

Denaturing gradient gel electrophoresis

To ensure reproducible separation of PCR products, each product was separated in two parallel wells. Dependent on the quantity of amplified DNA, 8-12 µl of each PCR product was mixed with gel loading dye in a 3:1 ratio and separated on a 6.5% acrylamide gel that incorporated a linear 45–65% denaturing gradient of urea and formamide. Electrophoresis was performed at 60°C and 75 V for 16-18 h in TAE buffer using a DCode Universal Mutation Detection DGGE System (Bio-Rad). After electrophoresis, nucleic acids were stained with 1x SYBR Gold in TAE for 30 min. The gel was rinsed with TAE and the banding pattern immediately photographed using a fluoroimager.
Intense bands were excised under low intensity UV light using sterile pipette tips and placed in 100 µl of sterile Milli-Q water overnight at 4°C to allow DNA to elute passively. The eluate was used as template for reamplification in a touchdown PCR reaction as described above, but using Hot Star *Taq* polymerase. An initial heat activation step at 95°C for 15 min was necessary, before continuing with the amplification profile as described above. Products from this reamplification were separated on a DGGE gel as described above, to ensure purity and correct mobility of bands within the gel.

Purification of PCR products

PCR products from reamplified band eluates were purified using the QIAquick PCR Purification Kit (Qiagen). For each PCR product, 35 µl was mixed with 175 µl of buffer PB and applied to a QIAquick column placed in a 2 ml collection tube. Sample was centrifuged for 1 min at 16000 x g, the flow-through discarded and column placed back into the collection tube. 750 µl of buffer PE was added, sample centrifuged for 1 min at 16000 x g and the flow-through discarded. Column was centrifuged for an additional 1 min at 16000 x g, then placed

into a fresh 1.5 ml polypropylene reaction tube. 50 µl of sterile Milli-Q was added to the centre of the column membrane and DNA eluted by centrifugation for 1 min at 16000 x g.

Sequencing and phylogenetic analysis

Purified PCR products were dried using a vacuum concentrator centrifuge and sent to Macrogen Ltd. (Seoul, South Korea) for sequencing. Products were sequenced with primer 1055F using an ABI3730XL automated sequencer. Chromatograms were checked using the Chromas software (Technelysium Software Pty Ltd.) for quality of the sequence result. Sequence data was aligned to the closest relative using the BLAST database algorithm (Altschul et al., 1997) with gaps not taken into account.

3.2 Artemia *experiments*

3.2.1 Application of antibiotics

Experimental setup

Artemia were on-grown from cysts as described in 3.1.1. For each antibiotic treatment, triplicate 50 ml polypropylene screw-cap tubes containing 30 ml of an *Artemia* culture aliquot were immediately aerated by application of a previously sterilized (overnight in 0.1 ppm chlorinated freshwater) plastic tubing system. Antibiotics were immediately added to the culture tubes with final concentrations of (i) 10 ppm streptomycin, (ii) 5 ppm oxolinic acid, (iii) 10 ppm tetracycline, and (iv) 25 ppm erythromycin, 25 ppm oxytetracycline, 10 ppm streptomycin, respectively.

For each sampling time point, five *Artemia* from each parallel were pooled in a 1.5 ml polypropylene reaction tube containing 150 µl of 0.22 µm filtered ASW. Pooled specimens were homogenized using a motor pellet pestle and 850 µl of 0.22 µm filtered ASW was added to obtain a final volume of 1 ml homogenate. A 1:10 dilution of this homogenate was prepared in 0.22 µm filtered ASW. Each 100 µl of homogenates was spread-plated on thiosulfate-citrate-bile-sucrose (TCBS) agar in triplicate. TCBS was originally developed for recovery of *Vibrio cholerae*, but is routinely used as a selective media for general marine *Vibrio* and *Aeromonas* organisms (Bolinches et al., 1988). Plates were incubated at room temperature.

Experiment 1. *Artemia* used in the experiment were on-grown for 7 days. All the antibiotics treatments outlined above, (i) to (iv), were tested. *Artemia* were sampled 2 h and 24 h after addition of the antibiotics. Samples were homogenized and spread-plated on TCBS as described above.

Experiment 2. The same hatch of *Artemia* as in Experiment 1 was used for this experiment, with the animals being on-grown for 3 additional days. Based on Experiment 1, the antibiotic treatment (iv) consisting of the mixture of erythromycin (25 ppm), oxytetracycline (25 ppm) and streptomycin (10 ppm final concentration) was selected for further studies. *Artemia* were sampled 1, 2, 4, 6 and 24 h after addition of the antibiotic mixture. Samples were homogenized and spread-plated both on TCBS and on Marine Agar 2216 (MA) as described above.

3.2.2 Application of *Sanolife Mic*

Sanolife Mic® Microbial Water Conditioner (INVE, Phichit, Thailand) is a commercially available probiotic product and was developed for improving microbial management in shrimp aquaculture. Sanolife contains three different *Bacillus* strains (*B. subtilis*, *B. licheniformis* and *B. pumilus)* at a concentration of at least 5 x 10^{10} CFU g^{-1}. The product is described to inhibit growth of several luminescent, pathogenic *Vibrio* strains commonly found in shrimp aquaculture facilities. The trial performed in this study was the first implementation of this product at AIMS. It evaluated the applicability and effectiveness of the product when added to the *Artemia* grow-out system.

Experimental setup

Artemia were on-grown from cysts as described in 3.1.1 and transferred to a 800 litre tank. Sanolife product (1 g) was added and the culture left for 2 days, after which the water flow was started. *Artemia* were enriched with the standard algae mixture for 8 more days under daily administration of 5 g of germinated product, prepared according to the manufacturer's instructions. Samples were taken immediately after starting the water flow (day 2 *Artemia*) and continued every second day (days 4, 6, 8 and 10 of *Artemia* age). For each sampling time point, 30 ml of *Artemia* culture was transferred into a 50 ml polypropylene screw-cap tube and the concentration of nauplii per ml determined. 200 µl of the culture aliquot was homogenized using a motor pellet pestle. 800 µl of 0.22 µm ASW was added to obtain a finale volume of 1 ml homogenate. A 1:10 dilution of this homogenate was prepared in 0.22 µm filtered ASW. Each 100 µl of homogenates was spread-plated on TCBS in triplicate and plates were incubated at room temperature.

3.3 Phyllosoma experiments

3.3.1 Phyllosoma collection

Captive-reared larvae were collected by Michael R. Hall (AIMS) and co-workers during a rearing trial within non-ozonated seawater in March 2005. Phyllosoma were randomly sampled from a 5,000 litre larval rearing tank containing several tens of thousands of phyllosoma. Sampling was performed within days 1-9 of the P1-P2 stage and days 1-13 of the P3-P4 stage, respectively.

Wild larvae for microscopical analyses were collected by Michael R. Hall (AIMS) and co-workers in April 2006 at Osprey Reef (14°S, 146°E) belonging to the outer reefs of the Coral Sea, off the Australian East coast (Figure 3.2). Wild larvae for isolation of potential bacterial probiotics were sampled at the same location in December 2006. During both sampling trips, phyllosoma were collected by overnight Isaac-Kidd trawling at various depths from 100 m to 10 m and a cruising speed of 2-3 knots.

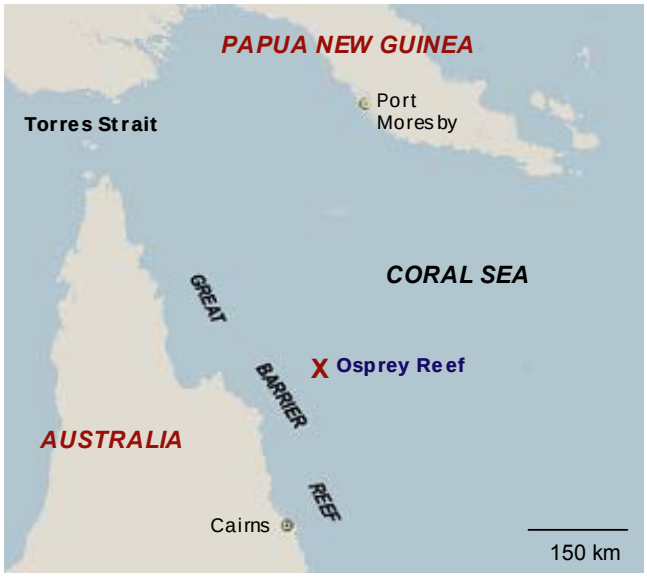


Figure 3.2 Location of Osprey Reef

The genus and larval stage of each collected specimen was determined using a dichotomous key (Baisre, 1994). For SEM and FISH analyses, phyllosoma were fixed according to the protocols described in 3.1.1, respectively. Phyllosoma for histological preparations were fixed in Davidson's fixative (30% ethanol / 22 % formalin / 11% glacial acetic acid) for 24 h and transferred to 70% ethanol for storage. Phyllosoma for the isolation of potential probiotics were processed immediately following their collection.

3.3.2 Microscopical analysis

Scanning electron microscopy. Fixed phyllosoma were dehydrated according to the method outlined in Table 3.2 and mounted on pin-type SEM mounts with their ventral side up. Specimens were sputter coated with platinum (reared) or gold (wild larvae) at 25 mA for 150 sec in a Balzars MFD 020 sputter coating unit. Phyllosoma were analyzed using a scanning electron microscope at 10 kV at the Advanced Analytical Centre (James Cook University, Townsville).

Histology. Fixed phyllosoma were dehydrated according to the method outlined in Table 3.3 and embedded in fluid paraffin. Using a microtome, hardened paraffin blocks were cut in sections of 5 µm that were placed onto microscope slides. Slides were incubated at 60°C for 3 h for fixation of sections. Paraffin was removed in xylene for 3 min and sample rehydrated through a 96% / 90% / 80% / 70% ethanol series to water (5 min each). Sections were stained within Mayer's Haematoxylin (8 min), blued with Scott's tap water substitute (30 sec) and counterstained with Young's Eosin (3 min). Samples were dehydrated through a 70% / 80% / 90% / 96% ethanol series (5 min each). Sections were cleared with xylene, mounted with DPX and a 24x60 mm coverslip and analyzed by light microscopy.

Table 3.3 Dehydration protocol for FISH and histology preparation

Solution	Incubation
70% ethanol	2 x 60 min
80% ethanol	60 min
90% ethanol	60 min
96% ethanol	3 x 60 min
Xylene	2 x 60 min
Paraffin	2 x 120 min (at 60°C; under vacuum)

Fluorescence *in situ* hybridization. Fixed phyllosoma were dehydrated according to the method outlined in Table 3.3, and embedded in fluid paraffin and sectioned as described above. Prior to hybridization, paraffin was removed by incubation in xylene for 20 min and sections dehydrated through a 50% / 75% / 85% / 96% ethanol series for 10 min each. Each section was surrounded with a water-repellent pen and covered with 18 µl of hybridization buffer that was previously mixed with 1 µl of fluorescent-labelled probe (Table 3.1). Hybridization and washing steps were carried out as described in 3.1.2. Fluorescence signals were recorded using a confocal laser scanning microscope at the Advanced

Analytical Centre (James Cook University, Townsville) and randomly selected 2D pictures generated.

3.3.3 Isolation of potential probiotics from wild phyllosoma

Selected phyllosoma were homogenized in 5 ml of 0.1 µm filtered ASW. Each 100 µl of undiluted, 1:10 and 1:100 diluted homogenate was spread-plated on MA in triplicate and plates were incubated at room temperature.

Candidate strains were selected on two growth characteristics; the first being slower-growing strains that appeared after more than 48 h of incubation. A corresponding colony colour in the range of red-orange-brown was regarded as a second positive indication, but this criterion was not always applicable.
Candidate strains were picked and restreaked on fresh MA plates to obtain pure cultures, which were maintained on MA until further analysis. Liquid cultures were grown from all isolates for 48 h in Marine Broth 2216 (MB) at 28°C and 160 rpm on a rotary shaker. In addition, glycerol stock cultures were prepared by combining 700 µl of liquid culture and 300 µl glycerol before thorough vortexing and immediate freezing at –80°C.

a. Probiotic activity assays

Potential probiotic strains were tested for probiotic activity against a *Vibrio harveyi* strain (C071). This strain was previously isolated from biofilm during a phyllosoma mass mortality event, and yielded increased larval mortality in small-scale challenge experiments (Bourne et al., 2006).
Besides the bacterial isolates from wild phyllosoma, four previously isolated strains were included in the screening. These included two *Roseobacter* strains (K2 and K19), previously isolated from the water column of *Panulirus ornatus* rearing systems; a *Vibrio alginolyticus* strain (C009), previously isolated from algae-enriched *Artemia*; and a *Sulfitobacter* strain (RR67), previously isolated from wild *Panulirus ornatus* phyllosoma. The strain labels refer to the original isolate labelling and do not incorporate taxonomical classification (Lone Høj, Matthew Payne; personal communication). In addition, the commercial probiotic product Sanolife Mic, incorporating three *Bacillus* strains, was also included in the probiotic activity assays. All candidate strains and the target strain were inoculated in MB and grown for 48 h at 28°C and 160 rpm on a rotary shaker.

Well diffusion agar assay. A well diffusion agar assay was performed as outlined by Hjelm et al. (2006). For the target strain, 60 µl and 300 µl of liquid culture was mixed into 10 ml of melted (43-44°C) M9GC-3 agar (M9 basic medium; supplemented with 0.4% glucose, 0.3% casamino acids, 3% NaCl and 1.2% bacteriological agar). Inoculated agar was poured into Petri dishes. After drying of plates, wells (Ø 3 mm) were punched into the agar and each 10 µl of a liquid candidate culture was applied to a well, respectively. Plates were incubated at 28°C and regularly monitored over a period of 2 weeks for candidate strain growth and the formation of inhibition zones.

Colony assay. Glycerol stocks from all isolates were used to inoculate candidate strains on MA plates, which were incubated for 48 h at room temperature.
M9GC-3 agar incorporating the target strain was prepared as described above. A single colony from each candidate strain was picked and streaked onto the agar using sterile toothpicks. Plates were incubated at 28°C and regularly monitored over a period of 2 weeks for candidate strain growth and the formation of inhibition zones.

Cell-free culture supernatant assay. Liquid cultures of all isolates, and M9GC-3 agar incorporating the target strain were prepared as described above. Cell-free culture supernatants were prepared by application of 250 µl of each liquid candidate culture to 96-well filter plates and centrifugation for 3 min at 1500 rpm. Antibiotic assay disks were dipped into the extracts and placed onto the agar. Plates were incubated at 28°C and regularly monitored over a period of 2 weeks for the formation of inhibition zones.

b. Taxonomical identification of strains

Extraction of DNA

Glycerol stocks were used to inoculate candidate strains in 5 ml MB and grown for 48 h at 28°C and 160 rpm on a rotary shaker. Extraction of cellular DNA was performed using the Wizard Genomic DNA Purification Kit (Promega) according to the instructions for Gram-negative bacteria.
For each candidate strain, 1 ml of liquid culture was transferred to a 1.5 ml polypropylene reaction tube and centrifuged for 2 min at 16000 x g. The supernatant was removed, 600 µl of Nuclei Lysis Solution added and sample gently mixed. Sample was incubated at 80°C for 5 min and cooled to room temperature. 6 µl of RNase solution (10 mg μl^{-1}) was added and sample incubated for 30 min at 37°C, then cooled to room temperature. 200 µl of Protein Precipitation Solution was added, sample vortexed and incubated on ice for 5 min before centrifuged for 3 min at 16000 x g and 4°C. The supernatant was transferred to a fresh 1.5

ml polypropylene reaction tube containing 600 µl isopropanol, the sample mixed and centrifuged as above. The supernatant was removed, 600 µl of 70% ethanol added and sample centrifuged as above. Ethanol was aspirated and pellet air-dried inside a laminar flood hood. DNA pellets were rehydrated in 100 µl sterile Milli-Q water at 4°C overnight. The quality and quantity of extracted DNA was checked by agarose gel electrophoresis as described above.

For isolates where this extraction protocol failed, the extraction was repeated using the instructions for Gram-positive bacteria, which included an initial lysis step. 1 ml of each liquid culture was centrifuged as above and pellets resuspended in 480 µl of 50 mM Na_2EDTA. For each sample, 120 µl of lysozyme (10 mg ml^{-1}) was added and sample incubated at 37°C for 1 h. After centrifugation for 2 min at 16000 x g, the supernatant was removed and 600 µl of Nuclei Lysis Solution added. Subsequent extraction was performed according to above instructions. The quality and quantity of extracted DNA was checked by agarose gel electrophoresis as described above.

Sequencing and phylogenetic analysis

Partial and complete 16S-rDNA sequences of bacterial isolates were obtained using the universal primers 27F (5'-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CACGGATCCTACGGGTACCTTGTTACGACTT-3') (Lane, 1990).
PCR was carried out in 0.2 ml tubes with a final volume of 30 µl. Each reaction mixture contained 1x PCR buffer, 100 µM each dNTP, 0.5 pmol each primer, 1.25 U *Taq* polymerase and 1 µl template in sterile Milli-Q water. For extracts where both the Gram-negative and Gram-positive extraction protocol failed, bacterial cell material from cultures on MA was suspended in sterile Milli-Q and this suspension was directly used as template.
The amplification profile consisted of an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 45 sec, 54°C for 45 sec and 72°C for 2 min. A final elongation was carried out for 7 min at 72°C. PCR products were separated by electrophoresis on a 0.8% agarose gel as described above.

16S-PCR products were dried using a vacuum concentrator centrifuge and sent to Macrogen Ltd. (Seoul, South Korea) for purification and sequencing with primer 27F or both 27F/1492R using an ABI3730XL automated sequencer. Chromatograms were checked using the Chromas software (Technelysium Software Pty Ltd.) for quality of the sequence result. Sequence data was aligned to the closest relative using the BLAST database algorithm (Altschul et al., 1997) with gaps not taken into account.

4. Results

4.1 Effect of seawater ozonation on development and composition of biofilm within larval rearing tanks

4.1.1 Fluorescence *in situ* hybridization

a. Effectiveness of biofilm embedding

Fluorescent probing of biofilm samples was tested with a modified FISH protocol according to the method outlined in Daims et al. (2006), which describes a preservation of biofilm 3D structures by embedding within a protective matrix (*3D-FISH*). This protocol was specifically tailored to preservation and examination of solubilized biofilm samples.

In this study, it was determined if embedding was also applicable to surface-bound biofilms formed on microscope slides. Tested procedures included embedding within 10% polyacrylamide (Daims et al., 2006) and within 0.5% and 1% agarose as suggested by Holger Daims (personal communication). Images from the respective X-Y series of each embedding method were compared to those from non-embedded samples.

Embedding in agarose or polyacrylamide did not interfere with hybridization stringency or signal strength. Increased background fluorescence was however observed with some samples, which was believed to be attributed to the embedding matrix. The application of the polyacrylamide embedding method proved to be complicated due to the very small amounts of solution required for individual microscope slide wells. Furthermore, it was not certain if removal of the coverslip would disturb the biofilm structure.
True 3D patterns within biofilm samples were seldom observed, and when a clear 3D structure was present, embedding did not provide improvement of its preservation. This was demonstrated by X-Y series of non-embedded samples, which confirmed that three-dimensional structures were intact and comparable to those observed after embedding. This was most likely due to the biofilm being firmly attached to the microscope slides and preserved by fixation, ensuring that its 3D structure was not affected during FISH processing. All biofilm samples collected during this study were subsequently processed according to a standard FISH procedure without prior embedding. Nevertheless, X-Y images stacks were taken and analyzed using the *daime* software, as this software allowed excellent visualization of the biofilm 3D structure *in situ*.

b. Course of biofilm development

Biofilm development in non-ozonated and ozonated water within larval rearing tanks was similar during the first three days of the experimental period. While no bacteria were detected on the surface on day 0 slides as expected, bacterial settlement was first observed on day 1 samples. Initial bacterial attachment appeared to be close to particulate matter also attached to the slide surface. By day 3, bacteria started to accumulate, with most cells appearing in smaller patches rather than being evenly distributed across the surface.

A distinct increase of bacterial proliferation was detected on day 5 for both water types, and enumeration of cells was commenced at this sampling time point. Cell coverage on the slides became evenly distributed at this time point, with colonization observed on previously blank surface areas. Despite this confluent colonization, areas with higher and lower rates of bacterial attachment to the slides were still clearly distinguished. Interestingly, the increase in cell numbers was greater for slides placed in ozonated water, with biofilm density on day 5 being approximately twice that of the biofilm density on slides in non-ozonated water.

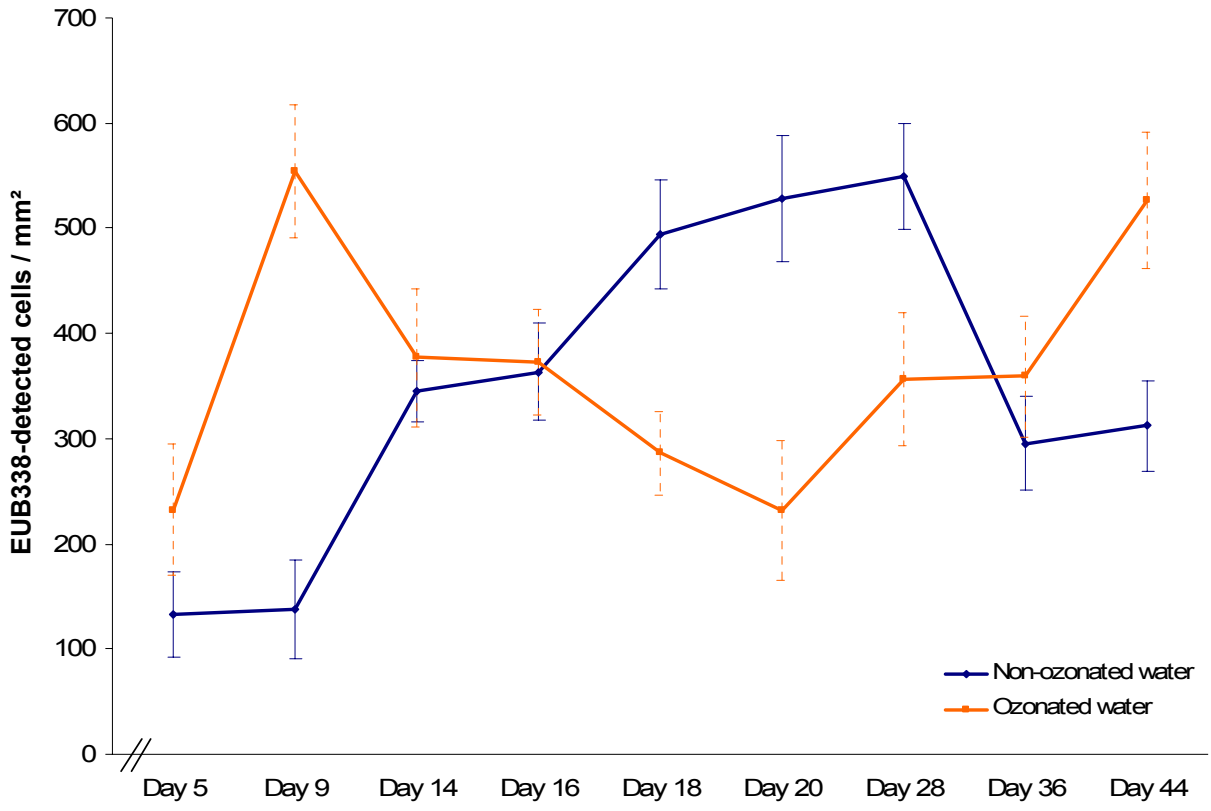


Figure 4.1 Development of larval rearing tank biofilms in non-ozonated and ozonated water, as detected with EUB338. Numbers describe cells per mm². Mean and standard deviation were calculated from direct cell counts on 2-3 randomly selected FISH confocal micrographs and were corrected by subtraction of Non-EUB338 counts.

