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APPLICATION OF POLY-β-HYDROXYBUTYRATE ACCUMULATING BACTERIA IN CRUSTACEAN LARVICULTURE

Thesis submitted in fulfilment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title: Toepassing van poly-β-hydroxybutyraat accumulerende bacteriën in de larvicultuur van crustaceeën.

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“To my family for their patience, sacrifice and unshakable support”

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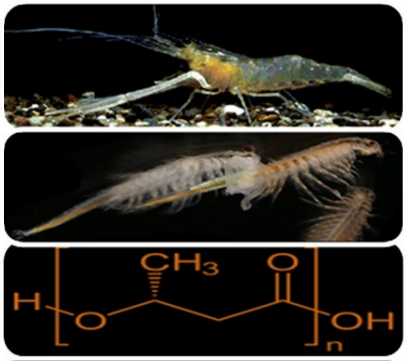
LIST OF ABBREVIATIONS AND UNITS

× *g*	Relative centrifugal force or G force
AHMA	Axenic hatching medium of *Artemia*
CDW	Cell Dry Weight
CFU	Colony Forming Unit
DAH	Day After Hatching
DHA	Docosahexaenoic Acid
EC	Enrichment Culture
FAASW	Filtered Autoclaved Artificial Seawater
FAO	Food and Agricultural Organization of the United Nations
HUFA	Highly Unsaturated Fatty Acid
LB	Luria-Bertani broth
LSI	Larval Stage Index
OD	Optical density
PHA	Polyhydroxyalkanoates
PHB	Poly-β-hydroxybutyrate
QS	Quorum Sensing
SCFA	Short Chain Fatty Acid
SPSS	Statistical Package for the Social Science
TCBS	Thiosulphate-Citrate-Bile Salt-Sucrose
TL	Total Length
TOC	Total Organic Carbon

CONTENTS



CHAPTER 1

LITERATURE REVIEW AND THESIS OUTLINE

1.1 The importance of aquaculture

Aquaculture includes all forms of culture of aquatic animals (fish, crustaceans, mollusks, etc.) and aquatic plants in fresh, brackish and marine waters (Pillay and Kutty, 2007). According to Subasinghe et al. (2009), aquaculture has been the fastest growing food-producing sector in the world. It is developing, expanding and intensifying in almost all regions of the world. Fish and fishery products, including those originating from aquaculture, represent 16.6% of the global population's intake of animal protein and 6.4% of all proteins consumed (FAO 2014a). The global capture and aquaculture production statistics of FAO (2014) from 1950 to 2012 (Fig. 1.1) show that the total capture fisheries production is more or less constant during the last decade of observation. The most recent tendency is that there even seems to be a decline illustrating that the wild fish stocks are being overexploited under the pressure of global population growth. On the other hand, the fisheries production by aquaculture steadily increased over the past years, with its output reaching 73% of the capture fisheries production in 2012 and being more or less equal to capture fisheries in 2015. Clearly, aquaculture will have a central role in the challenge to fulfill human food demand in the future. In addition, aquaculture has created much employment and trade because a large fraction of the global fish production is being traded internationally (Finegold 2009). Aquaculture becomes an important component for the poverty alleviation of rural areas where increasing population pressure, environmental degradation or limiting catch from wild fisheries is a large problem (Halwart 2005).

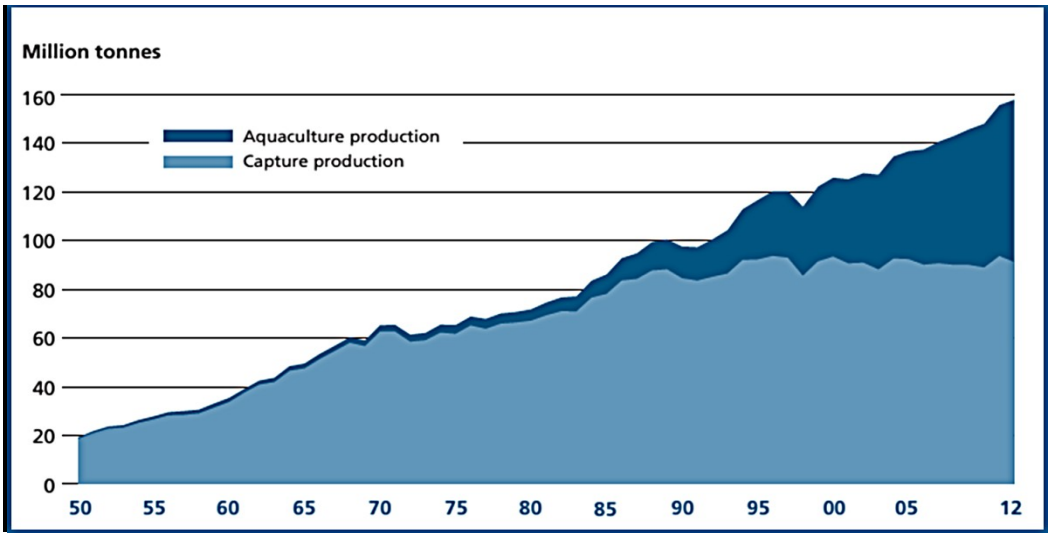


Figure 1.1 The global capture and aquaculture production (FAO 2014).

1.2 The immediate goals of the industrial aquaculture

Sustainable development is the most important target of industrial aquaculture in coming years. To accomplish that there are some priority needs which have been suggested by Sorgeloos (2013):

(i) *Domestication*: complete independence from natural stocks such as wild breeders or seed through domestication of aquatic species.

(ii) *Seed production*: improved or more cost-effective seed production through development of new hatchery practices (e.g., with regard to microbial steering) and applying innovative products (e.g., substrate for specific bacteria or signal molecules to disrupt virulence triggers).

(iii) *Species selection*: more selective in identifying suitable species for mass markets and niche species catering to local markets where value-added products might be in good demand.

(iv) *Selective breeding*: development of more efficient stock through selective breeding, especially genomic sequence comparisons with model species can help to identify best breeding goals.

(v) *Bacteria in aquaculture*: more microbial management for more sustainable production because water is an ideal environment for microbial development. The role of bacteria - beneficial and harmful – in aquaculture systems requires much more research attention.

(vi) *Health control*: more basic work using molecular tools should improve knowledge of activation, good functioning and disruption of the animal's immune systems, especially in invertebrates, crustaceans and mollusks.

(vii) *Ecological aquaculture*: more integrated farming of terrestrial and aquatic plants and animals for sensor-controlled nutrient dosing and heat, energy and water recovery.

(viii) *Marine aquaculture*: paying more attention to the marine environment. Oceans and and seas make up 70% of the global aquatic resources, yet only about 50% of our aquatic products is produced in marine environments (of which almost half are aquatic plants).

(ix) *Replacements for fishmeal and fish oils*: full independence from natural fish stocks for lipid and protein ingredients in production of aquatic feeds.

(x) *Stock enhancement programs*: more attention for integration of restocking activities with fisheries management in freshwater and marine environments.