Non-ozonated water. Filamentous bacteria were observed for the first time as members of the biofilm community on day 5. From day 5 to day 9, direct bacterial counts demonstrated no further increase in bacterial numbers in the biofilm (Figure 4.1). Between days 9 and 14 however, the bacterial density increased by a factor of approximately three and filamentous bacteria became a dominant part of the biofilm community. Circularly shaped structures were regularly observed (Figure 4.2 a), and protozoans occasionally seen (Figure 4.2 b). Highest bacterial counts occurred between days 20 and 28 with the biofilm dominated by single, and occasionally clustered, rod-shaped cells spreading across most of the surface.

Between sampling days 28 and 36, the bacterial counts dropped substantially, indicative of sloughing of the biofilm from the surface into the water column. The number of filamentous bacteria within the biofilm community was also reduced during this time period. On day 44, bacterial density marginally increased again. Upon draining the culture water at the end of the experimental period, the developed biofilm on the tank's walls was visible, and a smell of sulfide was observed.

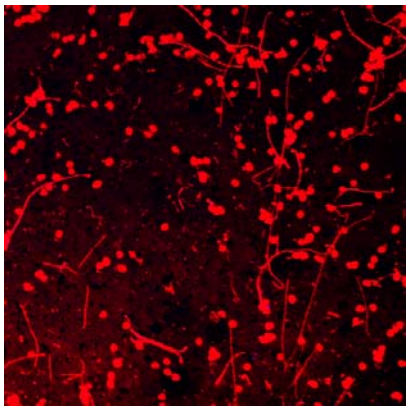


Figure 4.2 (a) Circular structures

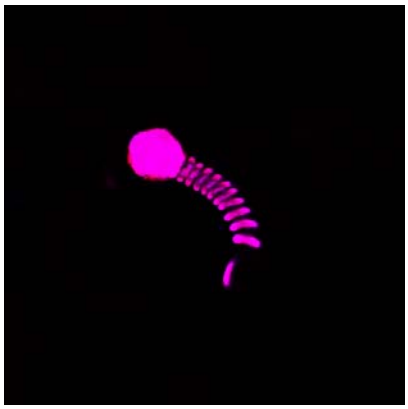


Figure 4.2 (b) Large protozoan

Ozonated water. In contrast to the biofilm from non-ozonated water, ozonated water biofilm samples demonstrated a peak in bacterial numbers on day 9, followed by a decline of bacterial density over the next ten days to a minimum of bacterial colonization on day 20. The sloughing of bacteria from the surface was accompanied by observations of a Cy3-fluorescent material partially covering the surface, prominently on days 14 and 16. Filamentous bacteria, which were virtually absent from the microbiota early in the biofilm development, became established on day 14. Circularly shaped organisms, as observed within non-ozonated water, were repeatedly present as well. Higher bacterial densities were observed in association with areas of high particulate matter attachment, and filamentous bacteria were occasionally interweaved with this material. As the experiment proceeded, the surface-covering matter tended to dissipate and subsequently disappear from the surface. On days 18 and 20, only scattered patches of matter were observed and large areas of the slides were clear of biofilm attachment. The biofilm present was predominantly characterized

by long filaments that occasionally featured a three-dimensional arrangement. Between days 28 to 44, the biofilm re-established in those areas where it had previously sloughed, and the bacterial density on day 44 was approximating that of day 9.

c. Bacterial community structure

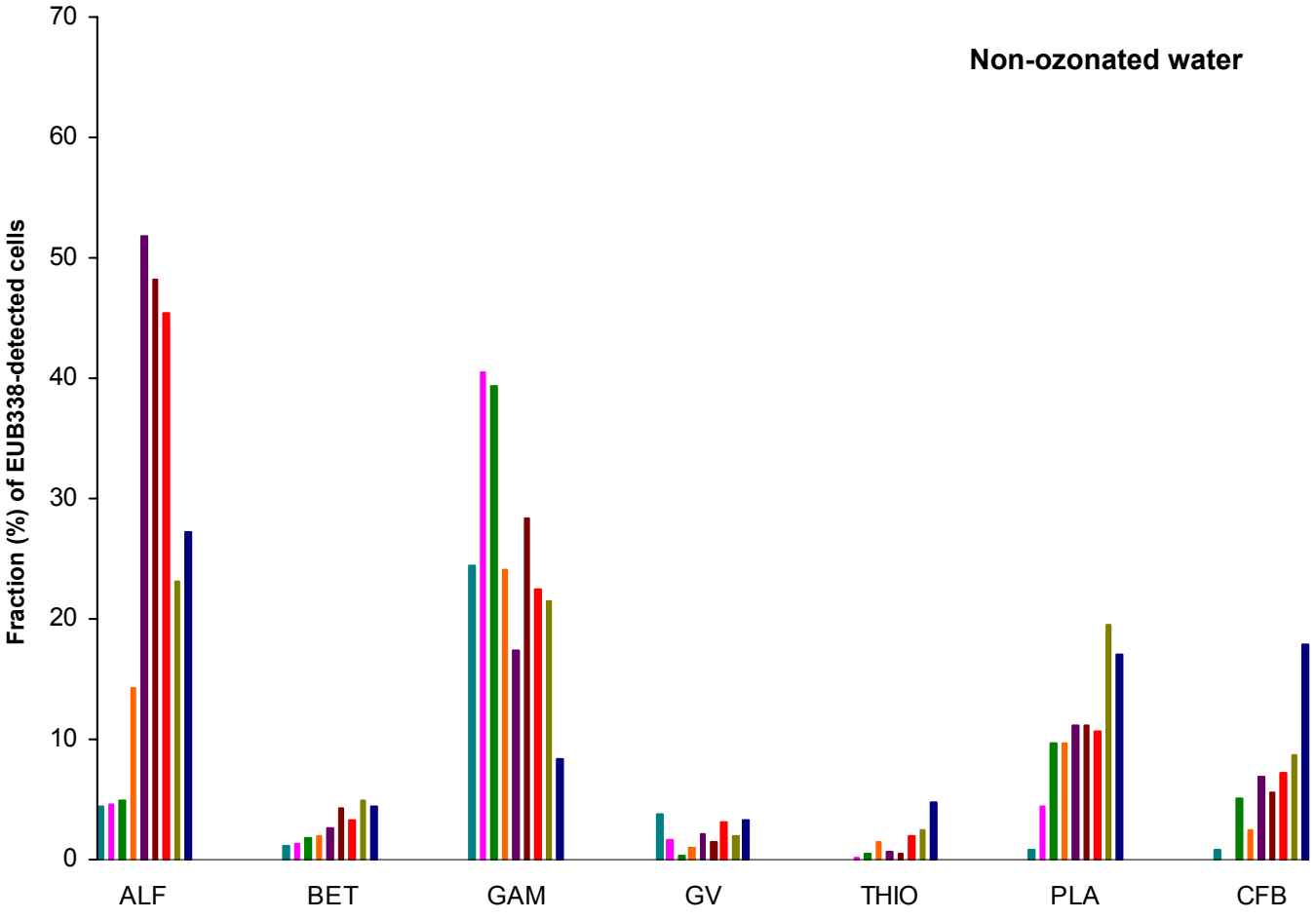
Table 4.1 and Figure 4.3 display the contribution of individual bacterial groups to the bacterial community as a fraction of EUB338-detected cells (see Figure 4.1).

Table 4.1 Fraction of bacterial groups in relation to the EUB338 count. ALF: α-*Proteobacteria*; BET: β-*Proteobacteria*; GAM: γ-*Proteobacteria*; GV: *Vibrionaceae*; THIO: *Thiothrix*; PLA: *Planctomycetes*; CFB: *Cytophaga-Flavobacterium* cluster of the CFB phylum. Mean and standard deviation (SD) were calculated from cell counts on each three randomly selected FISH confocal micrographs per tank.

NON-OZONATED WATER	**Fraction (%) of EUB338-detected cells (Mean ± SD)**						
	ALF	**BET**	**GAM**	**GV**	**THIO**	**PLA**	**CFB**
Day 5	4.4 ± 0.6	1.1 ± 0.7	24.5 ± 2.8	3.7[a]	0.0	0.9 ± 1.4	0.7 ± 0.5
Day 9	4.6 ± 3.6	1.4 ± 0.0	40.5 ± 5.1	1.7 ± 1.1	0.2 ± 0.0	4.5 ± 1.5	0.0[a]
Day 14	5.0 ± 0.4	1.8 ± 0.1	39.4 ± 3.6	0.3 ± 0.2	0.5 ± 0.1	9.7 ± 3.1	5.0 ± 2.6
Day 16	14.3 ± 3.3	1.9 ± 1.6	24.1 ± 2.7	0.9 ± 0.6	1.5 ± 2.0	9.7 ± 1.2	2.4 ± 2.0
Day 18	51.9 ± 4.8	2.6 ± 0.6	17.3 ± 2.7	2.1 ± 1.8	0.6 ± 0.1	11.2 ± 1.2	6.8 ± 0.9
Day 20	48.1 ± 3.6	4.3 ± 0.5	28.3 ± 2.6	1.5 ± 0.5	0.5 ± 0.5	11.2 ± 1.7	5.5 ± 0.8
Day 28	45.1 ± 5.4	3.3 ± 0.8	22.5 ± 0.4	3.2 ± 1.5	1.9 ± 1.0	10.7 ± 0.4	7.2 ± 4.6
Day 36	23.1 ± 0.3	4.9[a]	21.5 ± 1.2	2.0 ± 1.2	2.7 ± 0.7	19.5[a]	8.7 ± 2.5
Day 44	27.2 ± 2.9	4.3 ± 3.6	8.4 ± 0.8	3.2 ± 1.3	4.8± 2.0	17.0 ± 2.6	17.8 ± 2.8

OZONATED WATER	**ALF**	**BET**	**GAM**	**GV**	**THIO**	**PLA**	**CFB**
Day 5	12.3[a]	11.1 ± 5.2	21.6 ± 3.5	2.2[a]	1.3[a]	9.0 ± 1.4	14.8[a]
Day 9	9.2 ± 6.1	7.5 ± 5.7	43.4 ± 5.3	0.1 ± 0.1	0.3[a]	4.8 ± 6.4	15.3[a]
Day 14	7.6 ± 3.8	1.2 ± 0.1	62.5 ± 3.9	1.9 ± 2.2	0.3 ± 0.3	5.6[a]	13.0 ± 6.2
Day 16	7.0 ± 4.6	1.1 ± 1.3	60.3[a]	0.9[a]	0.9 ± 0.6	0.5[a]	9.7 ± 4.0
Day 18	7.1 ± 1.9	2.5 ± 1.7	60.0 ± 2.1	2.6 ± 3.8	0.9 ± 3.3	2.6 ± 1.2	7.7 ± 5.9
Day 20	6.8 ± 4.0	2.2 ± 0.9	64.8 ± 8.4	1.2[a]	1.4 ± 0.3	4.2 ± 3.4	7.3 ± 0.7
Day 28	23.6 ± 1.6	0.7 ± 0.2	34.0 ± 3.5	2.5 ± 1.4	2.5 ± 1.7	10.2 ± 2.9	9.3 ± 1.7
Day 36	36.9 ± 4.7	1.1 ± 0.2	37.5 ± 6.6	2.9 ± 1.5	2.5 ± 2.6	7.7 ± 3.2	7.6 ± 4.7
Day 44	21.3 ± 4.1	1.0 ± 0.4	51.3 ± 4.6	0.9 ± 0.4	2.8 ± 1.2	5.2 ± 0.3	7.0 ± 4.5

[a] No statistical analysis possible.


Non-ozonated water
Fraction (%) of EUB338-detected cells
70
60
50
40
30
20
10
0
ALF
BET
GAM
GV
THIO
PLA
CFB

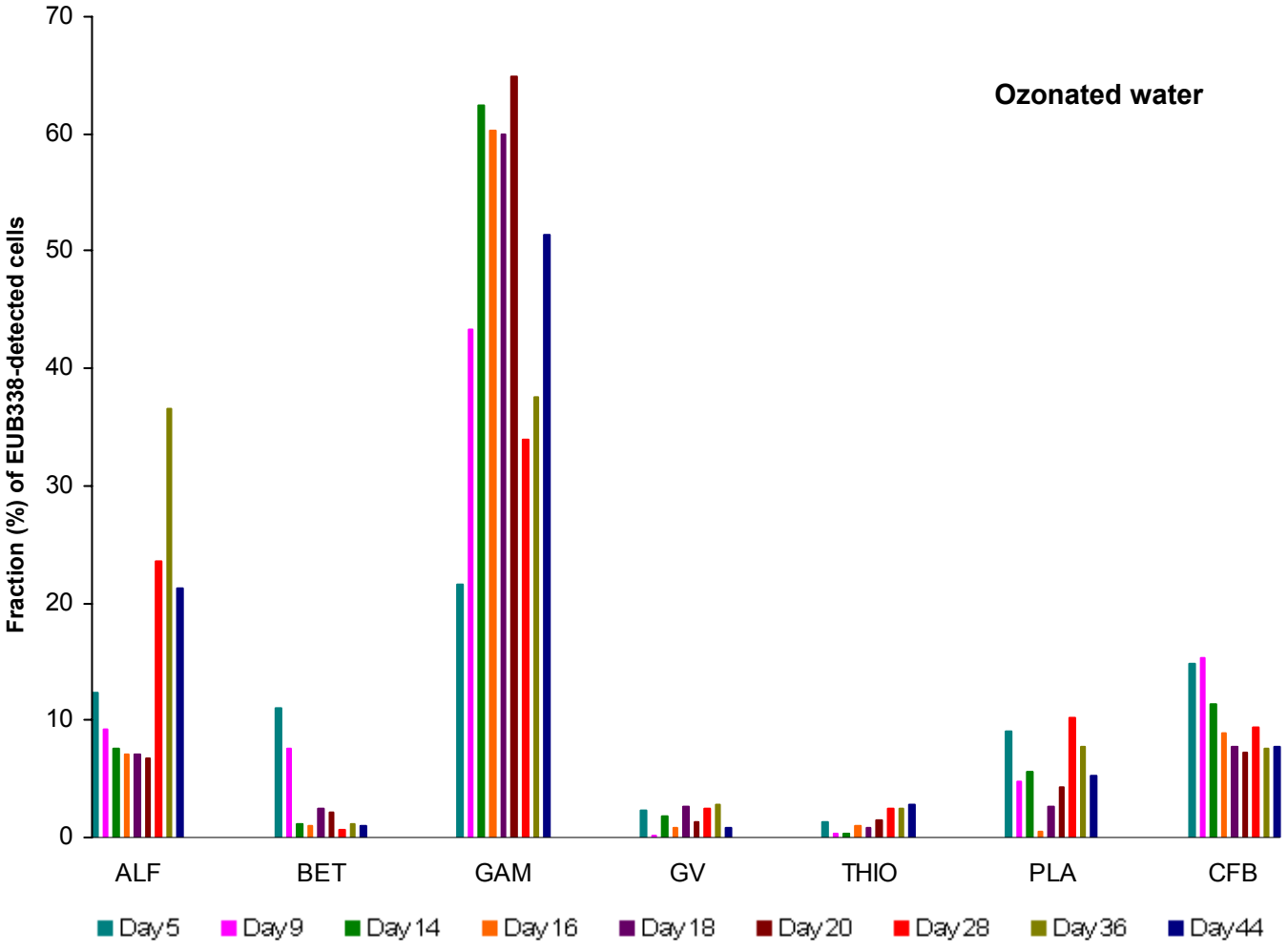

Ozonated water
Fraction (%) of EUB338-detected cells
70
60
50
40
30
20
10
0
ALF
BET
GAM
GV
THIO
PLA
CFB
Day 5
Day 9
Day 14
Day 16
Day 18
Day 20
Day 28
Day 36
Day 44

Figure 4.3 Fraction of bacterial groups of EUB338-detected *Bacteria* within larval rearing tank biofilms in non-ozonated and ozonated water during a period of 44 days, as determined by fluorescence *in situ* hybridization. ALF: α-*Proteobacteria*; BET: β-*Proteobacteria*; GAM: γ-*Proteobacteria*; GV: *Vibrionaceae*; THIO: *Thiothrix*; PLA: *Planctomycetes*; CFB: *Cytophaga-Flavobacterium* cluster of the CFB phylum.

Non-ozonated water. For the biofilm that developed within non-ozonated water, the percentage of each bacterial group increased over time. Exceptions were γ-*Proteobacteria,* whose initial dominance was reduced during the experimental period; and *Vibrionaceae*, which did not show a temporal trend in proliferation (Figure 4.3). The contribution of *Vibrionaceae* remained <5% during the entire biofilm development.

Between days 9-14, γ-*Proteobacteria* constituted approximately 40% of the EUB338-detected *Bacteria*. The relative contribution of this subclass subsequently decreased. By day 18, its contribution was reduced to below 20%, which coincided with a diversification of the bacterial community. Although the γ-*proteobacterial* contribution increased between days 20-36, a significant decrease was observed at the end of the experimental period with a contribution of only 8% to the bacterial biofilm population on day 44. Nevertheless, γ-*Proteobacteria* comprised the majority of filamentous bacteria during the entire experimental period.

Despite the diversification of the community structure, α-*Proteobacteria* emerged as the most dominant represented group. This subclass constituted approximately 50% of the EUB338 positive cells between days 18-28, with a maximum recorded on day 18. The α-*proteobacterial* population particularly comprised single-layer rod-shaped cells with an occasional clustered appearance (see 4.1 b) and determined the visual appearance of middle-age biofilm samples. On day 36, a reduction in the α-*proteobacterial* population was observed with cells decreasing from 45% to 23% of the EUB338-detected cells.

A distinct increase of both *Planctomycetes* and *Cytophaga-Flavobacteria* (CFB) bacterial groups was recorded during biofilm development. Starting from a base of only 1%, their proportions progressively increased and represented 17% and 18% of EUB338 positive cells on day 44, respectively. These groups therefore exceeded the contribution of the initially dominating γ-*Proteobacteria* on the final sampling day of the experiment.

Contributions of both β-*Proteobacteria* and *Thiothrix* represented <5% of the biofilm community, even though marginally increased towards the end of the experimental period. Morphological observations of *Thiothrix* demonstrated that the cells were rod-shaped in morphology rather then filamentous.

Ozonated water. The bacterial community of biofilm within ozonated water was dominated by γ-*Proteobacteria* being consistently greater than 20%, and more typically >35-40% of EUB338-detected cells. Maximum community contributions of 60-65% were recorded between days 14-20. The majority of filaments were represented by members of this subclass.

α-*Proteobacteria* were the second most dominant group, particularly in later days of biofilm development. In association with the reduction of γ-*Proteobacteria* on days 28 and 36, this subclass increased to >35% of the community and was approaching percentages of γ-*Proteobacteria*. On day 44, the α-*Proteobacteria* were reduced to 20% of the total population, which was associated with again increasing proliferation of γ-*Proteobacteria* that constituted >50% of the EUB338 positive cells.

Members of the CFB were well represented over the entire period. However, their contribution was gradually reduced from approximately 15% to <10% of the EUB338-detected cells. Later observed CFB were dominated by filaments and rod-shaped cells of comparatively large size.

Planctomycetes represented between 6 to 10% of the total bacterial community. Their relative contribution decreased on day 16 but re-established later in the experimental trial as the fraction of γ-*Proteobacteria* decreased. In later-age biofilm, percentages of *Planctomycetes* exceeded those of CFB, except for day 44 when the contribution of the group was reduced to approximately 5% of the EUB338 positive cells. The distinct amount of missing dual hybridizations, repeatedly observed for *Planctomycetes*, was due to the low coverage of EUB338 for this group (Neef et al., 1998). The *Bacteria* probe covers just 19 of the 2936 (= 0.6%) *Planctomycetes* genera comparing to e.g. 75% of the genera within γ-*Proteobacteria* (The Ribosomal Database Project II; http://rdp.cme.msu.edu).

β-*Proteobacteria* constituted 11% of the community on day 5, which was similar to the fraction of α-*Proteobacteria* on this day, though with a high standard deviation. During biofilm development, the relative fraction of β-*Proteobacteria* declined to 1% on day 44. A slight recovery occurred between days 16-20, though only by 1 to 2% of EUB338 positive cells. This subclass was the only tested bacterial group that did not show an increased representation in the bacterial community during the reduction of γ-*Proteobacteria*.

Vibrionaceae and *Thiothrix* spp. only represented a small proportion of the community with a maximum of 2-3%, respectively. *Vibrionaceae* showed nondirectional variations in contribution, whereas contribution of *Thiothrix* increased towards the final days. *Thiothrix* sp. were only characterized by rod-shaped morphology.

The development and composition of rearing tank biofilm in non- and ozonated water is illustrated in Figure 4.4, displaying representative biofilm features for specific points of development.