1.3 Importance of crustacean aquaculture

Crustaceans are a high value aquaculture product with a high global demand. The worldwide production of crustaceans keeps on increasing continuously and has grown from approximately 3 million tonnes in 2003 to 6.45 million tonnes in 2012 (FAO 2014). The most recent statistics (FAO 2014) show that the quantity of crustacean aquaculture products represented about 7% of the total quantity of aquaculture products produced worldwide in 2012 (Fig. 1.2). Despite this modest quantity, it represented a value of about 30.9 billion US dollars, which is 21.4% of the total value of the world aquaculture production.

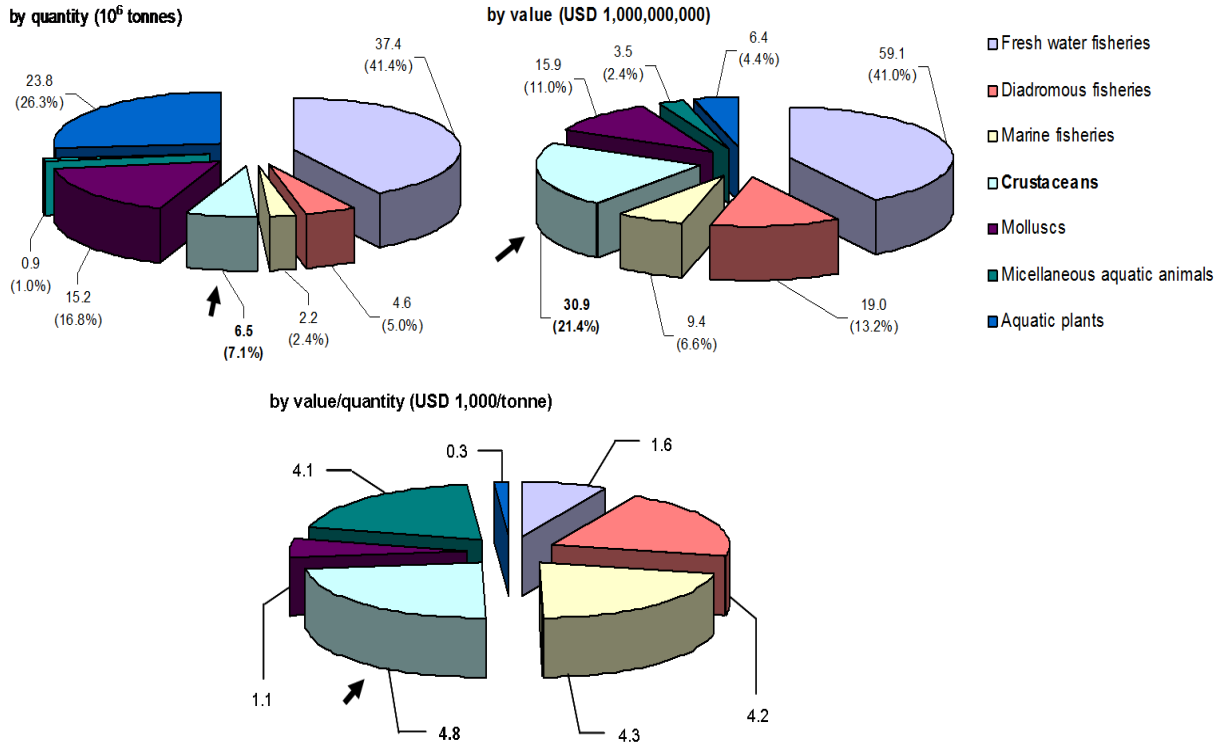


Figure 1.2 World aquaculture production of major species groups in 2012 by quantity and value: crustaceans are indicated with an arrow (FAO 2014).

1.4 Giant freshwater prawn (*Macrobrachium rosenbergii*)

1.4.1 Distribution, taxonomy and biology

The giant freshwater prawn, *Macrobrachium rosenbergii* (De Man 1987), is distributed in Southeast Asia, South Pacific countries, Northern Oceania, and Western Pacific islands as a native species (New 2002). Freshwater bodies are the normal environment where the adults of this species are found including the lower reaches of rivers and lakes, ditches, canals and pools connected to the sea. *M. rosenbergii* has had different names. In 1959 *M. rosenbergii* was accepted as a universal name.

The phylogenetic characterization of *M. rosenbergii* is:

Kingdom Animalia
Phylum Arthropoda
Subphylum Crustacean
Class Malacostraca
Order Decapoda
Suborder Pleocyemata
Infraorder Caridea
Family Palaemonidae
Subfamily Palaemoninae
Genus *Macrobrachium*
Species *M. rosenbergii* (De Man 1879)
English name Giant freshwater prawn

The morphology of adult *M. rosenbergii* is described by Nandlal and Pickering (2006). The main morphological characteristics are shown in Fig. 1.3:

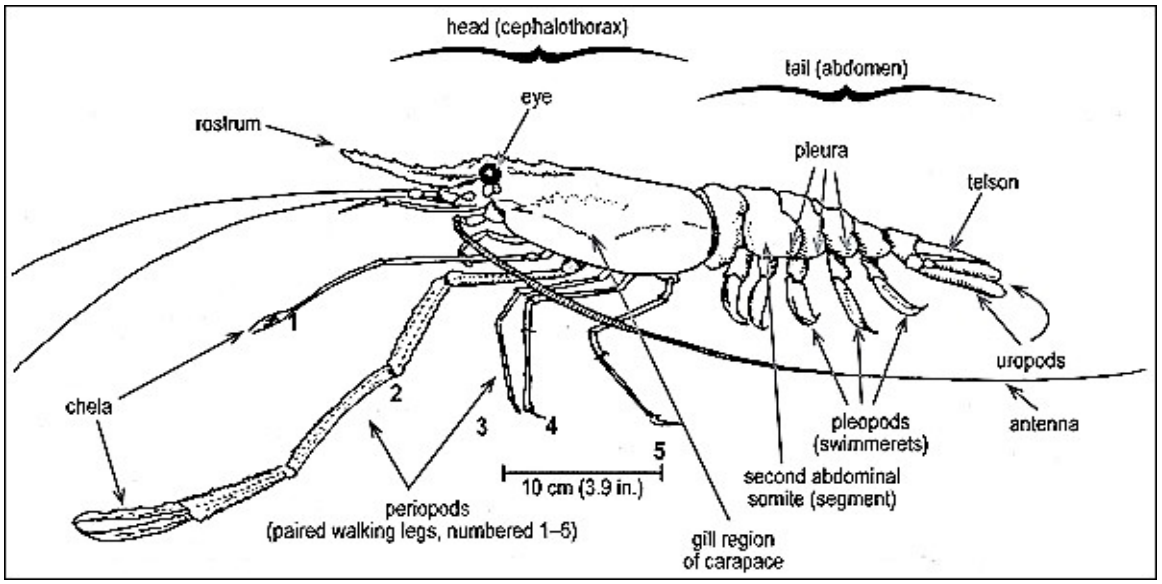


Figure 1.3 External anatomy of *Macrobrachium rosenbergii* (Nandlal and Pickering 2006)

Tropical freshwater environments, connected to adjacent brackish water bodies are the living environment of *M. rosenbergii*. Brackish water is the habitat of its larvae (Sandifer et al. 1975). Gravid females migrate downstream into estuaries, where free-swimming larvae hatch from the eggs and start a new life cycle (Fig. 1.4) (New and Valenti 2000). According to FAO (2002), at 28 ^{o}C, the eggs will hatch 18 – 23 days after spawning. The newly hatched larvae swim upside down and tail first, and move to brackish water with higher salinity (around 9 – 19 g/L) for optimum survival. The larvae pass through eleven stages during the metamorphosis into the postlarvae stage in 15 to 40 days (depending on temperature, quality and quantity of food, water quality, etc.). Zooplankton and larval stages of small aquatic invertebrates are the primary feed of *M. rosenbergii* larvae. Postlarvae can tolerate a range of salinities and have a more benthic lifestyle, and start to migrate upstream towards freshwater.

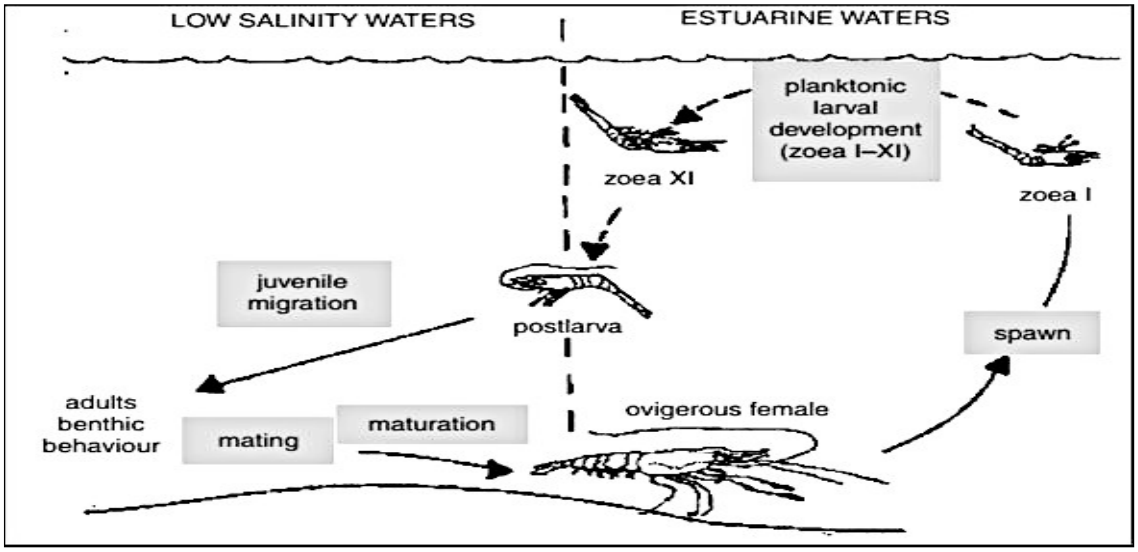


Figure 1.4 The life cycle of *Macrobrachium rosenbergii* (New and Valenti 2000)

1.4.2 The status of **Macrobrachium rosenbergii** *culture*

M. rosenbergii is not only a high value food source but also has a high economical value as an export product. Furthermore, *M. rosenbergii* farming does not need a high investment nor is it as technically demanding or capital intensive as the farming of black tiger or whiteleg shrimp. It is more environmentally sustainable due to lower culture density (Nandlal and Pickering 2006). Nowadays, the farming of *M. rosenbergii* is performed in many countries; China, India, Vietnam, Thailand, Bangladesh and Taiwan are the main producers of *M. rosenbergii* (New 2005). According to the FAO aquaculture production statistics for the year 2012, the global contribution of *M. rosenbergii* to aquaculture production has reached over 220,000 tonnes (Fig. 1.5).

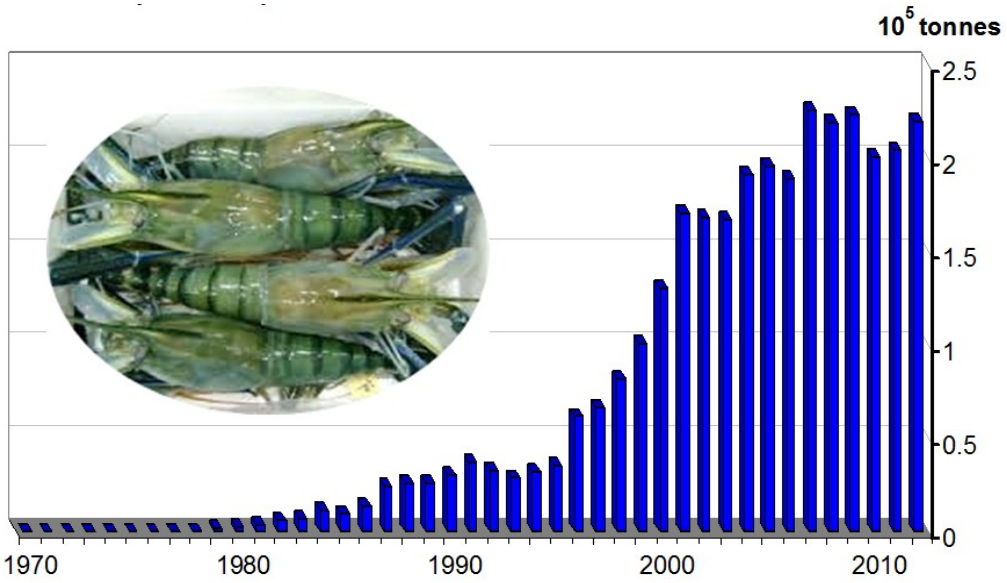


Figure 1.5 Global production of *Macrobrachium rosenbergii* from 1970 to 2012 (FAO 2014)

The commercial aquaculture of prawn depends on postlarvae from the wild and from hatcheries (Bashar et al. 2012). However, nowadays availability of wild seed is not sufficient to meet the demand of the prawn farming industry and seed is also not uniform in size (Soundarapandian et al. 2009). Reliable hatching techniques and management practices for the artificial propagation of prawn seed are thus very important to sustain farming of *M. rosenbergii* (Bashar et al. 2012).

1.4.3 Macrobrachium rosenbergii *culture practices*

Broodstock: Often only berried female prawns are kept in hatcheries until their eggs hatch. The term 'berried' or 'ovigerous' female is used to indicate an adult female carrying eggs under the tail. Berried females can be obtained from farm ponds or natural waters (e.g. rivers, canals and lakes) (Nhan 2009). The fecundity, egg hatchability and the overall quality of the larvae can be improved when the broodstock is fed with high levels of 18:2n-6 and n-3 highly unsaturated fatty acids (HUFA) (13 and 15 mg/g DW of food respectively) (Cavalli et al. 1999).