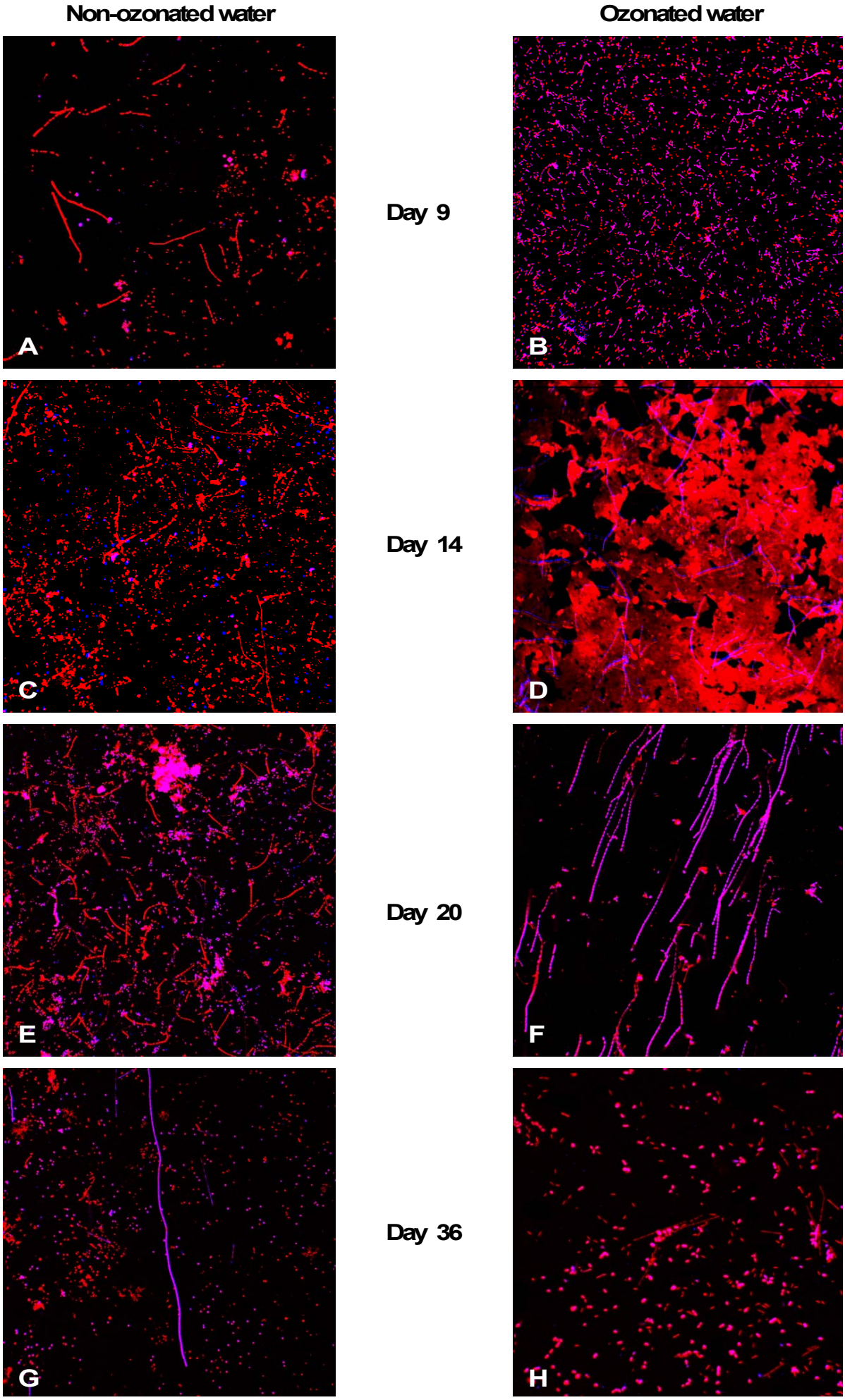

Non-ozonated water
Ozonated water
Day 9
Day 14
Day 20
Day 36
A
B
C
D
E
F
G
H

Figure 4.4 FISH confocal micrographs, illustrating the major effects of ozonation on structural biofilm appearance and composition of the bacterial community. Red signals originate from binding of Cy3-labelled EUB338, purple signals from additional binding of the respective Cy5-labelled group-specific probe yielding a merged signal. Remarks below in brackets indicate the oligonucleotide probe used in the hybridization. The dimension of each image is 205 x 205 µm. ***A:*** Non-ozonated water biofilm on day 9, showing the beginning of bacterial proliferation and establishment of filaments within the biofilm (ALF1b). ***B:*** Increased biomass within ozonated water biofilm on day 9. Rod-shaped γ-*Proteobacteria* were predominant (GAM42a). ***C:*** Growing biofilm on day 14 within non-ozonated water, featuring a distinct percentage of *Planctomycetes* (PLA886/PLA46). ***D:*** Surface coverage with unidentified Cy3 fluorescent matter and first prominent representation of filamentous bacteria within ozonated water biofilm on day 14. Filaments appeared to be interwoven with the material (GAM42a). ***E:*** Dominance of α-*Proteobacteria* within non-ozonated water biofilm on day 20, characterized by single and clustered rod-shaped cells. (ALF1b). ***F:*** Filamentous γ-*Proteobacteria* within ozonated water on day 20. Filaments appeared to be orientated to the water flow (GAM42a). ***G:*** Lower bacterial numbers after sloughing of non-ozonated water biofilm on day 36. The fraction of the *Cytophaga-Flavobacterium* cluster was increased during this phase, illustrated by a long filamentous bacterium and several rod-shaped cells (CF319a). ***H:*** Similar cell coverage as in non-ozonated water biofilm was observed on day 36 within ozonated water during re-establishment of the biofilm. α-*Proteobacteria* were approaching numbers of γ-*Proteobacteria* (ALF1b).

4.1.2 Scanning electron microscopy

SEM analysis of biofilm development revealed larger aggregates and particular matter attached to the surface of glass disks placed in both non-ozonated and ozonated water tanks. Limited bacteria could be detected on the surface. This was likely due to artefacts associated with the processing of the samples, and little information could be generated from the samples to support the observations of biofilm development.

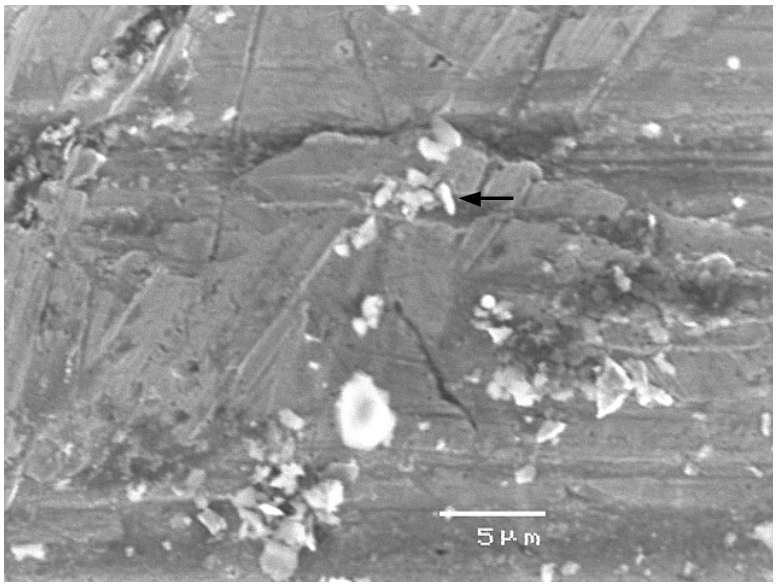


Figure 4.5 Surface coverage of glass disks as recorded by SEM. The arrow indicates a potential bacterial cell attached to the disk surface.

4.1.3 Denaturing gradient gel electrophoresis

DNA extracted from biofilm swab samples could not be amplified by PCR using the bacterial primers 1055F and 1392R-GC. Although sufficient amounts of extracted DNA was present, as confirmed by photometric analysis using the GeneQuant system (Amersham, Buckinghamshire, UK), it was suspected that co-extracted inhibitory compounds (for example humic acids and phenolics) prevented successful amplification. Purification of extracted DNA was performed and resulted in successful amplification of 16S-rDNA genes.

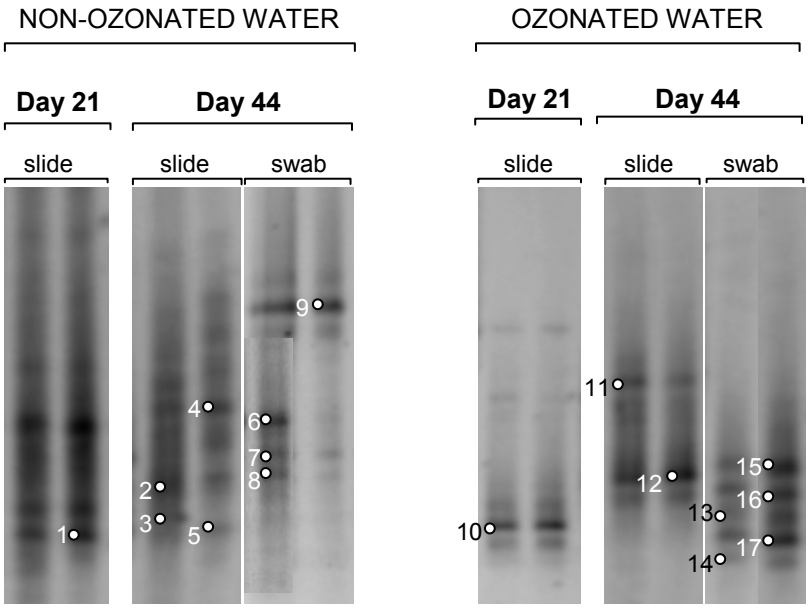


Figure 4.6 DGGE profiles of rearing tank biofilm on days 21 and 44 within non-ozonated and ozonated water. A total of 17 bands was excised and sequenced.

DGGE fingerprints were obtained from rearing tank biofilm formed in ozonated and non-ozonated water on days 21 and 44. Two PCR products from each extract were separated in parallel and 17 selected intense bands excised from the gel (Figure 4.6). Bands were sequenced to determine the phylogenetic identity of the respective rDNA and classify the bacterial community structure (Table 4.2).

The biofilm profile (Figure 4.6) differed both between the two water treatments and between the sampling days, respectively. In addition, biofilm from both water types on day 44 yielded a different bacterial DGGE profile depending on whether DNA was extracted from slide or swab samples. The profile of non-ozonated water biofilm obtained from slide on this day was not exactly reproduced in the two parallels. Changes in the bacterial community structure occurred over time, as demonstrated by variation in dominant bands and the affiliated

sequences retrieved. The DGGE profiles indicated a dynamic nature of the aquaculture microbial community, whose composition changed between the two sampling points.

Table 4.2 Phylogenetic identity of dominant bands from the DGGE biofilm profile, obtained for days 21 and 44 of biofilm development within non-ozonated and ozonated water. Non: non-ozonated water; Ozo: ozonated water.

Band	Water type	Sample (day)	Closest relative (Database accession number)	% Similarity	Category
1	Non	Slide (21)	Uncultured bacterium clone YE-DC-B50 (DQ438398)	93	γ-*Proteobacteria*
2	Non	Slide (44)	Uncultured organism clone ctg_CGOF052 (DQ395844)	90	α-*Proteobacteria*
3	Non	Slide (44)	No sequence retrieved.	----	----
4	Non	Slide (44)	Uncultured bacterium clone BBD_217_15 (DQ446131)	87	α-*Proteobacteria*
5	Non	Slide (44)	No sequence retrieved.	----	----
6	Non	Swab (44)	Uncultured clone Y30 (AB116485)	95	*Planctomycetales*
7	Non	Swab (44)	Uncultured bacterium clone C9-2-69R (DQ419258)	87	γ-*Proteobacteria*
8	Non	Swab (44)	*Pseudomonas* sp. gap-f-76 (DQ530477)	91	γ-*Proteobacteria*
9	Non	Swab (44)	*Rhodobacter* bacterium PH33 (AF513477)	96	α-*Proteobacteria*
10	Ozo	Slide (21)	Uncultured bacterium clone b1uc29 (EF206937)	95	γ-*Proteobacteria*
11	Ozo	Slide (44)	Uncultured bacterium clone b2ub58 (EF206984)	96	γ-*Proteobacteria*
12	Ozo	Slide (44)	Uncultured bacterium clone JTB23 (AB015248)	96	γ-*Proteobacteria*
13	Ozo	Swab (44)	Uncultured bacterium clone I3K-0188 (AY868184)	97	δ-*Proteobacteria*
14	Ozo	Swab (44)	Uncultured bacterium clone BBD_216_23 (DQ446091)	96	α-*Proteobacteria*
15	Ozo	Swab (44)	*Planctomycete* GMD14H07 (AY162124)	92	*Planctomycetales*
16	Ozo	Swab (44)	Bacterium c1cc72 (EF207154)	88	α-*Proteobacteria*
17	Ozo	Swab (44)	Bacterium c1cb34 (EF207125)	95	α-*Proteobacteria*

Bands with similar mobility in parallel replicate lanes were sequenced for verification of the reproducibility of extraction and profiling methods. Both the bacterial profiles and retrieved sequences were identical in the parallel samples, confirming the robustness of the methods (data not shown).

Non-ozonated water. Band 1 retrieved from the biofilm slide profile on day 21 had 93% sequence similarity to an uncultured γ-*Proteobacterium*. This uncultured bacterium itself was interestingly related to the sequence of band 11, retrieved from the profile of ozonated water slide biofilm on day 44.

Only two (bands 2 and 4) of the four bands retrieved from the biofilm slide profile on day 44 were successfully sequenced. These bands were affiliated with two uncultured clones within the α-*Proteobacteria*.

The biofilm swab profile on day 44 was dominated by four bands. Band 6 had 95% sequence similarity to an uncultured *Planctomycetes* clone. Band 7 showed only 87% similarity to its closest relative, an uncultured bacterium within the γ-*Proteobacteria*. Another γ-*proteobacterial* affiliated sequence, band 8, was related to a *Pseudomonas* sp. Band 9 was successfully sequenced and affiliated with an α-*Proteobacterium* being closely related to a *Rhodobacter* bacterium.

Ozonated water. Band 10 dominated the bacterial biofilm DGGE profile from slides sampled on day 21. This band demonstrated 95% sequence similarity to an uncultured γ-*proteobacterial* clone. Band 11 and 12 in the slide profile of day 44 affiliated with two other uncultured γ-*Proteobacteria.*

In contrast, the DGGE profile of biofilm swab on day 44 was missing γ-*proteobacterial* affiliated sequences, and was instead dominated by sequences affiliated with α-*Proteobacteria*. Bands 14, 16 and 17 were affiliated with uncultured clones of this subclass. The swab biofilm profile was further dominated by a band affiliated with an uncultured δ-*Proteobacterium* (band 13). The sequence of band 15 affiliated with an uncultured *Planctomycetes* clone.

4.1.4 *Artemia* mortality

Artemia mortality was monitored throughout the course of the biofilm experiment. Early in the trial, *Artemia* mortality in tanks containing non-ozonated water was low with approximately 10 to 20% after 12 h. However, mortalities of between 40 to 50% on day 18 within all three parallel tanks were observed after 12 h of incubation. In the following days, mortality levels further escalated, with relative daily rates rising up to 90 to 100% towards the final days of the experimental period. In contrast, *Artemia* mortalities in ozonated water tanks were consistently lower than observed in the non-ozonated water tanks. However, mortalities were intermittently recorded from day 21, reaching approximately 40 to 50% of *Artemia* after 12 h. From days 36-44, *Artemia* mortality within ozonated water was not observed at all.

4.2 Vibrio *load of* Artemia *and the effect of antimicrobial treatments*

4.2.1 Effect of the antibiotics streptomycin, oxolinic acid, tetracycline, and a mixture of erythromycin, oxytetracycline and streptomycin

The cultivable *Vibrio* load of *Artemia* after treatments with various antibiotics was determined in relation to colonies forming on TCBS agar and specified as colony-forming units (CFU) per *Artemia*.

Treatment of *Artemia* with antibiotics influenced their associated *Vibrio* load compared to non-treated animals (Table 4.3). The load increased for all treatments after 24 hours compared to the corresponding sample after 2 hours. However, the load after treatment with the mixture was low. During the incubations, the composition of the *Vibrio* load changed, represented by a variation in the dominant morphology of colonies forming on TCBS agar.

Table 4.3 *Vibrio* load of *Artemia* 2 h and 24 h past addition of antibiotics, determined as colony-forming units (CFU) per *Artemia* (rounded) ± standard deviation

	CFU/*Artemia* after cultivation for	
Treatment	***2 h***	***24 h***
Without antibiotics	0.5 ± 0.2	53 ± 6
Streptomycin	24 ± 1	>1000
Oxolinic acid	16 ± 2	33 ± 10
Tetracycline	0.3 ± 0.15	52 ± 7
Erythromycin, oxytetracycline, streptomycin	0.02 ± 0.03	0.4 ± 0.06

No antibiotic treatment resulted in a *Vibrio* load of 0.5 CFU/*Artemia* after 2 h; compared to a load of 53 CFU/*Artemia* after 24 h showing a hundredfold increase in the load. The composition of strains changed from a more diverse *Vibrio* community associated with 2 h *Artemia* that comprised similar proportions of strains forming green and yellow colonies, to a more uniform load dominated by strains forming yellow colonies when animals were incubated for 24 h.

Artemia treated with streptomycin had an increased *Vibrio* load relative to untreated *Artemia.* The load yielded 24 CFU/*Artemia* after a 2 h treatment and an estimated >1000 CFU/*Artemia* after 24 h, which was however difficult to determine since the plated homogenates yielded

95% agar coverage with small, evenly distributed colonies. The composition of the load was initially comparable to non-treated *Artemia* with strains forming both green and yellow colonies (2 h), but streptomycin excluded strains forming yellow colonies from the load after 24 h of treatment.

Compared to treatment with streptomycin, a 2 h treatment with oxolinic acid yielded a lower CFU value per *Artemia* (16), but a distinctly higher value compared to non-treated animals. In contrast, a 24 h treatment with oxolinic acid reduced the *Vibrio* load compared to non-treated *Artemia* at this time point and was determined as 33 CFU/*Artemia*. The load however had a different composition, and was dominated by strains forming green colonies on TCBS agar.

Tetracycline reduced the *Vibrio* load after a 2 h treatment of animals (0.3 CFU/*Artemia*), but the associate load after 24 h was distinctly higher (52 CFU/*Artemia*) and comparable to results from non-treated animals. For both time points, 90% of the strains formed yellow colonies on TCBS agar.

A distinct reduction of CFU values was attained by treatment of *Artemia* with a mixture of erythromycin, oxytetracycline and streptomycin, which was consistent at both sampling times (2 h and 24 h). Only two green, non-luminescent colonies were found on TCBS after 2 h treatment (0.02 CFU/*Artemia*) and six green, non-luminescent colonies after 24 h treatment (0.4 CFU/*Artemia*).

Treatment time optimization for the antibiotics mixture of erythromycin, oxytetracycline and streptomycin

Based on the previous experiment, treatment with the antibiotic mixture consisting of 25 ppm erythromycin, 25 ppm oxytetracycline and 10 ppm streptomycin was selected as the most applicable for an effective cleanup of *Artemia*. Further studies using 10-day on-grown *Artemia* were performed to optimize the treatment time for this mixture.

Increasing the exposure time of *Artemia* to the antibiotic mixture was found to increasingly reduce the associated *Vibrio* load (Figure 4.7). High *Vibrio* CFU counts after 1 h (116 CFU/*Artemia*) contrasted the result of the previous experiment for 7-day on-grown *Artemia*. However, increased exposure times subsequently reduced CFU numbers. The *Vibrio* load of *Artemia* was reduced by 60% after 2 h (50 CFU/*Artemia*) and by another 50% after 4 h treatment (22 CFU/*Artemia*). The load remained constant with *Artemia* treated for 6 h and was determined as 23 CFU/*Artemia*. Complete disinfection of *Artemia* was achieved after cultures were treated for 24 h (Figure 4.7).

A shift in colony morphology demonstrated that not only did CFU counts decrease, but that the composition of the *Vibrio* community altered with increased exposure to the antibiotic mixture (Figure 4.7). *Vibrio* strains associated with *Artemia* sampled after 1 h formed green

and yellow colonies, and these morphotypes were equally present. The *Vibrio* load of *Artemia* treated for 2 h was dominated by strains forming green colonies, whereas a treatment time of 4 h yielded a dominance of strains forming yellow colonies on TCBS agar. The associated *Vibrio* load after 6 h again comprised equal proportions of both morphotypes.

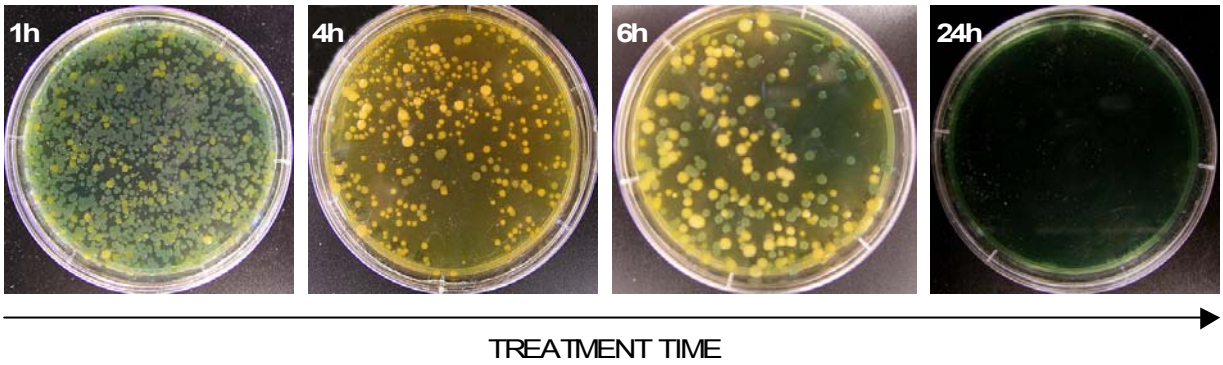


Figure 4.7 Reduction of the *Vibrio* load of *Artemia* with increasing exposure time to a mixture of erythromycin, oxytetracycline and streptomycin. The figure illustrates the shift in dominant colony morphology after treatment for 1 h, 4 h and 6 h and the complete disinfection of *Artemia* after treatment for 24 h. In addition, the subsequent increase in average colony diameter is shown.

Determination of CFUs on Marine Agar confirmed TCBS results of a high bacterial load associated with *Artemia*. Bacterial colonization of *Artemia* was not restricted to *Vibrio* species, with a diverse array of colony morphologies isolated. Interestingly, the CFU counts on MA for the 24 h treatment were high, despite no *Vibrio* CFUs being obtained. This suggested that the *Vibrio* load of *Artemia* was completely removed by that treatment, although Artemia still maintained a diverse associated microbial community.

4.2.2 Effect of *Sanolife Mic*

The cultivable *Vibrio* load of *Artemia* after treatment with the probiotic product Sanolife was determined in relation to colonies forming on TCBS agar and specified as both CFU ml^{-1} homogenate and CFU/*Artemia*. Although the concentration of *Artemia* in the culture was determined, no single animals could be sampled in the beginning of the experiment due to the small size of nauplii, and therefore a defined culture aliquot was homogenized instead. The increasing size of *Artemia* during the grow-out may have increased their load by providing a larger surface area for bacterial colonization, potentially biasing the CFU counts per ml of sample.

Table 4.4 *Vibrio* load of *Artemia* during a 10-day application of Sanolife to *Artemia* enrichment tanks, determined as colony-forming units (CFU)/ml homogenate and CFU/*Artemia*

***Artemia* age (day)**	**Nauplii ml^{-1}**	**CFU ml^{-1}**	**CFU/*Artemia***
2	170	50	0.29
4	60	1.9 x 10^4	316
6	16	1.5 x 10^3	93
8	12	2.2 x 10^4	1833
10	12	2.3 x 10^4	1916

Treatment of *Artemia* with Sanolife resulted in day 2 *Artemia* demonstrating a low *Vibrio* load. Only a few yellow, non-luminescent colonies were observed on TCBS agar. CFU counts (Table 4.4) distinctly increased with day 4 *Artemia* (1.9 x 10^4 CFU ml^{-1} and 316 CFU/*Artemia*, respectively), though these colonies were still restricted to yellow, non-luminescent strains (Figure 4.8).

6-day on-grown *Artemia* demonstrated reduced CFU counts (1.5 x 10^3 CFU ml^{-1} and 93 CFU/*Artemia*, respectively), and this was attributed to a decrease of the previously dominant strains forming yellow colonies. Strains forming luminescent, green colonies were establishing by this day (Figure 4.8).

The *Vibrio* load increased again on day 8 with CFU counts of 2.2 x 10^4 CFU ml^{-1} and 1833 CFU/*Artemia*, respectively. The number of strains forming yellow, non-luminescent colonies was comparable to counts with day 4 *Artemia*, but strains forming green, luminescent colonies increased and constituted approximately 20% of the total *Vibrio* load on this day.

Day 10 *Artemia* were mostly associated with strains forming green luminescent colonies, increasingly replacing strains of yellow colony morphology on TCBS agar (Figure 4.8). The *Vibrio* load represented as CFU ml^{-1} (2.3 x 10^4) was comparable to that observed after 4 and 8 days, and the value of CFU/*Artemia* similar to that of day 8 nauplii (1916 CFU/*Artemia*). The composition of this load however altered and nearly all colonies showed luminescence. The contribution of strains forming green, strong luminescent colonies increased to approximately 70% of the cultivable vibrios.

Furthermore, it was demonstrated that as *Artemia* aged, corresponding to an increase in animal size the ratio between CFU ml^{-1} and CFU/*Artemia* also changed (Table 4.4). The younger *Artemia* culture on day 4 had a CFU count of 1.9 x 10^4 ml^{-1}, corresponding to a CFU count of 316 per *Artemia*. The *Vibrio* load of day 8 *Artemia* yielded a similar CFU count of 2.2 x 10^4 ml^{-1}, but the CFU per *Artemia* increased to 1833. The similar number of colonies on the

plates was hence attributed to a higher *Vibrio* load of individual *Artemia*. It was however difficult to determine whether this increase of individual loads for the older *Artemia* was due solely to a larger animal surface allowing the colonization of a larger number of bacteria. Such an increase of vibrios per *Artemia* may not necessarily imply a decrease in the animals' health status. However, the increasing occurrence of *Vibrio* strains forming luminescent colonies on TCBS with older *Artemia* (days 8 and 10 samples) suggested that the increase in individual bacterial load was (in part) independent of the animal size. This increase in potential pathogenic strains may have started to induce diseased conditions associated with the animals.

In conclusion, the *Vibrio* load of individual *Artemia* increased over time, and a shift in community composition yielded mostly green, luminescent strains that outcompeted and replaced yellow, non-luminescent strains.

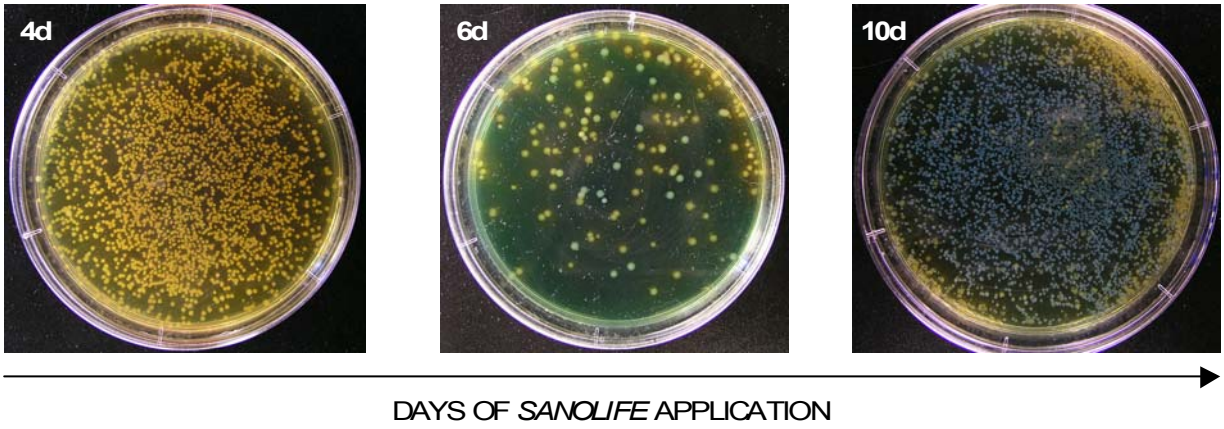


Figure 4.8 *Vibrio* colonies isolated from Sanolife treated *Artemia* during a 10-day application of Sanolife to *Artemia* enrichment tanks, illustrated by samples obtained after 4, 6, and 10 days (d) of cultivation. The figure illustrates the transition from non-luminescent, yellow strains (4d) to the reduction in CFU numbers and the first appearance of green, luminescent strains (6d) to the eventual dominance of green, strong luminescent strains over the yellow strains (10d). The blue colour from the majority of colonies in the 10d image probably originated from an optical effect during image acquisition caused by the strong colony luminescence.

4.3 Microbial colonization of phyllosoma

4.3.1 External microbial colonization of captive-reared phyllosoma

Phyllosoma from the P1 to P2 (days 1-9 of the total larval age) and P3 to P4 (days 16-28 of the total larval age) larval stages were examined for bacterial colonization.

Three body parts of phyllosoma were examined under the scanning electron microscope and included the presumptive high nutrient loading areas of the mouthparts and anus, and the low nutrient, 'neutral' surface of the eye. SEM micrographs of the mouthparts also focussed on the coxal and basal endite, as these mouthpart components handle and shunt food particles into the mouth palps where food is externally masticated. When random surveying of the general body revealed specific areas of interest, these were also investigated.

For each sampling day, the microbial colonization of randomly selected 6-8 phyllosoma was recorded. External bacterial colonization was assessed by direct cell count on SEM images and qualitative valuation of the overall colonization grade from 0 (no) to 10 (complete surface coverage). If a direct cell count was disabled, the evaluation was restricted to using the qualitative scale.

P1-P2 phyllosoma

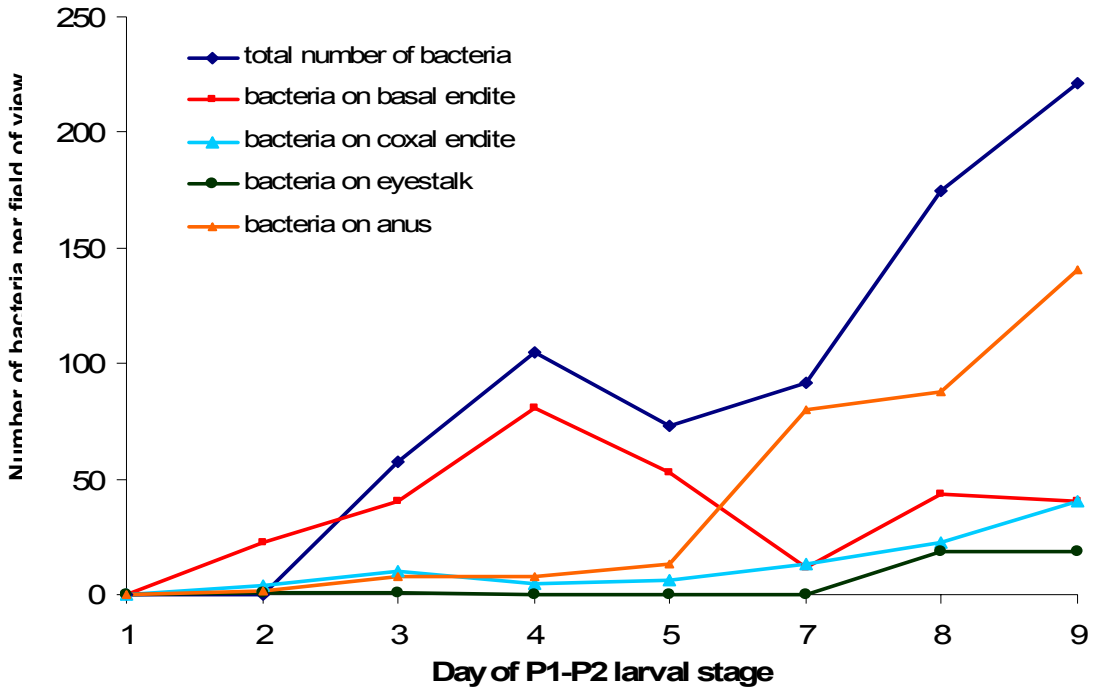


Figure 4.9 Progression of external bacterial colonization of phyllosoma within days 1-9 of the P1-P2 larval stage. Numbers relate to bacteria per field of view at 500x magnification.

P1-P2 larvae showed progressive external attachment of bacteria when reared within non-ozonated seawater (Figure 4.9). The total bacterial counts increased to a maximum of 220 bacteria per specimen on day 9 of the larval stage. Bacterial cells were well-defined, enabling a direct cell count. Colonization of the mouthparts started on day 2 of the P1-P2 stage and was mainly restricted to the basal endite. From day 4, colonization of the basal endite decreased. This corresponded to an increasing colonization at the anus, which from then on represented the area of maximum bacterial attachment. The coxal endite was not colonized as distinctly; with little colonization being recorded until day 5 after hatching and overall just to a lower extent. While most of the detected bacteria were of filamentous morphology, rod-shaped bacteria were commonly present as well. In addition, circularly shaped protozoans were repeatedly observed. Interestingly, similarly shaped protozoans were, although very occasionally, observed in the later days (36 to 44) of the biofilm development as analyzed by FISH (see 4.1).

P3-P4 phyllosoma

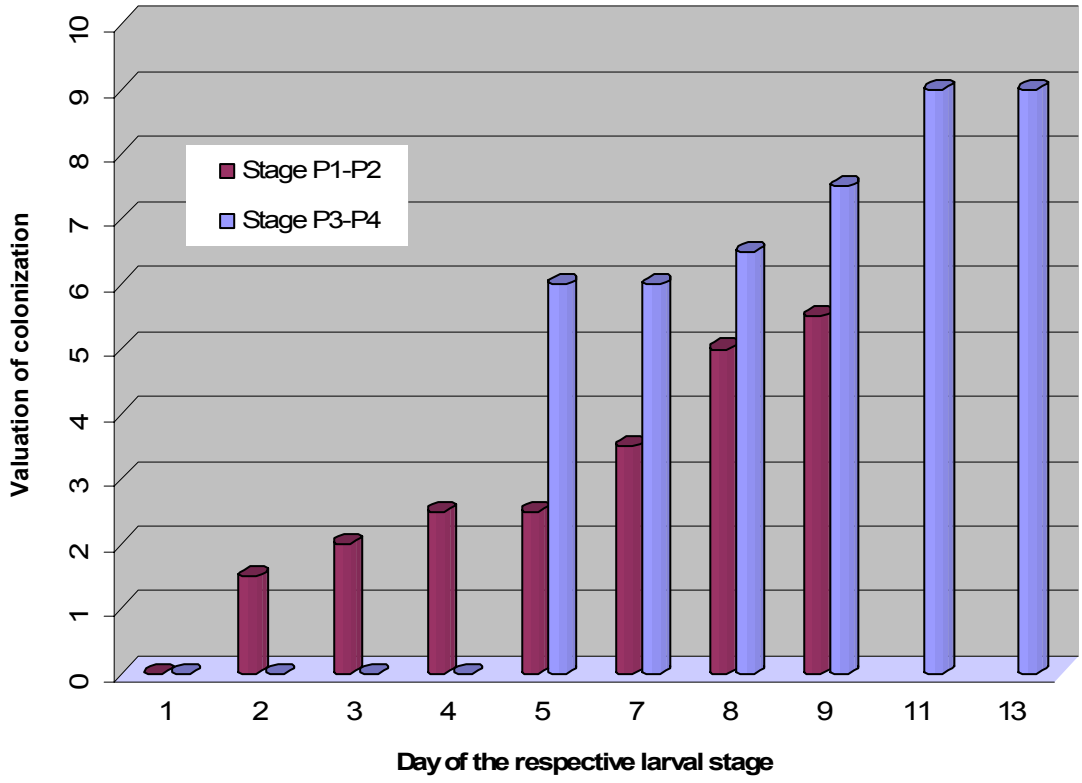


Figure 4.10 Qualitative valuation of external bacterial colonization comparing captive-reared P1-P2 and P3-P4 phyllosoma. Designation of days is respective for each larval stage.

Due to extensive bacterial colonization of the surface, external colonization of phyllosoma within the P3-P4 stage was assessed using the qualitative scale (Figure 4.10).

P3-P4 phyllosoma were free of external bacterial attachment until post-moult day 5. Bacterial densities recorded on day 5 of the P3-P4 stage already exceeded the highest densities observed at the end of P1-P2 stage. Such a high level of external colonization prevented accurate and robust direct cell counts.

External bacterial colonization on day 5 was only observed on the mouthparts, though when compared to P1-P2 phyllosoma, the degree of colonization was more extensive and included the coxal endite and the labrum or paragnath. These areas were only marginally colonized with P1-P2 phyllosoma. The morphology of bacteria colonizing the mouthparts of larvae mostly consisted of filamentous bacteria, which entangled the front parts of the endites that are directed to the pharynx. Bacterial proliferation on the anus was observed from day 7 and gradually increased. Anus colonization was dominated by rod-shaped cells, with occasional bacterial filaments spanning the anus. Filamentous structures could have also been represented by fungi (Lone Høj, personal communication). The colonization grade in the late days of the P3-P4 stage further increased, such that up to 90-100% coverage of mouthparts and anus was observed towards days 11 and 13. Furthermore, protozoa were increasingly observed both at the mouthparts and the anus. At the same time, specimens free of microbial colonization were observed with day 13 phyllosoma. This was likely due to the recent moult of these phyllosoma into the subsequent larval stage, during which the externally attached microbiota was removed with the shedding of the exterior surface.

Following figures 4.11 (a-h) are representative SEM micrographs of external microbial colonization of captive-reared phyllosoma of *Panulirus ornatus*, illustrating colonization within both the P1-P2 and P3-P4 larval stage.

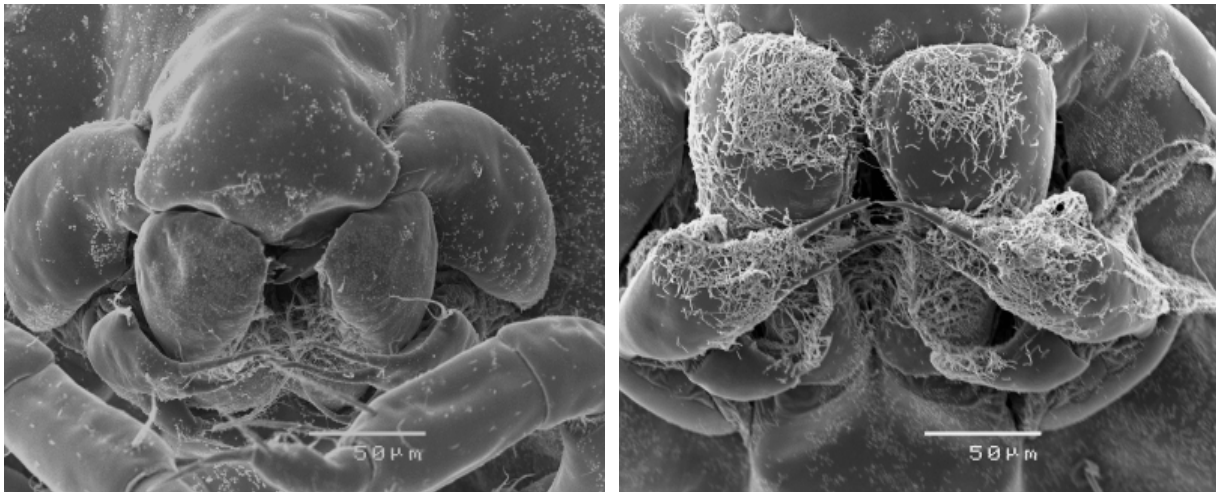


Figure 4.11 (a) Maximum degree of microbial colonization on mouthparts of aquaculture-reared phyllosoma. Left: Day 9 of the P1-P2 larval stage. Right: Day 13 of the P3-P4 larval stage.

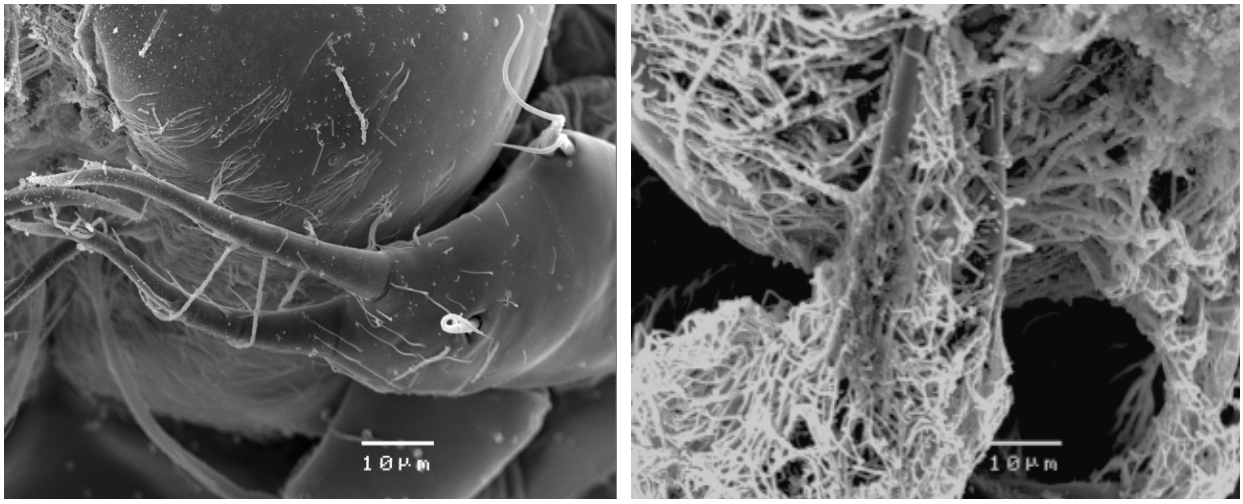


Figure 4.11 (b) Detailed views of the basal endite, illustrating the maximum degree of colonization which was significantly higher with P3-P4 (right) than P1-P2 phyllosoma (left).

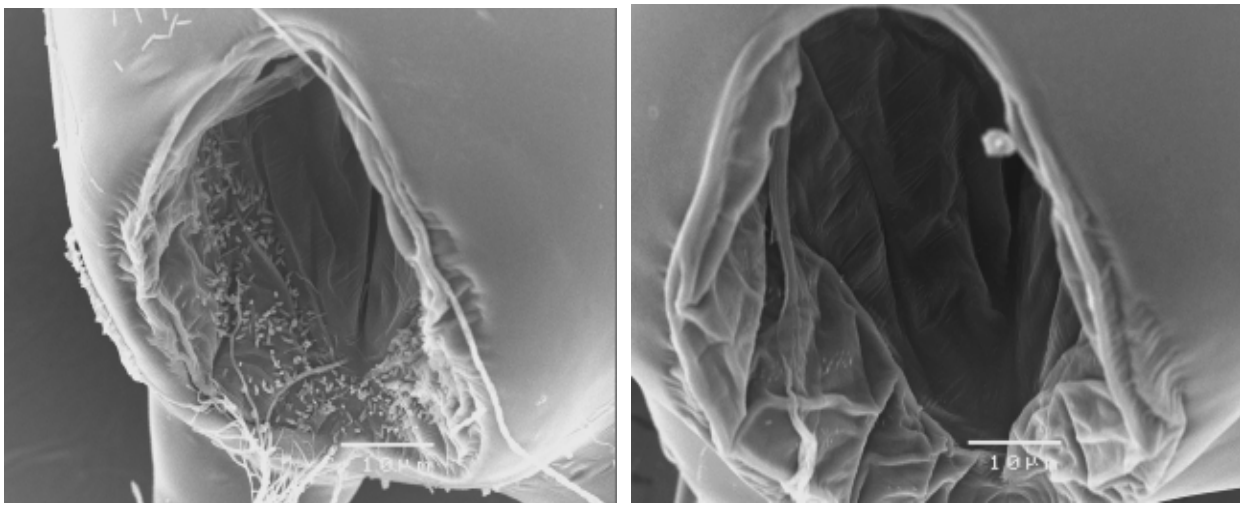


Figure 4.11 (c) Microbial proliferation on the anus on day 7 of the P1-P2 stage (left). At this time point within the P3-P4 stage, anus of phyllosoma was still free of microbial attachment (right).

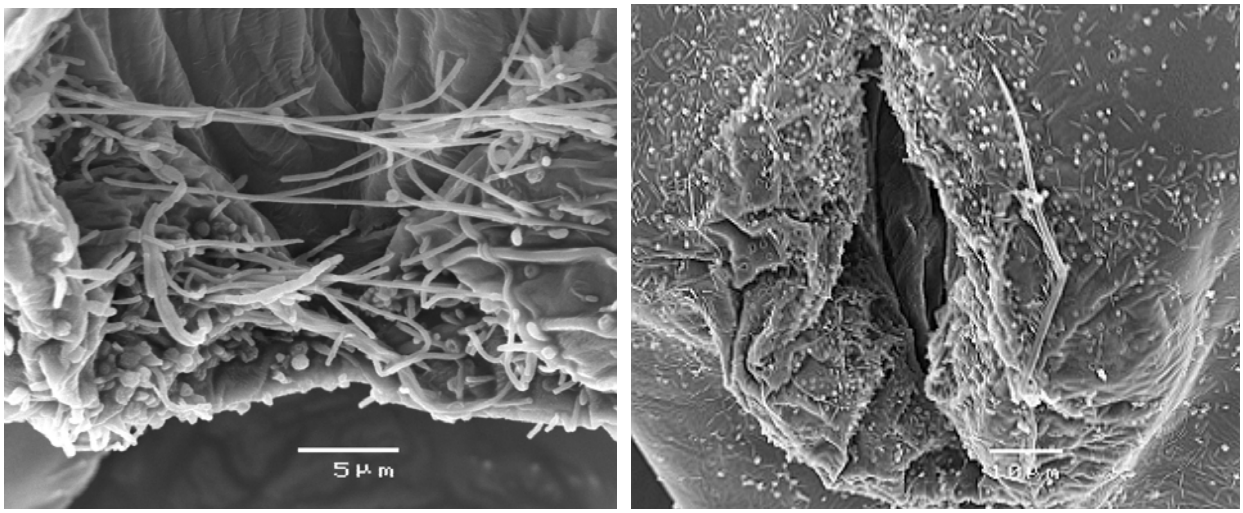


Figure 4.11 (d) Detailed view of anus colonization in late days of the first larval stage (Day 9, left). The anus colonization of P3-P4 phyllosoma was eventually (Day 9, right) exceeding dimensions observed with P1-P2 larvae.

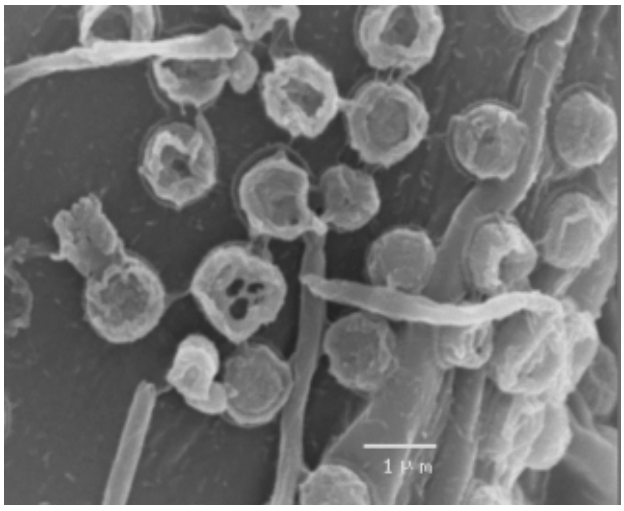


Figure 4.11 (e) Detailed view of protozoan and bacterial attachment above anus (Day 7 of the P1-P2 stage).