Egg incubation: Ovigerous female prawns are selected from the wild or farm ponds, then incubated in communal or separated tanks with brackish water until hatching. Different techniques are used depending on geographical location and the scale of the hatchery.
Larval rearing: The first larval stage (less than 2 mm long), which is collected after hatching of the eggs, is reared at an optimum temperature 28 – 31 ^{o}C and salinity of 12 g/L. The time for accomplishing the larval cycle is 25 – 30 days. There are two main culture methods in hatcheries: flow-through (the rearing water is continuously being renewed) and recirculating (using physical and biological filter system to minimize water use) systems (Nhan 2009). The food for larval prawn consists basically of newly hatched *Artemia* nauplii (Lavens et al. 2000). Currently, in the hatchery phase a variety of larval feeds are used apart from *Artemia* nauplii, such as fish eggs, squid flesh, frozen adult *Artemia*, flaked adult *Artemia*, fish flesh, egg custard, worms and commercial feeds (Nhan 2009). Several previous studies have shown that the growth of *M. rosenbergii* can be improved when the feed is enriched or supplemented with (n-3) HUFAs (Romdhane et al. 1995; Alam et al. 1995).

Grow-out: According to Nandlal and Pickering (2006), postlarvae of *M. rosenbergii* (15 – 20 mm, 0.015 – 0.020 g) are nursed in tanks or ponds to a size of 3 – 5 g before releasing them into grow-out ponds for culture (5 - 8 postlarvae/m^2) in monoculture. Beside the natural food sources such as small snails and shellfish, worms, grains, nuts, fruit, etc., supplementary (formulated) feeds are also used for the culture of prawns in grow-out systems. A typical formulated prawn feed contains 30 – 35% protein, 2 – 10% fat and 4 – 12% fibre. Diets are available in powder, meal, crumble or pellet form for different prawn stages. The duration of the pond grow-out phase is approximately 4 – 6 months. *M. rosenbergii* can also be cultured with other aquatic species in polyculture (Buck et al. 1981; Kunda et al. 2009).

1.4.4 Disease in **M. rosenbergii** *aquaculture*

The growing production of *M. rosenbergii* during the last decades has coincided with frequent outbreaks of diseases not only in grow-out ponds but also in hatcheries (New and Valenti 2000). These diseases can be caused by a large variety of pathogens as indicated in Table 1.1. Diseases have seriously influenced the quantity and quality of seed that is produced in prawn hatcheries (Shailender et al. 2012), and as such have negatively affected the commercial culture of *M. rosenbergii*. The infection of larval fish and shrimp by opportunistic pathogens, especially *Vibrio* spp. (Tongguthai 1995; Kennedy et al. 2006;

Jayaprakash et al. 2006, Sharshar and Azab 2008), has been described to result in high mortality (Skjermo and Vadstein 1999). Shailender et al. (2012) have stated that there are various ways for the pathogen to enter the hatchery system with the most important routes being feed, broodstock, instruments, water and unhygienic handling of workers.

Table 1.1 Common diseases and control measures in *M. rosenbergii* farming and/or hatcheries. In some cases antibiotics and other pharmaceuticals have been used as treatment but their inclusion in this table does not imply FAO recommendation (FAO 2002).

Disease	Agent	Type	Syndrome	Measures
MMV (*Macrobrachium* Muscle Virus)	Parvo-like virus	Virus	Infected tissue becomes opaque, with progressive necrosis; affects juveniles	Improved husbandry * (IH)
WSBV (White Spot Syndrome Baculovirus)	Baculovirus	Virus	White spot; affects juveniles	IH
Unnamed viral disease	Nodavirus	Virus	Whitish tail; affects larvae	IH
Black spot; brown spot; shell disease	*Vibrio* spp.; *Pseudomonas* spp.; *Aeromonas* spp.	Bacteria	Melanised lesions; affects all life stages, but more frequently observed in juveniles and adults	IH; oxolinic acid; nifurpurinol
Bacterial necrosis	*Pseudomonas* spp.; *Leucothrix* spp.	Bacteria	Similar to black spot but only affects larvae, especially stages IV and V	IH; nifurpurinol; erythromycin; penicillin-streptomycin; chloramphenicol

** Improved husbandry (IH): good management, hygiene, care of breeding, feeding activities and rearing water (e.g. no overstocking or overfeeding, and sanitary disposal of dead animals, remaining food, etc. during the culture period).*

Table 1.1 *(continued)*

Disease	Agent	Type	Syndrome	Measures
Luminescent larval syndrome	*Vibrio harveyi*	Bacterium	Moribund and dead larvae luminescent	HI; chloramphenicol ; furazolidone
White postlarval disease	Rickettsia	Bacterium	White larvae, especially stages IV and V	IH; oxytetracyline; furazolidone; lime, prior to stocking
Unnamed fungal infection	*Lagenidium*	Fungus	Extensive mythelial network visible through exoskeleton of larvae	IH; trifluralin; merthiolate
Unnamed fungal infection (often associated with IMN – see below)	*Fusarium solani*	Fungus	Secondary infection; affects adults	IH
Unnamed yeast infections	*Debaryomyces hansenii*; *Metschnikowia bicuspidate*	Fungi	Yellowish grayish or bluish muscle tissue in juvenile	IH
Protozoan infestations	*Zoothamnium*; *Epistylis*; *Vorticella*; *Opercularia*; *Vaginicola*; *Acineta*; *Podophyra*; etc.	Protozoans	External parasites that inhibit swimming, feeding and moulting; affect all life stages	IH; formalin; merthiolate; copper-based algicides

Table 1.1 *(continued)*

Disease	Agent	Type	Syndrome	Measures
IMN (Idiopathic Muscle Necrosis)	[environmental disease]	Unknown	Whitish color in striated tissue of tail and appendages; when advanced, necrotic areas may become reddish; affects all life stages	IH
MCD (Mid-Cycle Disease)	[undetermined aetiology]	Unknown	Lethargy; spiraling swimming; reduced feeding and growth; bluish-grey body color; affects larvae, especially stages VI and VII	IH; hatchery disinfection
EED (Exuvia Entrapment Disease), sometimes known as MDS (Moult Death Syndrome)	[undetermined aetiology]	Unknown but probably multiple causes, including nutritional deficiency	Localised deformities; failure to complete moulting; affects late larval stages; also seen in postlarvae, juvenile and adults	IH; dietary enrichment

1.5 The brine shrimp *Artemia*

1.5.1 Biology and ecology of Artemia

The brine shrimp *Artemia* (Leach 1819) is a small crustacean zooplankton species that lives in hypersaline biotopes throughout the globe in which the salt content may be up to 250 g/L (Sorgeloos 1980). The genus *Artemia* contains sexual species and parthenogenetic lineages. *Artemia* can reproduce in two ways (Fig. 1.6). If living conditions are favorable, the fertilized eggs in the brood pouch of the female develop into free-swimming *Artemia* nauplii (ovoviviparous reproduction). On the other hand, when living conditions deteriorate,

Artemia has the ability to produce dormant embryos of about 200 - 300 µm, cysts covered by a tough brown shell, that are in a state of obligate dormancy called diapause (Lavens and Sorgeloos 1987).