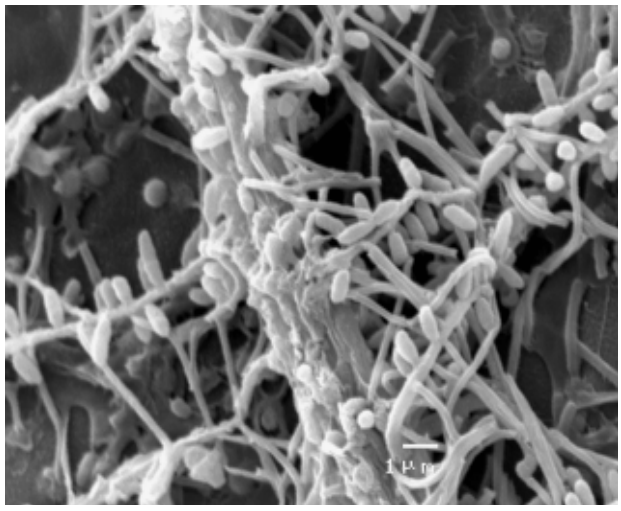


Figure 4.11 (f) Setae of the 2nd maxilliped, with attachment of both filamentous and rod-shaped bacteria (Day 11 of the P3-P4 stage).

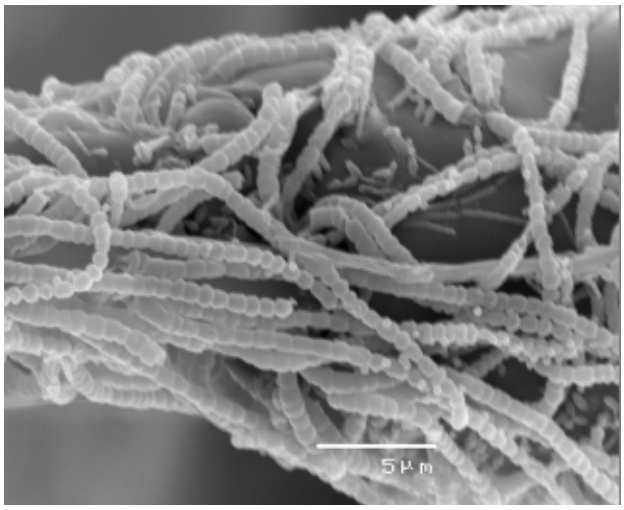


Figure 4.11 (g) Detailed view of significant attachment of filamentous bacteria to a leg (Day 8 of the P1-P2 stage).

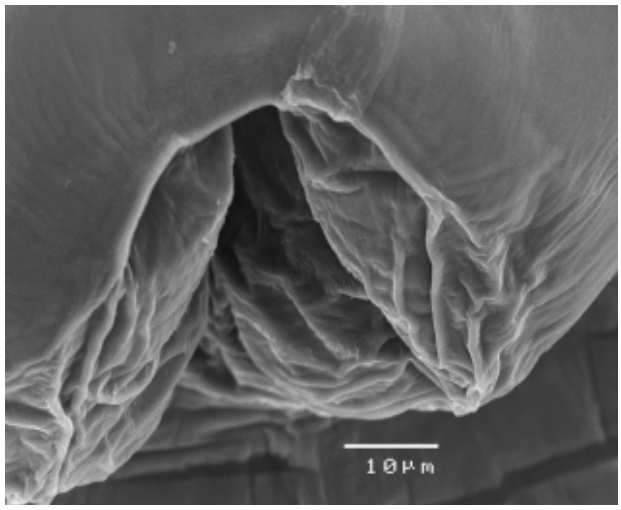


Figure 4.11 (h) Moult shedding removed the externally attached microbiota with the exterior surface, as shown on the anus (Day 13 of the P3-P4 stage).

4.3.2 Microbial colonization of wild phyllosoma

External colonization

Microbial colonization of wild-caught phyllosoma of the species *Panulirus ornatus* and *Panulirus longipes* was also investigated using SEM (Figures 4.12 and 4.13). No means was available for accurately determining the number of days passed the last moult of wild phyllosoma, and therefore it was assumed the observed colonization was representative of phyllosoma in their natural environment. Areas of investigation included the same as with the captive-reared larvae and were analyzed for microbial attachment on a total of 9 phyllosoma.

The examined specimens were of later developmental stages than those obtained from rearing trials (P8-9 for *P. ornatus,* P6 for *P. longipes*).

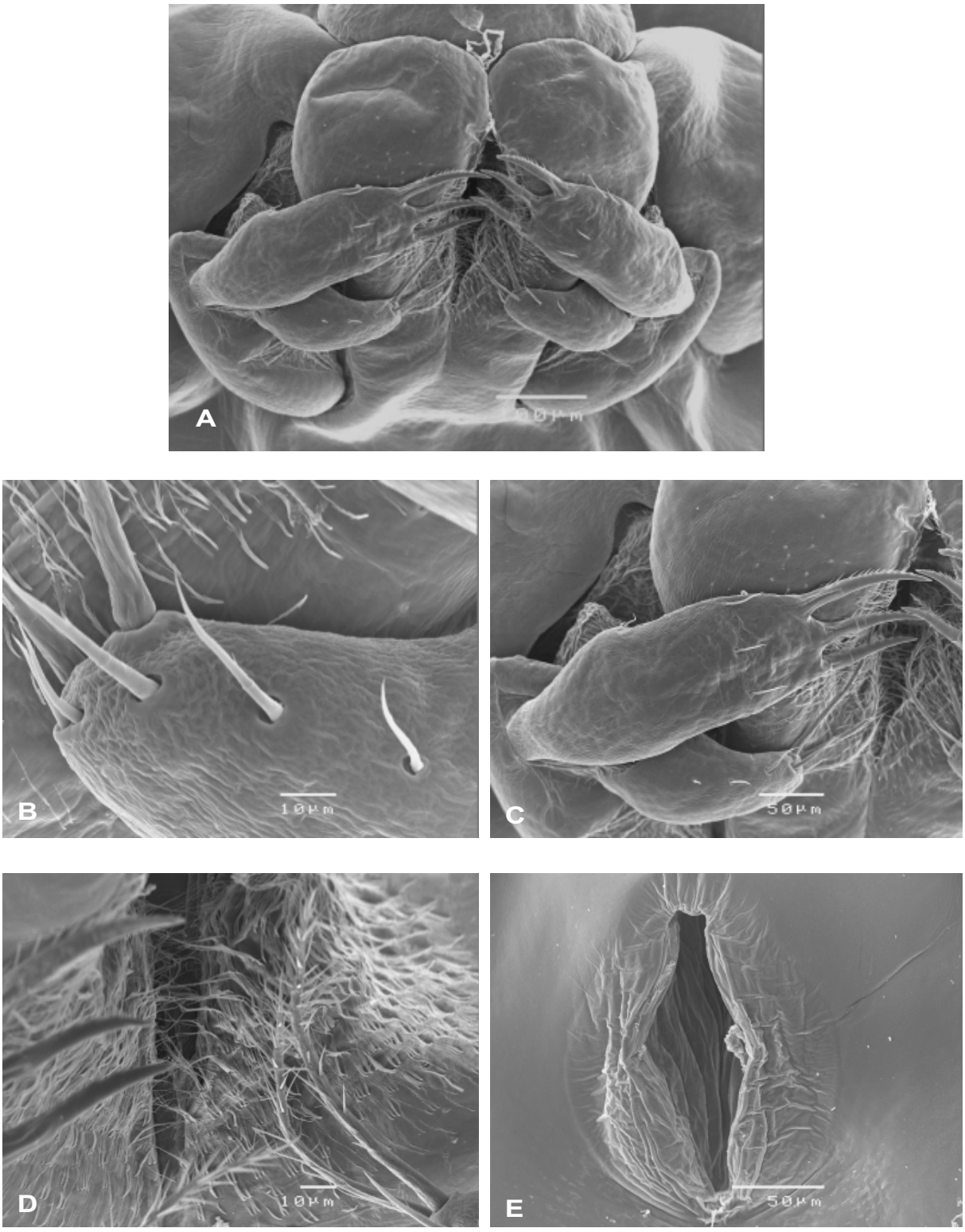


Figure 4.12 SEM micrographs of different areas of wild *Panulirus* phyllosoma. Pictures illustrate the virtually complete missing of microbial attachment to areas severely colonized on aquaculture-reared larvae. ***A:*** total view of mouthparts; ***B:*** basal endite; ***C:*** basal and coxal endite; ***D:*** pharynx; ***E:*** anus.

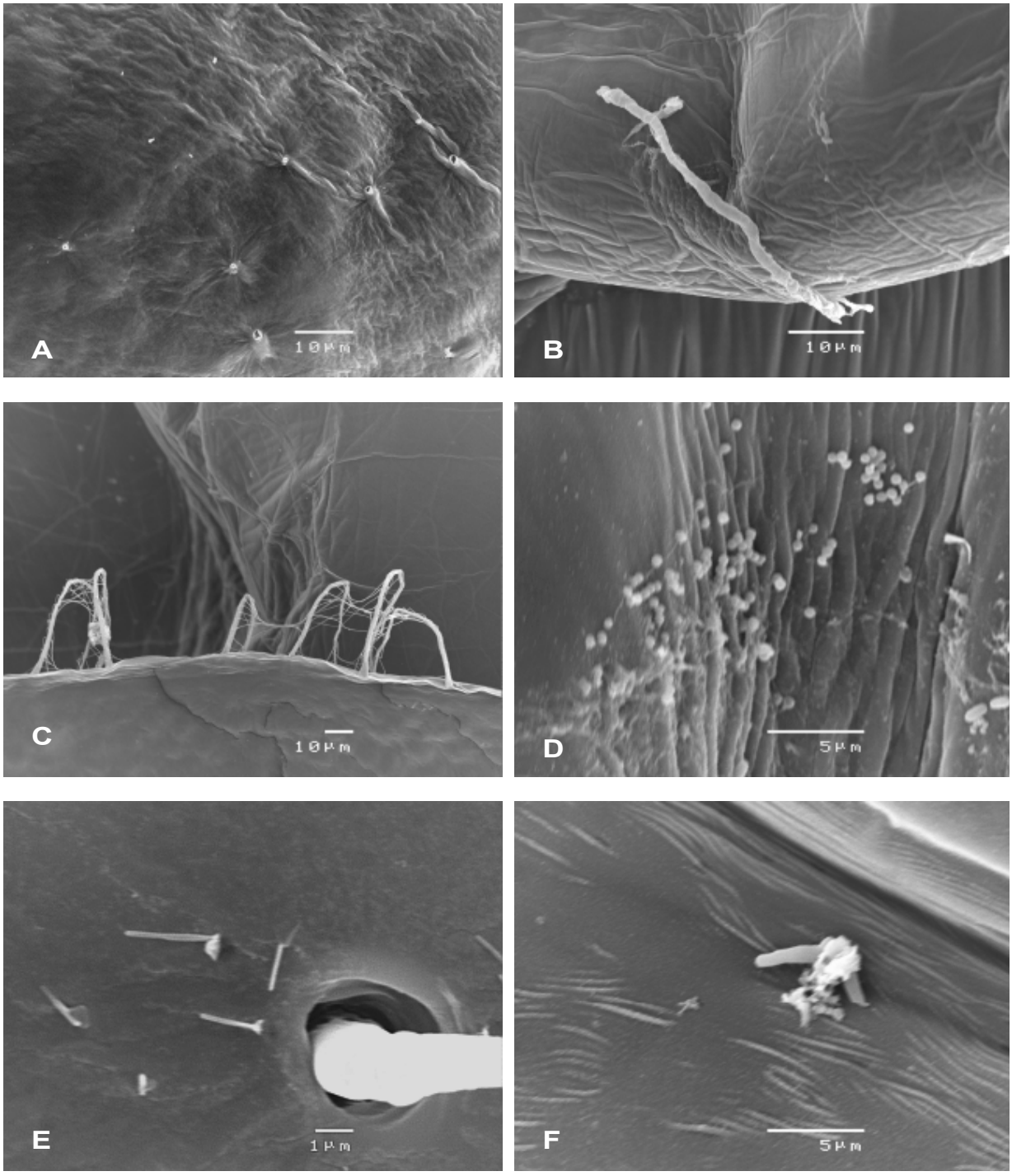


Figure 4.13 Combined display of the only examples of external microbial attachment found on wild *Panulirus* phyllosoma. ***A:*** sessile protozoa on the coxal endite; ***B:*** filamentous bacterium or fungus on the anus; ***C:*** fungus-like structures on the carapace; ***D:*** coccal bacteria above the anus; ***E:*** short filamentous structures on the coxal endite; ***F:*** short filamentous bacterium or fungus on the anus.

Internal colonization

Histology

Histology sections of wild phyllosoma demonstrated that the internal tract was intact and no tissue lesions or signs of infectious microbial colonization were present (Figure 4.14). The generated images illustrated minimal bacterial colonization within the animal tissue and confirmed subsequent FISH analysis (see below).

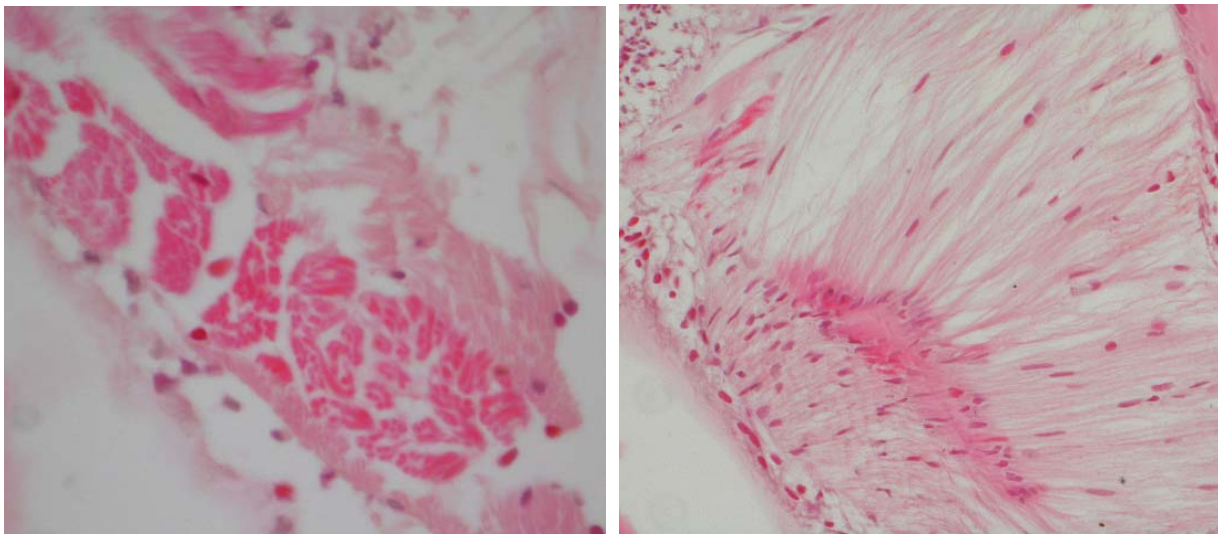


Figure 4.14 Light micrographs of representative histological sections of the intestinal tract of wild phyllosoma, illustrating intact hepatopancreas (left) and connective tissue (right) (40x magnification).

Fluorescence *in situ* hybridization

The degree of colonization varied between the investigated larvae, but overall few bacteria were visualized within the intestinal tract of wild phyllosoma. In addition, colonization varied across the tissue section; featuring areas without any proliferation, areas with low bacterial density and occasional cell accumulations. The estimated colonization, although difficult to assess, did not exceed dimensions of 10^3 bacteria per specimen. Due to increased Cy3 autofluorescence of phyllosoma tissue and repeatedly low bacterial signal from the EUB338 probe, cells were not always distinguishable from the tissue despite including a green channel scan to improve discrimination of background and probe fluorescence. The bacterial community structure was characterized by distinct numbers of α-*Proteobacteria* and γ-*Proteobacteria*. Members of the *Cytophaga-Flavobacterium* cluster and cell accumulations of especially β-*Proteobacteria* were repeatedly recorded. *Vibrionaceae* were demonstrated within the intestinal tract, but in low numbers. *Planctomycetes* were not detected in the wild phyllosoma. Fluorescent probes binding to the carapace lining did not detect external bacterial attachment. In wild phyllosoma, bacterial colonization was hence determined to be restricted to the digestive tract (Figure 4.15 a-c).

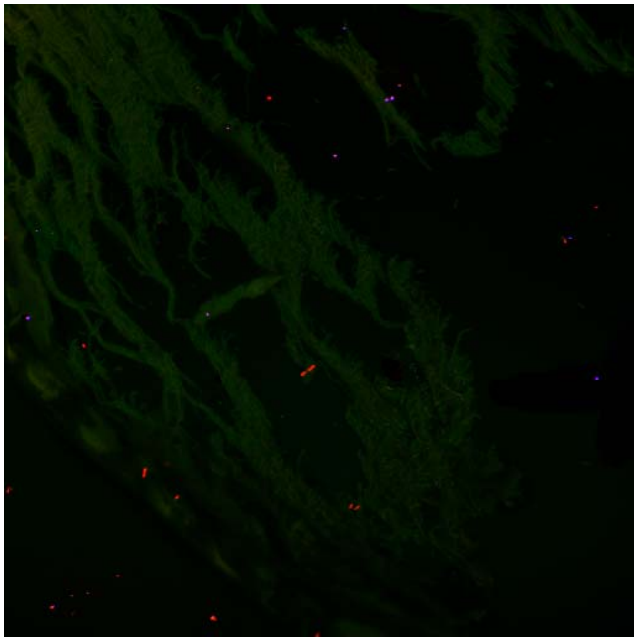


Figure 4.15 (a) γ-*Proteobacteria* (purple signals) and *Eubacteria* (red signals) colonizing internal connective tissue of wild Palinuridae phyllosoma.

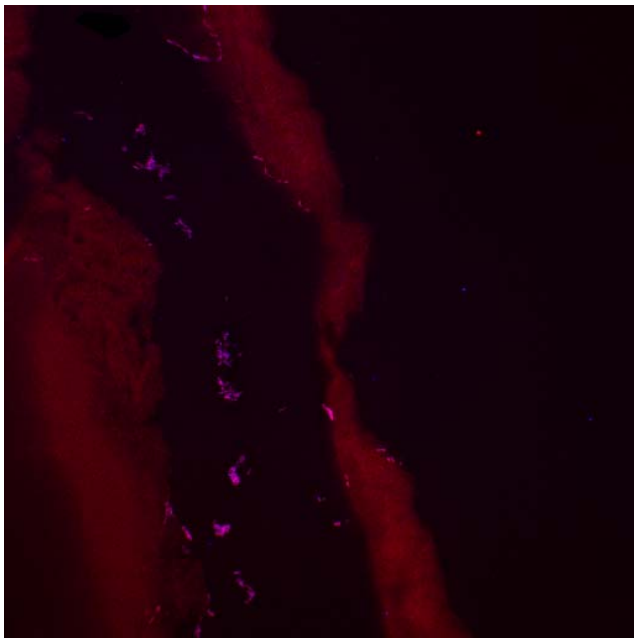


Figure 4.15 (b) Accumulation of β-*Proteobacteria* within the intestinal tract.

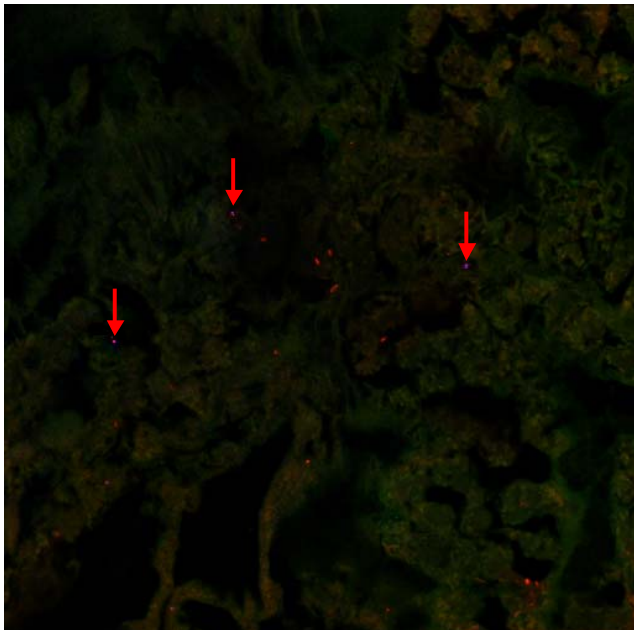


Figure 4.15 (c) Detection of *Vibrionaceae* in the hepatopancreas (arrows).

4.4 Isolation of potential probiotic bacteria from wild phyllosoma

4.4.1 Candidate strains

Thirty-five candidate strains were isolated from the total cultivable bacterial community of six wild Palinuridae and two wild Scyllaridae larvae.
All isolates and several other bacterial strains were screened, including two *Roseobacter* strains (K2 and K19), a *Vibrio alginolyticus* strain (C009), a *Sulfitobacter* strain (RR67) and the commercial probiotic product Sanolife Mic, incorporating three *Bacillus* strains. All candidates were tested for probiotic activity against a *Vibrio harveyi* strain (C071).

4.4.2 Screening for probiotic activity

Well diffusion agar assay

The ability of isolates to compete with and potentially inhibit a pathogenic strain was investigated using the well diffusion agar assay (WDAA).
With the lower pathogen concentration, 21 out of 35 phyllosoma-isolated bacterial strains demonstrated positive growth from wells into the surrounding agar, though in varying dimensions. Of the 21 positives, six strains displayed very strong growth even when inoculated against the higher pathogen concentration. For some of these strains, however, growth was not observed until five days after initial inoculation. The formation of inhibition zones was indicated for four strains (C4, C5, C16 and C22) seven days post incubation. The previously isolated *Roseobacter* (K2, K19) and *Sulfitobacter* (RR67) strains demonstrated positive growth, though only marginally and did not display pathogen inhibition. *Vibrio alginolyticus* C009 exhibited distinct growth and indicated clearing of pathogen after 7 days. Sanolife/*Bacillus* spp. showed the largest growth of all tested strains and yielded distinct clearing of the pathogen after six days of incubation.

Colony assay

In the WDAA, growth of the candidate strain into the agar could partly confuse the reading of clearing zones and the assessment of test strain inhibition. Hence, a colony assay was performed to complement the results of the WDAA.
With the lower pathogen concentration, 27 out of 35 of phyllosoma-isolated bacterial strains demonstrated positive growth on top of the agar. The higher pathogen concentration reduced the number of strongly growing strains to 16, though their growth was not observed until after

72 h post inoculation. Inhibition zones were formed by two strains, C1 and C12. This clearing of the target strain was observed after 24 to 48 h post inoculation, though did not increase further with longer incubations. The previously isolated *Roseobacter* (K2, K19), *Sulfitobacter* (RR67) and *Vibrio alginolyticus* C009 were demonstrating strong growth, but did not form inhibition zones of the pathogen. Sanolife/*Bacillus* spp. showed large growth as in the WDAA, but did not yield clearing as in the previous analysis.

Cell-free culture supernatant assay

This approach enabled the analysis of pathogen inhibition based on bacterial production of inhibitory compounds.
Only one strain, C27, distinctly yielded an inhibition of the target *Vibrio harveyi* strain. Three other strains (C17, *Sulfitobacter* RR67, Sanolife/*Bacillus* sp.) indicated a marginal inhibition effect.

4.4.3 Taxonomical identification of strains

Based on the above probiotic activity assays, 12 of the 35 bacterial strains isolated from wild phyllosoma were selected for sequencing of 16S rDNA genes to allow phylogenetic identification (Table 4.5). All strains were sequenced using the 27F bacterial primer. For several strains, sequencing was also performed using the reverse 1492R primer to obtain a longer sequence and improve the phylogenetic identification.

Table 4.5 Source, characteristics on Marine Agar and phylogenetic identity of bacterial isolates selected after probiotic activity assays.

Isolate label	Phyllosoma species	Colony morphology	Selected on the basis of	Closest relative (Database accession number)	Similarity (%)	Category
C1	*Panulirus ornatus*	yellow	Clearing of pathogen in colony assay	***Tenacibaculum mesophilum*** (AB032504)[a]	96	CFB
C4[b]	Scyllaridae	beige, faintly yellow	Significant growth and indication of clearing in WDAA	**Bacterium CWISO9** (DQ334347)[a]	100	γ-*Proteobacteria*
C5	*Panulirus longipes*	yellow-orange	Significant growth and indication of clearing in WDAA	***Pseudoalteromonas* sp.** (DQ985032)[a]	99	γ-*Proteobacteria*
C9	*Panulirus ornatus*	beige; granular colony surface	Significant growth	***Bacillus* spp.** (DQ234278)[c]	98	*Firmicutes*
C10	*Panulirus ornatus*	apricot	Significant growth	**Bacterium WP1OTU27** (DQ985896)	98	α-*Proteobacteria*
C12	Scyllaridae	dark green	Clearing of pathogen in colony assay	***Pseudoalteromonas citrea*** (X82137)[a]	97	γ-*Proteobacteria*
C16	*Panulirus longipes*	beige, faintly yellow	Significant growth and indication of clearing in WDAA	***Vibrio* sp.** (DQ868673)	100	γ-*Proteobacteria*
C17	*Panulirus longipes*	bright yellow	Indication of clearing in cell-free assay	***Cytophaga* sp.** (AB073588)[a]	99	CFB
C18	*Panulirus versicolor*	beige, faintly yellow	Significant growth	***Micrococcaceae* bacterium KVD-1921-02** (DQ490458)[a]	99	*Actinobacteria*
C19	*Panulirus versicolor*	yellow; very slow growing	Significant growth	***Micrococcus* sp.** (DQ238839)	91	*Actinobacteria*
C22	*Panulirus ornatus*	yellowish-beige	Significant growth and indication of clearing in WDAA	***Nocardioides* sp.** (DQ401093)[a]	99	*Actinobacteria*
C27	*Panulirus versicolor*	bright yellow	Clearing of pathogen in cell-free assay	***Winogradskyella poriferorum*** (AY848823)[a]	100	CFB

[a] Sequenced with 27F and 1492R. [b] Red labels designate sequences closely related to sequences previously found with wild phyllosoma. [c] No pure culture.

Four isolates (C4, C5, C12 and C16) affiliated with γ-*Proteobacteria*, while each three isolates (C1, C17 and C27) affiliated with the *Cytophaga-Flavobacteria* (CFB) group and another three isolates (C18, C19, C22) affiliated with *Actinobacteria* (High-GC Gram-positives). One isolate (C9) affiliated with *Firmicutes* (Low-GC Gram-positives) and the final isolate (C10) affiliated with α-*Proteobacteria* (Table 4.5).

Interestingly, 5 of the 12 isolates were closely related to 16S rDNA sequences previously found associated with wild phyllosoma (Payne et al., unpublished data).
Isolate C4 was closely related to an uncultured bacterial clone retrieved in the study of Payne et al. (unpublished) and showed further phylogenetic similarity to *Methylarcula* sp., a genus belonging to the *Roseobacter* clade. Isolate C9 was identified as being not pure and comprising several *Bacillus* strains, including *B. licheniformis*. This corresponded to the granular, compact structure of the isolate on Marine Agar that presumably represented a conglomerate of *Bacillus* spp. No single colonies were formed despite repeated streaking on Marine Agar, therefore not allowing separation and isolation of the individual strains.

The isolates within the group *Actinobacteria* included two *Micrococcaceae*-related strains (C18, C19) and a *Nocardioides* sp. related bacterium (C22). Whereas the *Micrococcaceae* affiliated isolates were represented by strong growth, the isolate with 16S rDNA sequence identity to *Nocardioides* sp. indicated an inhibitory activity towards *Vibrio harveyi*.

The isolates that distinctly inhibited growth of *Vibrio harveyi* in the probiotic screening (C1, C12 and C27) were identified as being closely related to *Tenacibaculum mesophilum*, *Pseudoalteromonas citrea* and *Winogradskyella poriferorum*, respectively. *T. mesophilum* and *W. poriferorum* are affiliated with CFB, *P. citrea* is affiliated with the γ subclass of *Proteobacteria*.

Besides *T. mesophilum* and *W. poriferorum*, the third isolate affiliated with CFB was closely related to *Cytophaga* sp. (C17). Sequence alignments interestingly showed a further phylogenic relationship to *Tenacibaculum cellulophagum*, *T. lutimaris* and also *T. mesophilum*.

Other γ-*proteobacterial* affiliated isolates besides *P. citrea* were a further *Pseudoalteromonas* sp. (C5) and two closely related, *Vibrionaceae*-affiliated strains (C4, C16). Both had been previously found associated with wild phyllosoma and had phylogenetic similarity to *Vibrio alginolyticus*, *V. parahaemolyticus* and *V. natriegens*.

5. Discussion

5.1 Effect of seawater ozonation on development and composition of biofilm within larval rearing tanks

The effect of seawater ozonation of biofilm composition and development in small-scale larval rearing tanks was studied using microscopical (FISH and SEM) and molecular analyses (DGGE).

The experiment was conducted outside reproduction and spawning periods of captive *Panulirus ornatus* broodstock. Although experimental tanks did not contain phyllosoma, they did contain *Artemia* live feed, which was added on a daily basis in the same manner as if phyllosoma would have been present. During a standard phyllosoma rearing trial, *Artemia* are added to each tank in an *Artemia*:phyllosoma ratio of 60:1, and hence the *Artemia* are by far the largest contributor to biological loading in the rearing tanks. As the research was primarily focussed on the differences in biofilm development after ozonation of seawater in the presence of biological loading, the absence of phyllosoma was not critical to the experimental outcomes. In addition, the study was focussed on the source of introduction of detrimental bacterial strains into the larval rearing system. This addressed both the role of the incoming seawater and the *Artemia* live feed, which are two critical potential vectors of pathogens entering into the larval rearing system (see 5.3.2).

5.1.1 Stages of development and trends in the community structure

In this study, fluorescent *in situ* hybridization techniques were used to follow the stages of biofilm development within larval rearing tanks. The dynamics of the biofilm bacterial community were elucidated using rRNA oligonucleotide probes targeting higher taxonomical groups of bacteria including the α-, β- and γ-*Proteobacteria*, *Vibrionaceae*, *Planctomycetes*, and the *Cytophaga-Flavobacterium* cluster of the CFB phylum. *Thiothrix* was the only genus specifically targeted in FISH. Molecular-based results derived from denaturing gradient gel electrophoresis, which discriminate at the genera and species level (see 5.1.2), were separated from this chapter to articulate the two hierarchical levels, but also since DGGE data was generated from only two sampling points during biofilm development.

Biofilm development in non-ozonated and ozonated seawater occurred in the five stages according to Characklis (1981). Ozonation influenced several aspects of the biofilm development dynamics within the larval rearing tanks.

Stages 1 and 2 – Matter transport and first bacterial adhesion. The initial stages of biofilm development occurred within two days of commencement of the experiment. In the first developmental stage, organic matter was transported to the surface of slides, which was represented by the observed attachment of particulate matter. Surface-attached particles likely facilitated initial bacterial cell attachment, mainly by reduction of micro-flow turbulences but potentially also by presenting a nutrient source. A potential nutrient source included *Artemia* faeces, though the nature of particles, whether organic or inorganic, was not evaluated. Stage 1 was rapidly followed by Stage 2 with adhesion of planktonic bacterial cells to the surface of slides. The transition from stage 2 to stage 3 was marked by the appearance of isolated, randomly distributed bacterial clumps on day 3. Ozonation influenced the biofilm development in following stages 3, 4 and 5 by impacting on the duration and time of initiation of these respective stages (Figure 5.1), as well as by modification of the microbial community structure. These stages will therefore be discussed in detail.

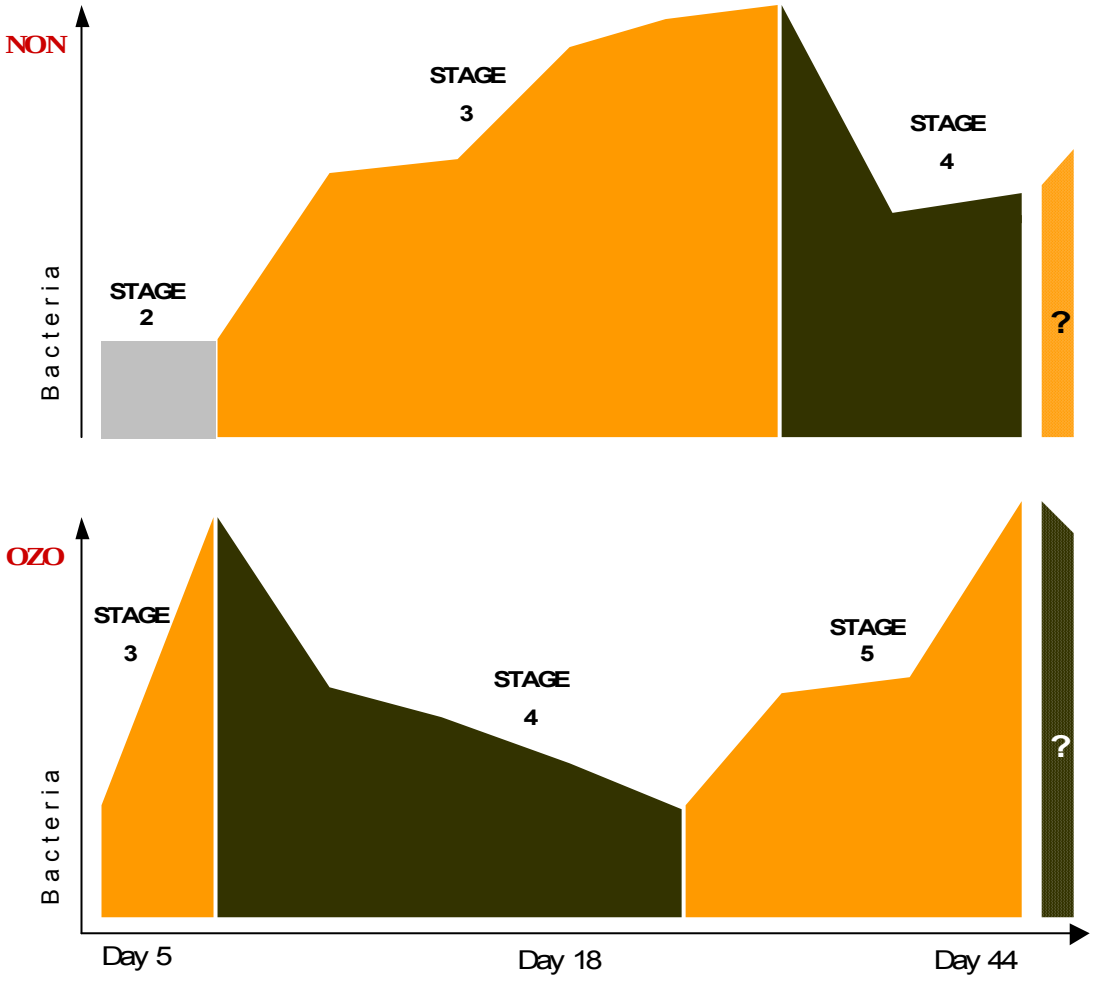


Figure 5.1 (modified after Fig. 4.1) Illustration of the effect of seawater ozonation (OZO, bottom) on biofilm developmental stages 3 to 5 in relation to non-ozonated water (NON, top) The column labelled with '?' indicates the proposed continuation of development, according to the cyclic pathway of biofilm formation.

Stage 3 – Reproduction. Stage 3 in non-ozonated water characterized the progress of biofilm build-up without a decontaminating water treatment. The process was illustrated by an increasing dominance of α-*Proteobacteria* in association with a slowly progressing build-up of biofilm. A dominance of α-*proteobacterial* affiliated bacteria supports previous observations of changes in bacterial diversity in 5,000 litre phyllosoma rearing tanks (Bourne et al., 2006). A significant presence of this subclass of bacteria has also been reported in other aquaculture facilities (Arias et al., 2006). One factor which likely promoted the biofilm establishment and growth of α-*Proteobacteria* is the ability of many marine α-*Proteobacteria* to produce quorum sensing compounds (Wagner-Döbler et al., 2005).
Ozonation of water restricted growth of α-*Proteobacteria*, and instead promoted proliferation of γ-*Proteobacteria* that increased massively after the early initiation of stage 3. The build-up of biofilm progressed faster after ozonation and high bacterial densities were obtained more rapidly. The constant biofilm density in non-ozonated water tanks during this phase suggested that the non-ozonated biofilm was still in stage 2 of development (Figure 5.1).
The original hypothesis of this study was that seawater ozonation would result in the lowering of bacterial densities in the seawater and therefore inhibition of biofilm development. Three concepts were possible to explain this apparent paradox.

(i) Ozonation yielded the breakdown of complex dissolved organics into smaller molecules (Janssen et al., 1984; Glaze, 1987), which were more readily assimilated by the bacteria as a primary nutritional source. At the same time, this increased the transport of organic matter to the surface, which likely facilitated attachment and proliferation of planktonic cells. In addition, ozonation changed the water chemistry – e.g. by its influence on nitrification – which altered the selection pressure and triggered different ways of bacterial growth and adaptation.

(ii) Ozonation could have further increased the organic load of the water through the release of nutrients from ozone-destroyed bacterial cells. This would have magnified the effects described in 1. and further promoted bacterial proliferation.

(iii) Ozonation removed most planktonic bacteria from the seawater. This could have benefited surviving populations or founder populations emanating from *Artemia* in surface attachment and subsequent reproduction. This was likely interconnected with the increased nutrient availability resulting from 1 and 2. The benefit could be compared to antibiotic-induced knock-out of bacterial communities and consequent promotion of 'ozone-resistant' strains. In contrast, non-ozonated seawater was probably incorporating a complex planktonic

bacterial community with intense competition, which restricted rapid growth of distinct genera or subclasses.

Interactions and feedback loops associated with the proposed effects of ozonation on stage 3 of biofilm development are likely. However, more detailed studies are required to elucidate the exact mechanism that lead to the promotion of γ-*Proteobacteria* during the build-up of biofilm in ozonated water. The above theories imply that ozonation also altered the first and second stage of biofilm development, even though this was not observed at these time points. It was likely that these early effects of ozonation promoted the significant growth of γ-*Proteobacteria*, which opportunistically took advantage of the changed growth conditions. Such growth characteristics could classify the involved strains as r strategists, which are characterized by high reproduction rates and both a rapid exploitation of resources and build-up of large, but unstable populations. Once attached in higher numbers to the surface, these bacteria effectively blocked settlement by other bacteria. It was possible that this effect was enhanced by the superior metabolic rates of the r strategists, which controlled the proliferation of planktonic bacteria.
An observable increase in surface-covering matter on the biofilm slides in ozonated water indicated increased bacterial production of polymers, such as EPS, within stage 3 of development. SEM analysis may have provided further supporting evidence for increased EPS attached to the surface, but this analysis failed (see 5.1.4). Although accumulation of polymers is generally thought to enhance protection of bacterial cells and promote further growth (Costerton, 2004), the observed gradual increase in surface coverage was interestingly associated with a continuous reduction of cell numbers. Stages 3 and 4, build-up and detachment, hence appeared to overlap after ozonation.

Stage 4 – Detachment. The early initiated detachment of ozonated water biofilm suggested that due to the fast build-up of biomass, limiting effects on the biofilm population were soon induced. This was possibly attributed to the increasing shortage of surface space and/or availability of nutrients. This highlighted the r strategy hypothesis of the biofilm-forming strains, characterized by rapid consumption of available resources and enhanced reproduction rates. At the same time, the resulting dominance of a single subclass was probably leading to a low community stability. The greater structural heterogeneity of non-ozonated water biofilm presumably stabilized and balanced the microflora, which could have been enhanced by metabolic interactions between sub-populations. Structurally more heterogeneous biofilm was characterized by a slower build-up (18 days) and by a longer period with high bacterial density (10 days). The slower progress of stages could classify the involved bacterial species in non-ozonated water biofilm as K strategists, which are

characterized by lower reproduction rates, more sustainable use of resources and build-up of stable populations. Although a dominance of bacterial subclasses were recorded as well, these were not as pronounced as observed with ozonated water biofilm samples.

Ozonation significantly delayed the onset of filamentous bacterial proliferation when compared to biofilm within non-ozonated water. Despite this delay, bacterial filaments did establish, and this occurred when biofilm detachment was beginning. Later dominance of filamentous bacteria indicated a beneficial growth mode during the detachment stage. Filaments withstood the ongoing biofilm detachment unlike rod-shaped bacteria that were largely eliminated from the community, together with most of the EPS matrix. Scattered patches of EPS and distinct numbers of long filaments remained after detachment.
It seems likely that the filaments were strongly bound to the surface, enabling them to withstand sheer forces and providing increased resistance against detachment. The filamentous, flexible morphology potentially further increased protection, as 3D *daime* image analysis revealed protruding cells which likely possessed the ability to bend with the water flow. In addition, filamentous growth potentially increased the nutrient uptake by presenting a larger surface area to the water flow. This could have allowed these bacteria to out-compete the small, two-dimensionally scattered rod-shaped cells.
With respect to *Thiothrix*, the increasing detection of single signals could have represented the initial stage of *Thiothrix* growth, which starts from spore-like, so-called gonidia (Larkin and Strohl, 1983). Ozonation appeared to limit their attachment, though low numbers of *Thiothrix* were observed. This indicated that the genus was able to attach to an inorganic surface despite their general association with biological surfaces (Polz et al., 1994). Although elongation and growth of filaments appeared not to be favoured in biofilm association, detachment of gonidia, colonization of the *Artemia* or phyllosoma carapace and following elongation was still possible.

Stage 5 – Re-establishment. Re-establishment of the biofilm community following detachment was only observed after the ozonation treatment. During this process, organic particles remaining on the surface of the slides may have served as a nutrient source and probably facilitated the attachment and reproduction of newly settled cells. This mirrored the observations from stage 1, where initial bacterial adhesion was increased in areas with surface-associated matter.
The re-availability of surface area permitted the attachment of planktonic populations which had been preliminary excluded from settling. This was illustrated by the community diversification during initial biofilm reconstitution, before γ-*Proteobacteria* again started to

proliferate. However, the dominance of this subclass during re-establishment of the biofilm was not as pronounced as in stage 3.
No distinct re-establishment of biofilm was observed within non-ozonated water. Since the progress of developmental stages was delayed with cell sloughing and detachment beginning later, it is likely that biofilm regrowth would have been observed within this water type as well, if the experimental period had been extended. This was supported by a slight increase in cell numbers from day 36 to 44.

5.1.2 Phylogenetic characterization of the community

Whereas FISH analysis surveyed the bacterial community structure on a higher taxonomical level, the molecular-based DGGE bacterial profiling and sequence analysis enabled allocation of dominant sequences to phylogenetic relatives on the genus level. Through DGGE, trends in structural composition could be assigned to discrete sequences, which were positioned in an upper hierarchical context.

General remarks

For both non-ozonated and ozonated treatments, the microbial community as classified by molecular analysis generally reflected the structure as determined by microscopical observations. The bacterial groups dominant in FISH analysis were also dominant in the DGGE bacterial profile, with only minor variations.
The biofilm bacterial profiles of non-ozonated and ozonated water sampled on days 21 and 44 were each dominated by each three bands affiliated with α-*Proteobacteria* and a further three bands affiliated with γ-*Proteobacteria*. FISH analysis detected a dominance of α-*Proteobacteria* for non-ozonated water and γ-*Proteobacteria* for ozonated water. This was not exactly reflected in DGGE; however DGGE analysis is limited by PCR bias and therefore does not provide quantitative indications of bacterial group dominances.
DGGE analysis retrieved a band affiliated with δ-*Proteobacteria*, though this subclass was not targeted with rRNA specific FISH probes. In addition, a dominant band was found in the biofilm of both water types that was affiliated with the *Planctomycetales* order. The retrieval of this sequence was not surprising within non-ozonated water, since FISH showed that *Planctomycetes* constituted one of the three most dominant groups at that time of biofilm development. However, the detection of a similarly dominating sequence in ozonated water biofilm was unexpected, since the contribution of *Planctomycetes* was below 10% at that time point. The sufficient coverage of the *Planctomycetes* targeting oligonucleotide probes used in this study (Neef et al., 1998) however precluded a misinterpretation of FISH results.

Slide and swab profiles from ozonated water on day 44 showed a clear distinction in the affiliation of the dominant bands. Whereas the profile from slide biofilm only comprised sequences affiliated with γ-*Proteobacteria*, the swab profile only comprised α-*proteobacterial* affiliated bands. This indicated the presence of distinct niches that favoured the proliferation of each subclass.
Interestingly, four of the five dominant retrieved sequences of the biofilm profile of ozonated water on day 44 were closely related to isolates from the Mediterranean coral *Oculina patagonica* (Karen and Rosenberg, unpublished data). The coral is studied for bleaching caused by infections of the species-specific pathogen *Vibrio shiloi* (Rosenberg et al., 1998). Bacterial infection as a cause for coral bleaching has also been suggested for tropical coral populations of the Great Barrier Reef. It was interesting that late-stage ozonated water biofilm was dominated by three bacteria affiliated with γ- and two bacteria affiliated with α-*Proteobacteria*, which were closely related to clones isolated from a single coral species. It was however unclear why these bacteria appeared to be promoted through ozonation.

The phylogenetic sequence relationships of bands from the biofilm profiles revealed interesting information which is presented in more detail in the following section.