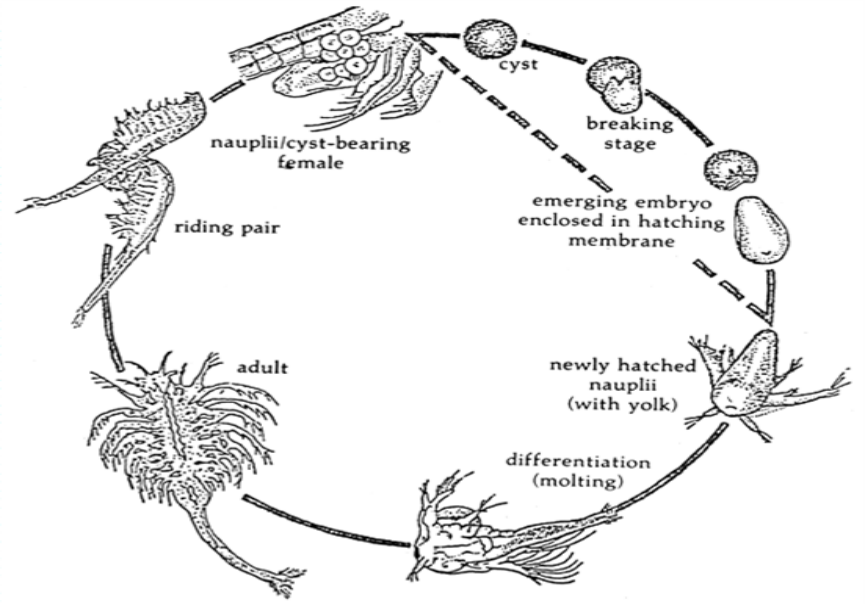


Figure 1.6 Life cycle of *Artemia*. Ovoviviparous offspring production is indicated by the dashed line, while oviparous offspring production is indicated by the full line (Jumalon et al. 1987).

The cysts will resume metabolic activity only when they have been completely dehydrated, later followed by a rehydration in externally suitable conditions (e.g. salinity, temperature, aeration etc.). During hydration, the aerobic metabolism comprises – among others – a trehalose-glycogen conversion and trehalose-glycerol conversion that ensure energy supply for respiration and hygroscopic compound accumulation for hatching, respectively (Clegg 1964; 1965; Van Stappen 1996). The appearance of an embryo with surrounding hatching membrane ('umbrella') occurs after approximately 8 − 20 h and just before the cyst shell bursts, after which the embryo (free swimming *Artemia* nauplius instar I) appears (Van Stappen 1996). The newly hatched nauplius of about 400 – 500 µm in length does not feed and relies on the energy stored in the yolk. The nauplius has a red nauplius eye in the head region and three pairs of appendages (Fig. 1.7) (Van Stappen 1996): the first pair of appendages (first antennae) have a sensorial function, the second appendages (second antennae) have locomotory and filter-feeding function, and the third appendages (mandibles) have a food uptake function. At about 8 h after hatching, the instar I nauplius develops into

the instar II (or metanauplius) stage and can start to take up exogenous particles (Campbell et al. 1993; Dixon et al. 1995; Van Stappen 1996; Sorgeloos et al. 2001).

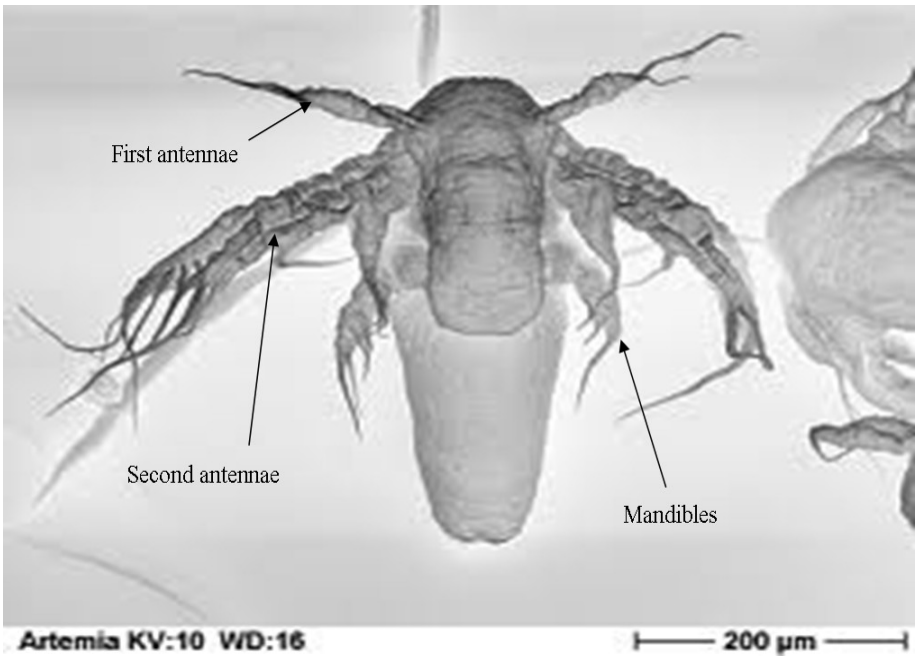


Figure 1.7 *Artemia* nauplius with three pairs of appendages
(Source: http://www.optics.rochester.edu/workgroups/cml/opt307/spr10/jonathan/)

Artemia metanauplii take up smaller food particles (e.g. algal cells, bacteria and detritus) with a size ranging from 1 to 50 µm from the water phase by a filteration process using the 2nd antennae, followed by ingestion of these particles into the functional digestive tract (Van Stappen, 1996). The preferred particle size range for *Artemia* nauplii is approximately 6.8 – 27.5 µm, with the optimum at about 16.0 µm (Fernández 2001). According to Roiha et al. (2010) the uptake of particles sized 4 – 10 µm by *Artemia* nauplii depends on the dose and exposure time. The morphology of the feeding appendages in *Artemia* metanauplii has been studied to understand the filtration process and it seems that the inter-setular distance is an indicator for the smallest size of food particles that can filtered (Marshall 1973). Makridis and Vadstein (1999) have shown that the maximum filtration rate of *Artemia franciscana* metanauplii increased during development and was found to be 50 - 60, 254 and 1480 – 2100 µL/ind./h in 2-, 4-, and 7-day-old metanauplii, respectively. The inter-setular distance in antennae and thoracopods was 0.20 ± 0.07, 0.16 ± 0.05 and 0.18 ± 0.04 µm in 2-, 4- and 7-day-old metanauplii, respectively, and accordingly independent of stage (Makridis and Vadstein 1999).

The instar I and II nauplius stages are most often used as a live food for the culture of larval shrimp and fish in aquaculture (Bengtson et al. 1991).