Non-ozonated water

Band 1, which was dominant on day 21, showed a 93% similarity to a bacterium found in the Great Barrier Reef, which indicated a regular occurrence of those related strains in Australian waters. Furthermore, this sequence was also affiliated with several isolates from the coral *Oculina patagonica* (see above), but not to clones obtained in bleaching studies of the species. The sequence was also positioned close to sulfur-associated bacteria by its phylogenetic relationship to *Acidithioophilus ferrooxidans* (Hallberg, unpublished data).
The closest relative of the *Planctomycetes*-affiliated band 6 was interestingly found abundant in a study on accelerated sulfur cycle in coastal marine sediment beneath areas of intensive shellfish aquaculture (Asami et al., 2005). However, no linkage of *Planctomycetes* and sulfur metabolism has been established to date. An involvement in sulfur metabolism was also indicated for the sequence retrieved from band 7, which demonstrated 87% sequence identity to *Rhodanobacter thiooxidans*. *R. thiooxidans* has previously been found associated with sulfur-autotrophic denitrification and was interestingly isolated from biofilm (Lee and Lee, unpublished data).
The dominance of the *Rhodobacter* affiliated band 9 in non-ozonated water biofilm on day 44 incorporated several interesting aspects. Genera from the *Rhodobacteraceae* family – which also comprises the *Roseobacter* clade – were investigated regarding their contribution to toxin production of a dinoflagellate (Green et al., 2004). This could suggest the involvement

of α-*Proteobacteria* in raising *Artemia* mortalities as they have been observed within non-ozonated water tanks. Furthermore, the involvement of *Rhodobacteraceae* in anoxic, nitrate-dependent sulfide oxidation in aquaculture systems has been proposed previously (Cytryn et al., 2005) linking the sequence retrieved from the biofilm to the sulfur-metabolizing bacteria. Interestingly, the BLAST alignment also showed 96% similarity to a *Rhodovulum* sp., of which a close relative was retrieved previously from the biofilm in a large-scale rearing tank (Bourne et al., 2006).

Ozonated water

The γ-*proteobacterial* affiliated sequence of band 10, besides to its relation to an isolate from *Oculina patagonica*, was also closely related to a *Pseudomonas* strain. This could classify the bacterial species as a potent biofilm-forming bacterium, since *Pseudomonas* is well-known for its biofilm formation potential (see 1.2.1).

The γ-*proteobacterial* affiliated sequence of band 12 retrieved from slide biofilm on day 44 was also closely related to two different agarolytic bacteria, which are capable of cleaving polysaccharide chains. This suggested that the bacterium corresponding to this DGGE band played a potential role in the earlier observed EPS polysaccharide degradation. This was highlighted by additional 95% similarity to a *Microbulbifer* sp. that was used for studies of a novel agarase gene (Han et al., unpublished data).

The α-*proteobacterial* affiliated sequences of bands 12 and 16 both showed close phylogeny to *Roseobacter* species. The colonizing characteristics of *Roseobacter*-affiliated strains positioned those sequences in proximity with potent biofilm formers, similar to the above *Pseudomonas*-related sequence. *Roseobacter*-affiliated species were also dominant in previous studies of biofilm composition in large-scale phyllosoma rearing tanks (Bourne et al., 2006). These sequences were furthermore related to several clones isolated from *Oculina patagonica*, respectively (Karen and Rosenberg, unpublished data).

The sequence of band 17, related to an uncultured α-*Proteobacterium*, was affiliated to a clone found associated with a diseased coral whose infection was significantly linked to proliferation of α-*Proteobacteria* (Sekar et al., 2006). Furthermore, the retrieved sequence also demonstrated 92% sequence similarity to *Hyphomicrobium sulfonivorans*, which is capable of utilizing methylated sulfuric compounds such as the algal osmolyte DMS (De Bont et al., 1981). As for non-ozonated water, the presence of a sequence affiliated with sulfur-metabolizing bacteria suggested an active sulfur metabolism for ozonated water as well. The less obvious smell of sulfide from biofilm in ozonated water tanks compared to biofilm from non-ozonated tanks still indicated the repression of microbial sulfur metabolism in ozonated water tanks.

5.1.3 Pathogenic bacteria

High mortalities of *Artemia* cultures recorded during the non-ozonated trial from day 18 onwards were likely attributed to a proliferation of pathogenic bacteria. Such high *Artemia* mortalities were not observed in ozonated water tanks, indicating some form of inhibition or reduction in pathogen load; or alternatively improvement in *Artemia* health. The continued high mortalities of *Artemia* throughout the experiment in non-ozonated water suggested that day-to-day variations in the general health status of *Artemia* cultures could not account for these results. Three bacterial groups could be considered as biofilm-residing pathogens of *Artemia*; α-*Proteobacteria*, γ-*Proteobacteria*, and *Vibrionaceae* (within the γ-*Proteobacteria*). An alternative explanation addressed differently progressing *a priori* infections of on-grown *Artemia.* This would have been feasible, since the same culture of 7-day on-grown *Artemia* was distributed to all rearing tanks and soon displayed a differentiated health status.

α-*Proteobacteria*

Biofilm-mediated evolvement of *Artemia* mortalities could have been caused by α-*Proteobacteria* whose increasing relative contribution in non-ozonated water biofilm corresponded to raising mortalities among *Artemia*. The distinct proliferation could have been enhanced by the simultaneous breakdown of competing strains, potentially represented by γ-*Proteobacteria* reaching an overall low in biofilm contribution during this phase. Suddenly lacking competition and increased availability of surface space could have enabled opportunistic bacteria to rapidly proliferate on free surfaces. The potential of opportunistic (pathogenic) bacterial strains to quickly react to and take advantage of favourable environmental conditions would have been underlined accordingly.

The distinct association of α-*Proteobacteria* with the biofilm suggested the mediation of pathogenicity via a surface-bound mechanism, possibly by a release of exotoxins. The limited information pathogenic α-*Proteobacteria* however pointed to their exclusion as the causative agents for *Artemia* mortalities. In addition, no linkage between pathogenic α-*Proteobacteria* and biofilms has been established to date. It was furthermore not clear why the later increase of this subclass within ozonated water biofilm did not similarly yielded *Artemia* mortalities. It could be hypothesized that ozonation prevented pathogenic α-*Proteobacteria* from exceeding a critical value within the biofilm.
Among the rare reports of pathogenic α-*Proteobacteria* is the description of *Aurantimonas coralicida* as the causative agent of a coral-associated plague type disease (Denner et al., 2003). Edgerton and Prior (1999) detected a rickettsial-like α-*Proteobacterium* in cultures of the Australian freshwater crayfish (*Cherax quadricarinatus*) that infected the hepatopancreas

of animals. Other classified pathogenic *Rickettsiales* comprise the fish pathogens of the *Piscirickettsia* group, as reviewed by Fryer and Hedrick (2003). However, for their obligate intracellular nature, *Rickettsiales* could be excluded as biofilm-associated microorganisms (Fryer et al., 1992).

Another, interesting interpretation positioned the pathogenic action of α-*Proteobacteria* in context with other, non biofilm-associated organisms. α-*Proteobacteria* were commonly found with toxic dinoflagellates which implied a direct or indirect pathogenicity by either toxin production or the contribution to such processes (Silva, 1982; Kodama et al., 1988). In this context, α-*Proteobacteria* of the *Rhodobacteraceae*, of which closely related strains were detected in non-ozonated water biofilm, were investigated regarding their contribution to dinoflagellate production (Green et al., 2004, see above) of the paralytic shellfish poison saxitoxin (Shimidzu, 1977). Hypothetically, *Rhodobacteraceae*-affiliated α-*Proteobacteria* could have indirectly caused mortalities by enhancing the toxicity of certain species. The necessary introduction of non-bacterial, toxin-producing organisms such as dinoflagellates would point to a co-cultivation mechanism via *Artemia*.

γ-Proteobacteria

The decreasing biofilm contributions of *γ-Proteobacteria* could have been attributed to the detachment of viable cells from the biofilm. The variation in biofilm contribution of *Vibrionaceae* could have (partially) expressed this *γ-proteobacterial* sloughing (see below). Planktonic *γ-Proteobacteria* could have provoked a direct effect on *Artemia* by their subsequent colonization.

Vibrionaceae

Although detected in low levels in the biofilm, the variations in relative contributions over time suggested an ongoing change of the survival strategy of vibrios by switching between a biofilm-associated and planktonic existence. This pointed to an increased adaptability and potential variability in the growth mode, thus indicating opportunistic characteristics.

Biofilm detachment increased their concentration in the water column and hence influenced other compartments. *Artemia* were shown to be prone to infection by *Vibrio* (see 4.2) and it seems likely that vibrios detaching from the biofilm could quickly colonize freshly added *Artemia*. Furthermore, also the distinct *Vibrio* load of on-grown *Artemia* was demonstrated. Therefore it was possible that ozonated water inhibited proliferation of pathogenic bacteria and expression of virulence determinants associated with on-grown *Artemia*. However, no such control was exhibited for non-ozonated water, and therefore mortalities of *Artemia* were higher.

5.1.4 Scanning electron microscopy

The inability to obtain data from electron microscopy samples was likely caused by artefacts associated with the preparation of the biofilm samples, which is often complicated due to high water content (Chenu and Jaunet, 1992). Inaccurate dehydration of biofilm could have yielded disturbance of samples. Nevertheless, successful experiments have already been performed using the same fixation and preparation protocol (Bourne et al., 2006), which were however conducted within circular 5,000 litre tanks. The lower water mass and decreased flow rate within the 16 litre raceway rearing tanks probably facilitated surface settlement of particles, leading to the observed effect.

5.1.5 Conclusions

Ozonation of seawater impacted on three aspects of biofilm development and composition within small-scale larval rearing tanks. The results of the present study supported previous observations of biofilm formation in large-scale 5,000 litre rearing tanks (Bourne et al., 2006) and suggested a general pattern of biofilm formation in association with the establishment of a specific microbial community composition, if no pre-treatment of the culture water is performed.

(i) Stages of development

Although the biofilm development within both non-ozonated and ozonated water appeared to proceed according to the cyclic pattern, ozonation distinctly influenced developmental stages 3 to 5. Ozonation resulted in early on-set of bacterial reproduction and rapid biofilm build-up (stage 3). The biofilm reached its maximum bacterial density on day 9 and accumulation of this biomass was occurring within 4 days, whereas biofilm in tanks containing non-ozonated water took a build-up period of 11 days to establish a comparable biomass and had its highest density on day 20.

Following the peak of bacterial density, biofilm within ozonation water tanks detached (stage 4), a process observed over an 11 day period. Following this detachment or sloughing, the biofilm re-established (stage 5). In contrast, biofilm development within non-ozonated water tanks displayed very different characteristics. Stage 4 of biofilm development was beginning later and the detachment or sloughing was delayed by approximately 15 days compared to a previous study within non-ozonated water (Bourne et al., 2006). This could potentially be attributed to a decreased water flow within the raceway rearing tanks used in this study creating less physical shear to remove established biofilms. However, when detachment did occur it progressed faster than in the ozonated water, lasting only 8 days before the

commencement of further biofilm growth. However, this re-establishment of new biofilm could not be confirmed due to the termination of the experiment.

(ii) Biofilm appearance and bacterial community structure

Ozonation distinctly changed the structural appearance of the biofilm. Whereas the biofilm was initially dominated by rod-shaped cells, filamentous bacteria established on day 14 and dominated the observed bacterial morphologies during the following phase of lowest bacterial density, until rod-shaped bacteria re-established at stage 5. Within non-ozonated water, filaments established in the biofilm 9 days earlier, but did not dominate observed bacterial morphologies, which also featured similar proportions of rod-shaped cells.

Ozonation distinctly modified the bacterial community structure, as represented by a dominance of γ-*Proteobacteria* throughout the entire experimental period. This was reflected by molecular analyses, which showed similar dominant bands affiliated with the second most represented group, α-*Proteobacteria*. However, no retrieved DGGE sequences which dominated the biofilm in this study were related to previously retrieved sequences from biofilm studies of phyllosoma larval rearing tanks (Bourne et al., 2006).

It is suspected that ozonation treatment caused changes in the planktonic community structure, which subsequently affected the biofilm formation both directly and indirectly. Direct effects included the removal of most viable bacteria and promotion of 'ozone-resistant' strains. These probably formed an unstable population of mainly r strategists, which were able to rapidly exploit the higher load of organics and nutrients provided through ozonation. Furthermore, founder populations introduced by *Artemia* could have proliferated differently between the two water types. Indirect effects based on changes in the water chemistry, which altered the selection pressures, likely triggering different ways of bacterial adaptation mechanisms.

In contrast, biofilm in non-ozonated water tanks was characterized by a greater homogeneity in the community structure, and hence consisted of a presumably more balanced community. The slow build-up of non-ozonated water biofilm suggested a majority of K strategists. Nevertheless, the dominant subclasses observed by FISH analyses were represented by r strategic bacteria such as γ-*Proteobacteria* in early- and α-*Proteobacteria* in middle-age biofilm. This was also reflected by molecular analysis, demonstrating dominating sequences of both subclasses in the biofilm profile.

(iii) Establishment of pathogens

Ozonation potentially removed or inhibited strains with pathogenic capabilities, since *Artemia* mortalities were higher in non-ozonated water. The pathogenicity could have been related to the biofilm, accounting for the proliferation of α-*Proteobacteria* that released exotoxins or the

sloughing of viable pathogenic γ-*Proteobacteria* into the water column that subsequently infected *Artemia*. Furthermore, bacterial interactions with toxic microorganisms as well as the *Vibrio* load of on-grown *Artemia* themselves can be considered as a cause for the emerging *Artemia* mortalities.

Although *Vibrionaceae* were minor components of the biofilm both within non-ozonated and ozonated water, their presence showed the general potential of biofilms to harbour potential pathogens. However, no distinct increase of *Vibrionaceae* in the biofilm, as observed by Bourne et al. (2006) in later days of the experiment, was observed in the current study. This difference could potentially be due to the presence of phyllosoma in rearing trials of the previous study, with *Vibrio*-affiliated sequences demonstrated to dominate around mass larval mortalities. Massive proliferation of opportunistic vibrios was presumed to occur around induced stress periods of cultured phyllosoma. This suggests that the external factors in the current study were different and did not induce a massive proliferation of potential pathogenic vibrios. Even if vibrios accounted for the *Artemia* mortalities in this current study, total numbers may still have been low and were therefore not detected readily, especially in the biofilm.

5.2 Microbial colonization of phyllosoma

External colonization of reared phyllosoma

High larval mortality associated with early stage cultured *Panulirus ornatus* phyllosoma is a major hurdle in the development of a successful aquaculture industry for these animals. External microbial colonization of the larval surface is believed to play an important role in larval mortalities (Bourne et al., 2004). Therefore, this study aimed to investigate the microbial colonization of captive-reared, early-stage (P1 to P2 and P3 to P4) phyllosoma; and to compare the degree of larval colonization in captivity with that found with wild phyllosoma.

The microscope-based analysis of reared phyllosoma highlighted the complex microbiota and extensive problems affiliated with external colonization of larvae reared in non-ozonated water. The bacterial colonization was enhanced at areas with high nutrient loading, and this included mouthparts entangled by filamentous bacteria and attachment of rod-shaped bacteria at the anus. Moult shedding was demonstrated to be effective in the removal of externally attached organisms, and the newly developed carapace demonstrated to be free of colonization.

Comparison of captive-reared P1-P2 and P3-P4 larvae revealed vast differences in the type and extensiveness of bacterial colonization. It has been observed that later-stage *Panulirus ornatus* larvae were capable of establishing innate immune mechanisms (Michael R. Hall, personal communication). Accordingly, delayed initiation of colonization within the P3-P4 stage phyllosoma could be linked to (i) the production of antimicrobial compounds, (ii) an improved immune system and/or (iii) internally associated, beneficial bacteria of the older phyllosoma. The latter has been reported for larvae of the Atlantic halibut (*Hippoglossus hippoglossus*), where larva-associated bacteria were inhibiting growth of several pathogens (Bergh, 1995). If such mechanisms exist for P3-P4 phyllosoma, these could however not prevent the P3-P4 larvae from featuring higher colonization than P1-P2 larvae at the end of the larval stage. This was probably attributed to the progressively increasing bacterial load of non-ozonated water, which was shown in previous analyses of the water column (Payne et al., 2006) and also indicated through conclusions drawn from biofilm development in the present study.

It was valuable to compare the external colonization of captive-reared larvae with that of wild larvae. The observations of a nearly completely missing external microflora on wild larvae essentially highlighted the connection between the closed rearing environment and the enhancement of external microbial colonization.

Internal colonization of wild phyllosoma

This study investigated the bacterial colonization of the digestive tract of wild, presumably disease-free phyllosoma. The internal bacterial density was very low comparing to previous studies with cultured larvae, where severe proliferation of particularly *Vibrionaceae* within the intestinal tract was linked to hepatopancreatic lesions and mass mortalities (Bourne et al., 2004; Webster et al., 2006).

The composition of the intestinal microflora could have reflected the bacterial community in the respective sampling environment. However, phyllosoma presumably encounter varying microbial communities during their extensive pelagic drift. It may be hypothesized that wild phyllosoma acquire specific microbial groups that benefit animal health, and that therefore these animals possess a typical microflora. This process would be based on the larval characteristics as a bacterial habitat, which may be more suitable for distinct bacterial groups while excluding colonization by others; for instance planktonic bacteria not adaptable for evading from the water column. The establishment of a typical microflora implies the possible beneficial effects on the larval health, and may identify the involved bacteria as probiotic.

The previous observations of α-*Proteobacteria* dominating the bacterial flora of wild phyllosoma (Payne et al., unpublished data) was only partially reproduced by the FISH analyses of wild phyllosoma sections. Although the presence of α-*Proteobacteria* was demonstrated, the community was diverse and comprising similar numbers of γ-*Proteobacteria*. In addition, *Cytophaga-Flavobacteria* and β-*Proteobacteria* were observed, though lower in numbers. It was plausible that the FISH-based observations would not exactly reflect the results by Payne et al., since the previous study was mostly based on analysis of clone libraries.

Fluorescence *in situ* hybridization detected *Vibrionaceae* within the hepatopancreas of wild larvae, which supported the proposed association of this genus with the natural microflora. *Vibrio* affiliated sequences were also retrieved from clone libraries (Payne et al., unpublished data), although in minor proportions which was in accordance to their low abundance demonstrated by FISH. The occurrence of *Vibrio* sp. with wild, disease-free larvae underlined the dual character of the genus; as a general member of the microbiota in the natural environment, but also as an opportunistic potential pathogen under stressful conditions. Nevertheless, it is possible that a significant proliferation of opportunistic *Vibrionaceae* could also occur in the wild, if for example phyllosoma are affected by external injuries. However, the likelihood of such events is enhanced within rearing systems, attributed to the higher stocking densities and increased physical competition between cultured larvae.

5.3 Options for microbial management in aquaculture

5.3.1 Ozonation

It has to be considered if ozonation would truly improve microbial management in aquaculture. As shown in this study, ozonation largely prevented *Artemia* mortalities, which is indicative of a positive effect despite the overall high bacterial densities. It was however not certain if the higher bacterial load in early-stage biofilm would possibly interfere with phyllosoma development, especially regarding newly hatched or recently moulted larvae within the initial larval stages. Ozonation substantially modified the community structure; and it may be questioned if the changed community structure consisting of a significantly promoted bacterial subclass and the simultaneous reduction in community diversity would represent a balanced and beneficial microflora.

One managerial option could be to run an ozone-treated system for a short period without addition of larvae, aiming at the stabilization of the bacterial community structure before larvae are added. The accelerated initiation of biofilm developmental stages suggested that

the early and most distinct effects of ozonation – as they have been probably observed in this study – could be followed by the establishment of a more diverse and presumably more beneficial community. However, no considerable diversification was attained within an experimental period of 44 days. As such prolonged adjustment times would be highly contradictory for commercially viable phyllosoma rearing.
Ozonation was however demonstrated to be successful in the control of filamentous bacterial growth in biofilms. This study therefore supported the introduction of ozonation as a means of restricting the biofilm as a reservoir for filaments that may subsequently colonize phyllosoma. The observable presence of filamentous bacteria was delayed for 14 days, before establishing in the biofilm. At this time point, most phyllosoma would have been moulted into the P3-P4 cycle, and therefore external filamentous attachment of earlier stage (P1-P2) phyllosoma would have been prevented. In contrast, non-ozonated water biofilm comprised significant numbers of filaments 9 days earlier, which underlined the potential for a filamentous colonization of P1-P2 animals when no water treatment is performed. However, since filaments were hardly removed even during biofilm detachment, it has to be considered if biofilm-associated filaments in fact represent the major source of phyllosoma colonizers.

Ozonation interfered with bacterial sulfur-metabolizing pathways in larval rearing tanks. A strong smell of sulfide was associated with the biofilm of non-ozonated water tanks, which indicated the presence of an active sulfur cycle. The phylogenetic relationship to sulfur-metabolizing bacteria, shown for three sequences dominant in non-ozonated water biofilm on day 44, supported the putative presence of an active, biofilm-associated sulfur cycle within this water especially towards the end of the experimental period. This could be associated with the continuous increase of *Thiothrix* within the biofilm, along with the establishment of anaerobic zones. It was likely that increasingly anoxic conditions were occurring in non-ozonated water, which were presumably located near the tank walls and hence in proximity to the biofilm. Such interfaces of oxic and anoxic zones would have been suitable for increasing proliferation of *Thiothrix*, since it requires both H_2S and O_2. The necessary production of hydrogen sulfide was most likely due to sulfate-reducing bacteria that established in anaerobic niches.

5.3.2 *Artemia* as a vector of pathogens and the effect of antibiotics

The *Artemia* disinfection protocol used at AIMS at the beginning of this study included the application of both formalin and streptomycin. Streptomycin is a multi-spectrum antibiotic and inhibits growth of both Gram-negative and Gram-positive bacteria (Schatz et al., 1944).

However, this treatment did not substantially reduce *Artemia* contamination, since the load of luminous *Vibrionaceae* demonstrated after cleanup was high (Matthew Salmon, personal communication). Therefore, the evaluation of an alternative disinfection protocol was required.

Only one of the antibiotic treatments tested in this study demonstrated a significant reduction in vibrios associated with *Artemia*. In addition, changes within the *Vibrio* community occurred when *Artemia* were exposed to the range of antibiotics.

The initiation of rapid growth and community alteration was most likely due to antibiotic knock-out of some bacterial strains followed by proliferation of resistant vibrios. This essentially highlighted the problem of antibiotics resistance and underlined the inadequacy of antibiotics, suggesting their exclusion from future protocols if alternative decontaminating options can be evaluated. Changes in the composition of the *Vibrio* community were however also seen for non-treated *Artemia*, which demonstrated that also mechanisms of competitive exclusion accounted for the diversification of the *Vibrio* load.

The reduction in the *Vibrio* load by treatment with the mixture of erythromycin, oxytetracycline and streptomycin was expected. A combination of several antibiotics targeted different biochemical pathways and was thereby effective against a wider range of bacteria. A maximum positive effect of the antibiotics mixture was however only observed in the first experiment, which tested the range of antibiotic treatments on 7-day on-grown *Artemia*. In the second experiment, the *Vibrio* load of *Artemia* was higher from the beginning, even though it could be reduced to a minimum after a prolonged time of incubation with the antibiotics. The two experiments were performed with the same hatch of *Artemia* and without changes in the *Artemia* grow-out protocol during the three days between the experiments. This consequently suggested the significant proliferation of vibrios in the *Artemia* culture and progressing colonization of nauplii during this period.

A mixture of antibiotics possibly circumvented the problem of resistance, since to date multiresistant strains are rarely detected in Australian aquaculture (Akinbowale et al., 2006). However, resistance can be developed as shown by recent work on a *Vibrio alginolyticus*, isolated from moribund larvae of *Artemia*-fed Giant freshwater prawns, which possessed significant resistance against both erythromycin and oxytetracycline (Jayaprakash et al., 2006).

Later experiments, which were not part of this study, reported a constantly reduced *Vibrio* load when *Artemia* were treated with the antibiotic mixture (Matthew Salmon, personal communication). Most notably, in subsequent experiments strains forming luminescent colonies were mostly excluded by this treatment. Thus, the improvement in the *Artemia* treatment protocol resulting from these studies had a large practical significance for

managing the microbial status of *Artemia*. Nevertheless, it was suggested that *Artemia* on-grown for 10 days posed an increased risk for introducing potential pathogens as compared to younger *Artemia*, even when implementing the improved cleanup protocol. This was highlighted by the Sanolife application. The increasing individual *Vibrio* load per animal when ageing, in association with a higher contribution of luminescent vibrios, was indicative of a subsequently increasing risk of pathogen transfection.

5.3.3 Perspectives for the application of probiotics in larval rearing

The use of probiotics is increasingly considered as an effective, environmentally sensitive means of disease control in aquaculture. Various studies have tested the probiotic activity of a broad range of microorganisms, including numerous Gram-positive and Gram-negative bacteria but also microalgae (e.g. *Tetraselmis*) and yeasts (*Phaffia* and *Saccharomyces*). Introduction of probiotics would bear significant advantages over other antimicrobial treatments, in particular as the usage of antibiotics incorporates serious health and environmental concerns. Two approaches were undertaken in this study for the identification of potential probiotic strains; (i) the screening of recognized probiotic strains for activity, and (ii) the selective isolation of candidates from wild, disease-free larvae.

a. Screening of recognized probiotics

The screening of bacterial strains with approved probiotic activity – such as commercially available probiotics or bacteria described in the scientific literature – could also lead to the identification of strains beneficial for phyllosoma health. Screening of a commercial probiotic was attempted by analyzing the *Bacillus* product Sanolife regarding its potential (i) to reduce *Vibrio* infection of *Artemia* in enrichment tanks, and (ii) to inhibit a pathogenic *Vibrio harveyi* strain under laboratory conditions. *Vibrio alginolyticus*, which indicated an inhibiting activity on pathogenic vibrios (see 1.4.2), and *Roseobacter* (K2, K19) and *Sulfitobacter* (RR67) strains were tested under similar prerequisites. *Roseobacter* and mainly *Sulfitobacter* were included as a result of their prolific association with wild phyllosoma (Payne et al., unpublished data).

Bacillus spp. in the commercial Sanolife product were demonstrated not to prevent infection of on-grown *Artemia*, despite the product being suitable for decontamination of a related species within the crustaceans. The missing antimicrobial effect suggested that a wider range of potential probiotics are required to be tested to evaluate suitable strains for a

distinct culture organism. Only the additional use of a broadly targeting antibiotic mixture yielded the reduction of luminous, potential pathogenic *Vibrio* strains.
In contrast, Sanolife/*Bacillus* spp. exhibited a distinct ability to cause clearing of a pathogenic *Vibrio harveyi* strain in the probiotic screening experiment. Presumably, the positive effect of the product was linked to more controlled conditions within the experimental setup. In the screening, *Bacillus* was just exposed to a single *Vibrio* strain and growing on agar, whereas it was confronted with a diverse bacterial community in the *Artemia* experiment and growing in liquid medium. The missing effect of Sanolife despite its effectiveness under controlled conditions underlined the presence of a competitive microbial community within the grow-out system.
The other candidate strains – *Vibrio alginolyticus*, *Roseobacter* and *Sulfitobacter* – never resulted in inhibition of the pathogenic *V. harveyi*, even under laboratory conditions. Although these strains showed distinct growth and potential clearing ability in some instances, they were excluded as representing promising probiotics.

b. Isolation of candidate strains from wild phyllosoma

The excellent characteristics of *Roseobacter* strains as potential probiotics (see 1.4.2) was the basis for attempts to isolate probiotic active strains from wild phyllosoma. Several *Roseobacter* species were shown to be excellent biofilm forming bacteria, which could imply a role in biofilm exclusion of pathogenic strains and the suppression of biofilms as a reservoir for pathogens. Furthermore, the involvement of *Roseobacter* in the sulfur cycle (see 1.4.2) suggested a potential interference with growth of potential phyllosoma-colonizing *Thiothrix* or sulfide-producing strains.

General remarks

The culture-based isolation of potential probiotics from the total cultivable, heterogeneous bacterial community represented the most stringent approach. A similar approach yielded the identification of several *Roseobacter* strains inhibitory towards pathogenic vibrios (Hjelm et al., 2006). It was therefore shown that active probiotic strains could be obtained using this approach, able to separate promising candidates from the broad background bacterial diversity. It has however to be considered that the isolated phyllosoma microflora could have been disturbed, for instance if stress during the collecting process caused the larval vacation of the intestinal tract. However, for some collected specimens the gut was still containing prey, identifiable due to the translucence nature of the phyllosoma. Accordingly, the

phyllosoma-associated intestinal flora as isolated on medium was regarded as mostly undisturbed and reflecting its natural composition.

10 of the 12 bacterial isolates of the cultivable flora of wild phyllosoma, selected for sequencing after their screening for probiotic activity, were affiliated with three bacterial groups; γ-*Proteobacteria*, *Cytophaga-Flavobacteria* (CFB) and *Actinobacteria*. CFB and particularly γ-*Proteobacteria* affiliated bacteria were also detected in the intestinal tract of wild phyllosoma, as demonstrated by fluorescence *in situ* hybridization (see 4.3.2). It would be interesting to test the abundance of *Actinobacteria* which could be performed e.g. by using horseradish peroxidase-labelled probes in CARD-FISH, since standard fluorochrome-labelled FISH probes mostly fail to detect *Actinobacteria* due to their presumably low ribosomal rRNA content (Pernthaler et al., 2002).
Interestingly, 5 of the 12 bacterial isolates were closely related to bacterial sequences found in a previous study of bacterial diversity of wild Palinuridae and Scyllaridae phyllosoma (Payne et al., unpublished data). The study by Payne et al. was principally based on the analysis of clone libraries constructed from the microbiota, and in parts from standard culture-based techniques.
Molecular rRNA approaches are markedly superior to culture-based analyses due to the non-cultivability of many microorganisms (Amann et al., 1995), and selectivity of the isolation medium is likely to bias the actual composition of the microflora. The Marine Agar used as the isolation medium is selective for fast growing organisms able to utilize high nutrient levels. In contrast, it is not ideal for the isolation of most slow growing strains. These are commonly involved in nutrient cycling, as e.g. *Roseobacter* and *Sulfitobacter* that play a role in the marine sulfur cycle. A minor, but readily cultivable member of the community could hence be interpreted as an important member and/or potential probiotic, even if its competitiveness may be marginal within a heterogeneous community.
In this context, four of the five sequences obtained in the current study were similar to sequences from previous culture-based isolation, and only one isolate was closely related to a 16S rDNA sequence retrieved from a clone library. Although culture-based analyses of the bacterial flora are inferior, the multiple retrieval of sequences being closely related to previous isolates nevertheless indicated the presence of a phyllosoma-typical microflora, which also would be according to the interpretations in 5.2.

Probiotic active isolates

The three phyllosoma-isolated strains which distinctly showed clearing of the pathogenic *Vibrio harveyi* C071 were identified as being closely related to *Tenacibaculum mesophilum*

(96% similarity), *Pseudoalteromonas citrea* (97% similarity) and *Winogradskyella poriferorum* (100% similarity).

An extensive assay on the inhibitory action of the genus *Pseudoalteromonas* suggested that most of the affiliated species efficiently produce bioactive compounds against settlement of bacteria and fungi (Holmström et al., 2002). It was further proposed that this production by organism-associated *Pseudoalteromonas* species may aid the host against colonization of its surface. The probiotic capability of *Pseudoalteromonas* sp. was supported by studies in Oyster aquaculture, reporting increased larval survival and growth when *Pseudoalteromonas* was applied in combination with algae (Douillet and Langdon, 1993; 1994).

The limited information on potential competing, inhibiting, or probiotic capabilities of *Tenacibaculum mesophilum* and *Winogradskyella poriferorum*, both affiliated with CFB, is mainly due to their only recent identification. *Tenacibaculum mesophilum* was isolated from sponge and green algae from coastal environments of Japan and Palau (Suzuki et al., 2001). The Gram-negative *Winogradskyella* is an only recently established genus. The three other recognized members of the genus were isolated from algal frond surfaces in Japanese waters, whereas *Winogradskyella poriferorum* was first isolated from a tropical sponge (Lau et al., 2005).

5.3.4 Future options

a. Biofilm control

Quorum sensing

An interesting future approach for microbial control is represented by the implementation of quorum sensing-controlling protocols. The observation that quorum sensing is linked to the production of virulence factors and biofilm formation suggests that many virulent Gram-negative organisms could be rendered to a non-pathogenic phenotype by inhibition of their signalling pathways (Hartman and Wise, 1998). These authors stated that ‘research into quorum sensing, and inhibition thereof, may provide a means of treating many common and damaging chronic infections without the use of growth-inhibitory agents, such as antibiotics and disinfectants, that unavoidably select for resistant organisms’.

It has been reported that the red macroalga *Delisia pulchra* produces a range of halogenated furanone compounds (de Nys et al., 1993), which display antifouling and antimicrobial properties (Reichelt & Borowitzka, 1984; de Nys et al., 1996). This is attributed to an interference of these furanones with bacterial signalling by specifically targeting QS mechanisms (Whiteley et al., 1999). In addition, furanones increase the effect of antibiotic

treatments, possibly by circumventing bacterial resistances (Hentzer, 2003). In reference to the same publication, 'blocking of AHL-mediated gene expression represents an effective approach to interfere with surface colonization and to attenuate the virulence of opportunistic bacterial pathogens'. This would make quorum sensing a promising novel drug target. QS-targeting protocols could represent a highly applicable solution for biofilm-associated pathogen control in larval rearing systems, especially with respect to the high bacterial densities shown within the tank biofilms suggesting the occurrence of density-dependent population control. Recent work in fact revealed an increase of AHL molecules during mass mortality events of phyllosoma. This indicated a role for QS in mediating pathogenicity and highlighted the potential of disease control by this means (Bourne et al., 2006). Further research is underway to determine possible ways of biofilm control in phyllosoma rearing systems by interference of these pathways. First applications in aquaculture underlined the potential of such protocols, as the attenuation of virulence by the use of QS inhibitors was achieved for a strain of *Vibrio harveyi* pathogenic for the Black tiger prawn (Manefield et al., 2003).

Bacteriophage therapy and PACT

The application of bacteriophages may provide biofilm control, based on their encoding of depolymerase enzymes that are enabled to degrade polymerics of the biofilm EPS matrix (Hughes et al., 2001). Such approaches have been proposed for the control of clinical infections such as cystic fibrosis, but could be hypothetically applicable for aquatic biofilms as well.

Photodynamic Antimicrobial Chemotherapy (PACT), as reviewed by Matchett et al. (2001), is technology based on the sensitivity of bacteria to the photosensitizing action of a variety of light-absorbing dyes. This therapy uses the localized ability of light-activated molecules to initiate redox reactions and/or form highly reactive singlet oxygen. The mode of action is hence comparable to the mechanism of ozone.

b. Feed enhancement

Alternative decontaminants

Despite the establishment of an antibiotic treatment that evidently reduced the *Vibrio* load of *Artemia* nauplii, use of such compounds in aquaculture should only be considered as a temporary solution. The development of a commercial scale phyllosoma rearing industry

would require the development of alternative management regimes, which do not rely on the use of antibiotics.

Until reliable protocols are established, alternative options for microbial management should be considered. Some of these have already been tested at AIMS, including treatments with Virkon S and carbon dioxide (unpublished data), however the treatments have not improved *Artemia* health. Since further potential decontaminants are available, these could be tested for practicability. Gimenez et al. (2006) reported 90% survival of *Artemia* associated with up to 94.5% reduction of heterotrophic bacteria and a further 82.8% of *Vibrionaceae*, after application of the hydrogen peroxide-based product Ox-Aquaculture©. At the same time, no oxidation of polyunsaturated fatty acids or changes in the fatty acid composition was recorded; therefore the nutritional profile of *Artemia* was not altered. In addition, the study demonstrated the need of only a short exposure time to the chemical and an easy removal.

Variation of the algal enrichment

Skjermo et al. (2006) have shown a immunostimulatory effect of a dietary supplement, an algal glucan derived from the diatom *Chaetoceros mülleri*, on larvae of the Atlantic cod (*Gadus morhua*) when fed by glucan-enriched rotifers. This represented an interesting observation, since a *Chaetoceros* species is already included in the *Artemia* enrichment at AIMS. In this context, concepts on the potential use of so-called 'green', i.e. algal-enriched water, were already outlined. This is especially interesting regarding the possible implementation of algae in probiotic application (see below).

Artificial diets

The development of artificial diets as larval feed, completely substituting *Artemia*, would represent another possible goal. The microbial load of feed could hence be tightly controlled. In order to evaluate suitable dietary formulations, knowledge of the natural prey and nutritional requirements of phyllosoma would be beneficial. A possible experimental layout would address the DNA analysis of the gut contents of wild larvae. The feasibility to analyze ingested biomass by a molecular approach was confirmed for several Palinuridae and Scyllaridae phyllosoma (Suzuki et al., 2006), but excluding *P. ornatus*. Investigations by the above authors linked phyllosoma feeding on Urochordata and Cnidaria, which were thought to be prey animals for middle- or later-stage phyllosoma. This interesting result indicated the value of such an approach. Molecular prey analysis of wild *P. ornatus* larvae could be used towards the development of dietary formulations.

c. Probiotics

The identification of potential probiotic strains would consequently imply the need of a realizable application protocol. In parts based on the present analyses, two approaches would be feasible to incorporate a potential probiotic in the rearing process.

Probiotics and matured water

A possible approach for probiotic implementation was the use of so-called matured water (Skjermo and Vadstein, 1999). A water maturation process includes the controlled incubation of seawater for weeks up to several months, aimed at the establishment of a stable microbial flora potentially incorporating balanced interactions and integrated nutrient utilization. Particularly the enrichment of persistent, slower-growing bacterial strains during the maturation process may impair the possibility of opportunistic (pathogenic) bacterial strains to later proliferate within this water.

Algae and probiotics

The typical association of *Roseobacter* with algae indicated its eligibility as a potential probiotic in phyllosoma rearing, and partly motivated the attempt to detect such strains with wild larvae. This had suggested both a general 'phyllosoma compatibility' and the prospect of using the algae as a vehicle for the probiotic.

However, only one isolated strain was affiliated with the *Roseobacter* clade. In contrast, three isolates were affiliated with the *Flavobacteriaceae* and two strains with the *Pseudoalteromonadaceae* family, of which three strains were displaying distinct inhibitory activity. As *Roseobacter* sp., both families were identified as algal colonizers. This suggested a future outlook on studies on their potential for an algae-based administration, especially regarding the widespread probiotic activity found with several *Pseudoalteromonas* strains (Holmström et al., 2002).

Initial accumulation of a probiotic via the association with algae and further concentration during the *Artemia* enrichment with probiotic-'loaded' algae could prepare a concentrated probiotic flora within the nauplii. Additionally, such a treatment would potentially reduce potential pathogens associated with *Artemia* by subsequent uptake and enrichment of probiotics within the *Artemia* digestive tract. Thus, their co-cultivation potential would have been minimized. Transfer of these probiotic-'loaded' *Artemia* to phyllosoma rearing could transmit a probiotic flora to the tanks, given that no additional clean-up via antibiotics or disinfectants would be performed. In context with the other beneficial effects of algae, enrichment via the steps *probiotic* → *algae* → Artemia → *phyllosoma* could represent a

promising method of application. *Artemia* hence would have been transformed from a vector of pathogens to a vector of beneficial microorganisms. Phyllosoma feeding on ‘probiotic’ nauplii could therefore assimilate probiotic strains from the earliest stage, and internal colonization of the probiotic in the phyllosoma digestive tract could be facilitated. Due to the improvement of the phyllosomas’ intestinal microbial flora, culture larvae may exhibit a superior health status, increased fitness and lowered susceptibility to infections. Even if pathogens continued to be present, improved control of the microbial flora could stabilize the system and prevent opportunistic pathogens from massive proliferation.

6. Summary

The presence of a challenging microbial flora in larval rearing systems of the ornate rock lobster, *Panulirus ornatus*, requires the evaluation of suitable options for microbial management. Larval rearing on a commercially viable scale can only be established with a controlled microbial environment, since the proliferation of pathogenic and/or biofouling bacteria contribute to high mortalities of captive-reared larvae. In the present study, the bacterial colonization of three microbial compartments of an aquaculture larval rearing system of *Panulirus ornatus* was investigated and microbial management options for each compartment analyzed.

Biofilm. Ozonation significantly modified the temporal progression of biofilm development and favoured the dominance of γ-*Proteobacteria* within the biofilm community, of which some dominant sequences showed most similarity to coral-associated bacterial strains. Ozonation delayed the establishment of filamentous bacteria, and also repressed the proliferation of pathogens and sulfur-metabolizing bacteria. The various effects of ozonation illustrated the presence of complex interactions during the process of bacterial colonization. Thus, the experiment highlighted the dynamics within the microbial community.

Artemia. The distinct *Vibrio* load of *Artemia* demonstrated their potential as a vector of pathogenic bacteria to phyllosoma rearing. This potential was shown to be increased with older animals. Only a mixture of three different antibiotics reduced their load with cultivable vibrios. Concurrently, the problem of antibiotics resistance was underlined. The analysis accented the inadequacy of antibiotics for effective microbial management. At present however, alternative treatments cannot rely on commercial probiotics, whose poor efficiency was demonstrated likewise.

Phyllosoma. The bacterial colonization vastly differed between cultured and wild phyllosoma. This confirmed the enhancement of biofouling processes in closed rearing environments, which was illustrated by the increased external attachment of filamentous and rod-shaped bacteria at the mouthparts and anus. The bacterial colonization of wild, healthy larvae was low, and comprised a diverse community including the *Vibrionaceae*. Thus, a presence of *Vibrio*-affiliated strains was separated from the occurrence of disease. Three bacterial isolates from wild larvae were demonstrated as inhibitory towards a pathogenic *Vibrio* strain and could represent probiotic candidates for implementation in phyllosoma rearing.

Die Gegenwart einer problematischen mikrobiellen Flora in Larven-Kultivierungsanlagen der Schmuck-Languste, *Panulirus ornatus*, erfordert die Evaluierung geeigneter Optionen für mikrobielles Management. Eine wirtschaftliche Zucht kann nur unter Kontrolle des mikrobiellen Milieus etabliert werden, da die Vermehrung pathogener und/oder übermäßig oberflächen-besiedelnder Bakterien zu einer hohen Sterblichkeit gezüchteter Larven beiträgt. In der vorliegenden Arbeit wurde die bakterielle Besiedlung dreier mikrobieller Kompartimente einer Aquakultur-Zuchtanlage von *Panulirus ornatus* untersucht und Maßnahmen des mikrobiellen Managements für jede Abteilung analysiert.

Biofilm. Ozonierung modifizierte signifikant den zeitlichen Ablauf der Biofilm-Entwicklung und begünstigte die Dominanz von γ-*Proteobakterien* innerhalb der Biofilm-Gemeinschaft, von denen dominierende Gensequenzen größte Ähnlichkeit mit korallen-assoziierten Bakterien zeigten. Ozonierung verzögerte die Etablierung fadenförmiger Bakterien, und schränkte die Vermehrung von Pathogenen und schwefel-metabolisierenden Bakterien ein. Die verschiedenen Effekte der Ozonierung implizierten das Vorhandensein von komplexen Interaktionen während des bakteriellen Besiedlungsprozesses. Das Experiment unterstrich daher die Dynamik innerhalb der mikrobiellen Gemeinschaft.

***Artemia*.** Die ausgeprägte *Vibrio*-Last von *Artemia* zeigte deren Potenzial als Vektor von pathogenen Bakterien in die Phyllosoma-Zucht. Das Potenzial stieg dabei mit fortschreitendem Alter an. Nur ein Gemisch dreier Antibiotika reduzierte die Last kultivierbarer Vibrios. Gleichzeitig wurde die Problematik der antibiotischen Resistenz unterstrichen. Die Analyse verdeutlichte die Uneignung von Antibiotika für ein effektives mikrobielles Management. Zum jetzigen Zeitpunkt können alternative Behandlungen jedoch nicht auf kommerziellen Probioten beruhen, da deren mangelhafte Effizienz ebenfalls gezeigt wurde.

Phyllosoma. Die bakterielle Besiedlung unterschied sich deutlich zwischen kultivierten und wilden Phyllosoma. Dies bestätigte die Verstärkung bakterieller Fouling-Prozesse innerhalb einer geschlossenen Aquakultur, was durch die erhöhte externe Anheftung faden- und stäbchenförmiger Bakterien an den nährstoffreichen Regionen der Mundwerkzeuge und des Anus veranschaulicht wurde. Die bakterielle Besiedlung wilder, gesunder Phyllosoma war gering, und bestand aus einer diverse Gemeinschaft inklusive der *Vibrionaceae*. Die Präsenz *Vibrio*-angegliederter Stämme wurde folglich vom Auftreten eines Krankheitsbildes gelöst. Drei bakterielle Isolate wilder Larven inhibierten einen pathogenen *Vibrio*-Stamm und könnten probiotische Kandidaten für den Einsatz in der Larvenzucht darstellen.

7. Appendix

7.1 Supply and processing of seawater

Water supply and preparation

Seawater at AIMS is won through dual pipelines, which are fastened to the sea bed and extend 200 m off the beach line adjoining the institute. The intake heads of these pipelines are anchored in 5-6 m of water depth. Seawater is pumped directly into a 455,000 l concrete settlement tank. From the primary settlement tanks, seawater is gravity fed to the institute. The supply is split between the aquaculture facilities, the outdoor aquarium and the AIMS laboratories. All incoming seawater for the larval rearing facilities and live feed production is pre-settled in a 130,000 litre dam. Following settlement, water is pumped from the dam through a floating suction line, which draws water from just below the surface. Settled water is initially passed through 2 x 5 µm bag filters (X100 Convertible Filter Housing, Australian Filter Specialists) and then through a manifold of 5 and 1 µm cartridge filters (P5.19 and P1.19, Walker). Each of these filters has a 400 kpa (60 psi) liquid filled pressure gauge which is monitored to assess the performance of the filters.

Ozonation

Ozone is formed in a corona discharge (Bablon et al., 1991) caused by the interaction between an electrical discharge and oxygen molecules. An alternating current is applied across the two electrodes. One of the electrodes is coated with ceramic dielectric material to evenly distribute the current over the surface.

Ozone is applied to the water with a oxidative redox potential (ORP) setting of between 290 and 320 mV through an venturi eductor-operated injector system which is powered by a booster pump. After the process, residual ozone is removed from the water prior to its use within the larval rearing and live feed production facilities.

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