1.5.2 The role of **Artemia** *in aquaculture*

1.5.2.1 The supply and demand of cysts

Dehydrated *Artemia* cysts are mainly harvested from the wild in inland salt lakes with the Great Salt Lake (Utah, USA) as the largest producer in the cysts market. The global annual production is about 2,500 – 3,000 tonnes, used in aquaculture worldwide (FAO 2011), in the culturing of shrimp and fish in fresh, brackish and as well as marine water regions (Bengtson et al. 1991). Along with the development of the industrial aquaculture in the world, the demand of *Artemia* cysts will increase strongly in coming years. The production of *Artemia* cysts from inland salt lakes is not constant and the demand of this product for hatcheries sometimes exceeds the supply. Trying to meet the demands, the culture of *Artemia* has been introduced throughout the world, such as in Brazil (Camara and Tackaert 1994), Thailand (Vos and Tusutapanich 1997), the Philippines (De los Santos et al. 1980), Vietnam (Baert et al. 1997), Pakistan (Sultana et al. 2011) and India (Sivagnanam et al. 2011). Among these countries, the production of *Artemia* cysts in the Vinh Chau and Bac Lieu districts of the Mekong Delta, Vietnam was approximately 30 tonnes in 2012 (Toi 2014).

1.5.2.2 *Artemia* as live food

In industrial aquaculture, *Artemia* are known as an excellent live food for most larvae of fish and crustaceans in the hatchery (Sorgeloos et al. 1977), particularly in the early stages. It offers several advantages such as sustainable nutritional composition, appropriate size, ease of ingestion and long term storage capacity (Lavens and Sorgeloos 1996; Sorgeloos et al. 2001). Furthermore, when *Artemia* nauplii molt into the second instar stage, being non-selective particle feeders, simple methods have been developed to incorporate different kinds of products into the *Artemia* prior to feeding to predator larvae (Sorgeloos et al. 1998). Léger et al. (1987) have reported that highly unsaturated fatty acids (HUFAs) enriched nauplii can improve the nutritional composition, *i.e.* they have higher energy content and contain all essential fatty acids including 22:6n-3, which are naturally limited in nauplii from most strains. Through enrichment techniques other nutrients, prophylactics, and therapeutics may be passed to the predator via *Artemia* nauplii as well (Campbell et al. 1993). *Artemia* nauplii

have been used as a killed bacteria vaccine carrier for oral vaccination of fish fry by incubating *Artemia* nauplii with a *Vibrio anguillarum* antigen. Other studies showed that growth, disease resistance and stress tolerance is increased when *Peneaus monodon* postlarvae are fed with enriched *Artemia* nauplii. The enrichment can consist of HUFAs, probionts and herbal products (Immanuel et al. 2007).

Besides the use of *Artemia* nauplii as live food, biomass of ongrown *Artemia* is also used as a protein and lipid source in the feed for aquatic animals (Anh et al. 2009; Dhert et al. 1993). According to Anh et al. (2011a), nursery stage *Scylla paramamosain* crabs can be fed with *Artemia* biomass (live and frozen forms), which were collected in solar salt works when the production season of *Artemia* cysts ends. This resulted in a higher survival as compared to crab fed with fresh shrimp meat. Alternatively, *Artemia* biomass can be cultured in tanks using rice bran and microalgae *Tetraselmis suecica* as food. This results in a biochemical composition of the *Artemia* biomass that is similar to the biomass of *Artemia* adults collected from nature (Teresita et al. 2005). *Artemia* biomass is also specifically fed to large larvae of carnivorous species such as those from lobster (Shleser and Gallagher 1974), mud crab (Mann et al. 2001) or post-larval giant tiger prawn (*Penaeus monodon*) (Anh et al. 2011b).

1.5.2.3 Risks associated with the use of *Artemia* as live food in aquaculture

There are certain risks associated with the use of *Artemia* nauplii as live food for the larvae of fish and shrimp. As mentioned above, the early live stages of fish and shrimp can suffer highly from the uncontrolled interference of opportunistic pathogens. During the incubation of cysts for the production of *Artemia* nauplii, the cyst shell can become loaded with bacteria (such as *Vibrio parahaemolyticus* (Orozco-Medina et al. 2002)), protozoa or fungi. The concentration of contaminating bacteria can reach more than 10^7 CFU/mL in the hatching medium (New and Valenti 2008). In that way, the feeding of *Artemia* nauplii to the larvae can be a main route for the introduction of pathogenic bacteria such as *Vibrio* spp. (López-Torres and Lizárraga-Partida 2001 and Interaminense et al. 2014). The growth of opportunistic bacteria is proportional to the density of *Artemia* nauplii production (Interaminense et al. 2014). In the laboratory, *Vibrio campbellii* and *Vibrio harveyi* are usually used as pathogens for *Artemia* nauplii in gnotobiotic conditions (Defoirdt et al. 2005; Marques et al. 2006).

Normally, to control the bacterial input from *Artemia* in hatcheries, the chemical hypochlorite is widely applied for *Artemia* cyst disinfection (Sorgeloos et al. 2001). Most commercial *Artemia* strains are completely disinfected by this treatment, following the standard disinfection procedures, as described by Sorgeloos et al. (1977). However, the hatching medium of disinfected *Artemia* will be recolonized fast by bacteria during the incubation process. It may pose a threat to the health of the larvae feeding on the *Artemia* in case of pathogenic bacteria (Sorgeloos et al. 2001). Specialized products such as INVE's Sanocare ACE (replacing the former Sanocare Hatch Controller) have been developed to minimize the growth of pathogenic bacteria in the hatching tank (Delbos and Schwarz 2009). Being a proprietary product of INVE Aquaculture NV (Belgium), the active ingredient is not known although it is stated that its composition is mainly of herbal origin.

1.5.2.4 *Artemia* as a model test organism

Artemia has been extensively used in aquaculture research as a model animal for crustaceans. In comparison with target aquaculture animals, trials using *Artemia* have a much higher throughput because of the small scale of the set-up allowing a high number of replicates and/or treatments, the short production time of *Artemia* nauplii out of cysts (ca. 18-24 hours), the short generation time of ca. 2-3 weeks for the production of live offspring by adults, and the possibility to work with sterile *Artemia* nauplii (Marques et al. 2004a; 2004b; 2005). Bacteria free nauplii of *Artemia* can be obtained by decapsulation and sterilization of the cysts followed by incubation under axenic conditions (Sorgeloos et al. 1977; Verschuere et al. 1999; 2000a; b). This explains why many researchers have used *Artemia* as a model organism for example to examine disease infection in penaeid shrimp, lobsters and other crustaceans (Overton and Bland 1981; Criado-Fornelio et al. 1989; Verschuere et al. 1999; 2000a). Using the *Artemia* model the effects of food composition, host microbe interactions, heat shock proteins, antimicrobial agents, etc. on the survival and growth in both challenge and non-challenge tests can elegantly and efficiently be investigated (Marques et al. 2004a; 2004b; 2005; Soltanian et al. 2007; Baruah et al. 2010; Defoirdt et al. 2007b; Cam et al. 2009).

1.6 Measures to control diseases in aquaculture

Most attempts to control disease in aquaculture are directly targeting the pathogens within the host. The use of antibiotics has been the most straightforward strategy for a long time. Antibiotics have been used for various goals, including the treatment of sick animals

(therapeutic), as pre-emptive treatments (prophylactic) and as feed additive for increasing the growth performance (Gunal et al. 2006). The frequent use of antibiotics in a prophylactic way, particularly at suboptimal doses, in aquaculture systems, has resulted in the development of antibiotic-resistant pathogens and in an increased risk of resistant plasmid transfer to pathogens of humans and domesticated animals (Khachatourians 1998; Willis 2000; Das et al. 2009). In this context, the development of alternative approaches to substitute antibiotics has become a challenge for the researchers throughout the world.

1.6.1 Water control

In primary instance, diseases can be controlled at the level of the environment. Disinfection of the water in aquaculture systems is applied as a method to prevent the invasion and persistence of pathogens in the water. It is assumed that as this method eliminates the causative agent, diseases can be avoided (Summerfelt et al. 2009). Lime and hypochlorite were traditionally used to disinfect the culture water (Cruz-Lacierda and De Le Peña, 1996; Tonguthai 2000), to totally remove all living organisms (thus including potential pathogens) usually just used before stocking animals.

Ozone (O_3) was introduced as an alternative approach to control pathogenic bacteria and fungi since it destroys the outer membranes by its powerful oxidizing characteristic (Gräslund and Bengtsson 2001). However, it is commonly applied within a recirculation system during culture of the animals as opposed to lime and hypochlorite application. Therefore, the application of O_3 should be performed with care because next to harmful bacteria it also kills the ones that are beneficial, and even necessary, for the animals during growth. In addition, O_3 also represents a high cost of investment (Gräslund and Bengtsson 2001) while it may also be toxic for the animals (Tango and Gagnon 2003).

Ultraviolet (UV) irradiation is used as an alternative to O_3 to kill pathogens in the water of the recirculation systems by denaturing the DNA of microorganisms (Summerfelt 2003; Liltved 2002). However, the efficient penetration of UV irradiation depends on the turbidity of the water and, similar to ozone, this method does not only target the pathogens, but also the beneficial bacteria (Summerfelt 2003; De Schryver 2010a).

1.6.2 Immunostimulation and vaccination

A second approach to control diseases is the stimulation of the immune system of the cultured animals. Bricknell and Dalmo (2005) stated that "immunostimulants are naturally occurring compounds that increase the host's resistance against disease agents by modulating the immune system". According to Smith et al. (2003) immunostimulants can comprise live bacteria, killed bacteria, glucans, peptidoglycans and lipopolysaccharides. Immunostimulation is not only possible in fish but also in crustaceans although these invertebrates possess a non-specific immune defense mechanism in which circulating haemocytes play an important role (Smith et al. 2003). In fish, immunostimulants activate lymphocytes, enhance phagocytic cell activities and sometime also boost antibody production (Sakai 1999). In crustaceans, immunostimulants arouse responses such as changes in the number and activity of haemocytes, which are extremely important by direct sequestration and killing of infectious agents, the synthesis and exocytosis of a battery of bioactive molecules, and by executing inflammatory-type reactions such as phagocytosis, production of reactive oxygen metabolites and the release of microbicidal proteins (Smith et al. 2003). Immunostimulants have been shown to provide protection for crustacean species such as *Penaeus indicus* (Alabi et al. 1999), *Penaeus monodon* (Thanardkit et al. 2002) and *Artemia* nauplii (Soltanian et al 2007) against luminescent vibriosis. They have also been shown to improve the immunity of *Penaeus monodon* in challenges with white spot syndrome virus (Chang et al. 2003).

Alternative to immunostimulation, vaccination would be a very good method to prevent diseases which are causing losses in aquaculture such as vibriosis and pasteurelosis (Press and Lillehaug 1995). The goal of vaccination is based on the stimulation of long term specific immunity against a specific pathogen by applying a specific antigen (Ellis 1988). The vaccination of crustacean species is therefore more difficult than of fish because all invertebrates are generally thought to lack any form of immunological memory (Rowley and Pope 2012). Nonetheless, several studies increased survival of shrimp, preventing pathogenic growth in experimental "vaccination" trials with white spot syndrome virus or with vibrios (Mavichak et al. 2010; Chotigeat et al. 2007; Powell et al. 2011).

1.6.3 Quorum sensing interference

Another example of a sustainable disease control strategy is quorum sensing (QS) regulation. QS is known as a process of bacterial cell-to-cell communication (Waters and Bassler 2005). The mechanism of this process is that bacteria coordinate the expression of certain genes in response to the presence or absence of small signaling molecules (Defoirdt et al. 2007b), which are called acylated homoserine lactones (Camilli and Bassler 2006) or autoinducers (Defoirdt et al. 2006a; Natrah et al. 2011). In aquaculture, the disruption of QS as a new anti-infective strategy is currently still in the experimental phase. The research to control infections based on the QS mechanism has focused on (*i*) the inhibition of signal synthesis, (*ii*) the application of QS antagonists, (*iii*) the chemical inactivation of QS signals by oxidized halogen antimicrobials, (*iv*) signal molecule biodegradation by bacterial lactonases and by bacteria and eukaryotic acylases and (*v*) the application of QS agonists (Defoirdt et al. 2004). Several of the studies on QS biocontrol have focused on crustacean species such as *Artemia* (Defoirdt et al. 2006a) and *M. rosenbergii* larvae (Nhan et al. 2010a; Pande et al. 2013).

1.6.4 Probiotics and prebiotics

A lot of studies on the control of disease in aquaculture are focusing on the use of probiotics and prebiotics. Verschuere et al. (2000b) proposed a modified definition of probiotics for use in aquaculture, based on the important differences between terrestrial animals and aquaculture farmed species and defined them as "live microbial adjuncts which have a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment". Probiotics modulate the growth of intestinal microbiota, suppress potentially harmful bacteria and reinforce the body's natural defense mechanisms (Giorgio et al. 2010). The action of probiotics is said to be based on several possible modes of action against pathogens, including (*i*) production of inhibitory compounds, (*ii*) competition for nutrients, (*iii*) competition for adhesion sites in the gastrointestinal tract, (*iv*) enhancement of the immune response and (*v*) production of essential nutrients such as vitamins and fatty acids, and enzymatic contribution to digestion (Verschuere et al. 2000b; Vine et al. 2006). A wide

range of microalgae (*Tetraselmis* sp.), yeasts (*Debaryomyces* sp., *Phaffia* sp. and *Saccharomyces* sp.), Gram-positive bacteria (*Bacillus* sp., *Lactococcus* sp., *Micrococcus* sp., *Carnobacterium* sp., *Enterococcus* sp., *Lactobacillus* sp., *Streptococcus* sp., *Weissella* sp.) and Gram-negative bacteria (*Aeromonas* sp., *Alterromonas* sp., *Photorhodobacterium* sp., *Pseudomonas* sp. and *Vibrio* sp.) have been termed as probiotics (Gatesoupe 1999; He et al. 2011; De et al. 2014). A high number of studies have been executed on different kinds of probiotic bacteria in the production of aquaculture species. Currently, liquid or powder (spore and freeze-dried) forms are available as commercial product, and various technologies have been developed to improve the production process with the aim to enhance the functionality of probiotics and to improve the performance (Cruz et al. 2012). In practical application, probiotics can be provided to the host in several ways: (*i*) addition via live food (Gomez-Gil et al. 1998); (*ii*) bathing (Austin et al. 1995; Gram et al. 1999); (*iii*) addition to culture water (Moriarty 1998; Spanggaard et al. 2001); (*iv*) addition to artificial diet (Rengpipat et al. 2000). In Table 1.2 examples are given focusing on crustacean species. The studies on probiotics should not only assess the effects on disease resistance and growth performance, but also the persistence of the probiotic in the intestinal tract of the host. The latter should be an important factor as it determines the endurance of a treatment which in turn has an effect on the cost of applying probiotics (Gatesoupe 1999).

Ringø et al. (2010) has defined prebiotics as non-digestible components that are metabolized by specific health-promoting bacteria while limiting potentially pathogenic bacteria. They act by stimulating the beneficial microorganisms in the gastrointestinal tract and as such improving the intestinal health of the host. More specifically, their use aims at reducing the presence of intestinal pathogens and/or change the production of health related bacterial metabolites (Manning and Gibson 2004) and directly enhancing the innate immune system (Song et al. 2014). Prebiotics are carbohydrates which can be classified according to their molecular size or degree of polymerization (number of monosaccharide units) into oligosaccharides or polysaccharides (Ringø et al. 2010). The acidification of the colonic environment is of specific importance. The production of short-chain fatty acids (SCFAs), which are generally considered to be positive for gut health, is promoted due to the fermentation of these prebiotic compounds in the gastrointestinal tract (Bongers and Van den Heuvel 2007). Some of the most common prebiotics that have been investigated since their introduction in aquaculture of crustaceans are listed in Table 1.2.

Table 1.2 Examples of biocontrol measures against crustacean disease in aquaculture (after De Schryver 2010a)

Crustacean species	Antagonist/active compound	Disease	Probiotic/prebiotic effect	Reference
Antagonistic activity of probiotics against pathogens:				
Shrimp	*Bacillus sp.*	Luminescent vibriosis	Increase in survival up to 80 – 100%	Moriarty (1999)
Black tiger shrimp *(Penaeus monodon)*	*Bacillus S11*	Vibriosis	Growth and survival of shrimp	Rengpipat et al. (2003)
Black tiger shrimp *(Penaeus monodon)*	*Bacillus subtilis* BT23	*Vibrio harveyi*	Antagonistic effect in *in vitro* assay against *Vibrio* sp. isolated from *Penaeus monodon*	Vaseeharan and Ramasamy (2003)
Whiteleg shrimp *(Litopenaeus vannamei)*	*Bacillus subtilis* UTM126	Vibriosis	Protection against disease	Balcázar and Rojas-Luna (2007)
Macrobrachium rosenbergii	*Bacillus subtillis*	*Aeromonas hydrophila*	Increase in survival	Mehran and Masoumeh (2012)
Juveniles of *Macrobrachium rosenbergii*	*Bacillus* NL110 and *Vibrio* NE17	Undefined cause of mortality	Improvements in water quality, growth, survival, SGR, FCR and other immune parameters	Rahiman et al. (2010)
Whiteleg shrimp (*Litopenaeus vannamei*)	*Pediococcus pentosaceus* and *Staphylococcus hemolyticus*	WSSV and IHHNV	Decrease in the prevalence of WSSV	Leyva-Madrigal et al. (2011)

Table 1.2 *(continued)*

Crustacean species	Antagonist/active compound	Disease	Probiotic/prebiotic effect	Reference
Antagonistic activity of probiotics against pathogens:				
Whiteleg shrimp (*Litopenaeus vannamei*)	*Bacillus subtilis* and *Bacillus megaterium*	Undefined cause of mortality	Increased stress tolerance	Olmos et al. (2011)
Swimming crab (Zoea)	*Pseudoalteromonassaliena*	Undefined cause of mortality	Mortality rate reduced	Morya et al. (2013)
Prebiotic application:				
Whiteleg shrimp (*Litopenaeus vannamei*)	(Short-chain) fructooligosaccharides	Undefined cause of mortality	Increase in survival up to 19%	Zhou et al. (2007)
Tiger shrimp (*Penaeus semisulcatus*)	Mannanoligosaccharides	Undefined cause of mortality	Increased growth, feed conversion and survival	Genc et al. (2007)
Pacific white shrimp	Isomaltooligosaccharides	White Spot Virus	Improved disease resistance by enhancing immunity and modulate microbiota in the gut	Li et al. (2009)

1.6.5 Alcaligenes *spp. and* Bacillus *spp. as probiotics*

Alcaligenes spp.: Members of the genus *Alcaligenes* are ubiquitous, non-fermentative, Gram-negative rods occurring naturally in both marine and fresh water, soil and sewage (Tilton 1981). Several studies have considered this genus as a pathogen of man, animals or plants since the isolation of this genus has been reported occasionally from diseased tissues (Tilton 1981). However, the role of this group of bacteria as a pathogen is not well understood (Tilton 1981). *Alcaligenes eutrophus* (also known under the names *Ralstonia eutropha*, *Cupriavidus metallidurans and Cupriavidus neccator*) is a non spore-forming bacterium found natively in soils that can utilize a wide array of carbon sources for growth, and can accumulate carbon intracellularly in the form of polyhydroxyalkanoates (Brigham et al. 2012). This bacterium was originally considered for its potential as single cell protein (SCP) in the 1970s, for which its efficient accumulation of non-nutritive polyhydroxybutyrate (PHB) in the cytoplasm was undesirable (Kunasundari et al. 2013). For this reason it quickly lost interest as SCP, and became the most widely used organism for the production of PHB because it is easy to grow, and because it can synthesize high amounts of PHB in simple media (Kim et al. 1994). The genus of *Alcaligenes* has the typical feature of metal resistance because it contains genes for multiple resistance to heavy metals in one or two megaplasmids (Collard et al. 1994). In aquaculture research, the addition of freeze-dried *Alcaligenes eutrophus* combined to an algal diet was shown to increase the survival of blue mussel (*Mytilus edulis*) larvae (Hung et al. 2015).

Bacillus spp.: *Bacillus* species are rod-shaped, endospore-forming aerobic or facultatively anaerobic, Gram-positive bacteria, and many *Bacillus* species can live in every natural environment based on a wide range of physiological abilities (Peter and Turnbull 1996). A high number of *Bacillus* species are not harmful to mammals, including humans, and are commercially important as producers of a high and diverse amount of secondary metabolites such as antibiotics, bio-insecticides, biosurfactants and enzymes (Hong et al. 2005). As an example relating to aquaculture, poly-β-hydroxybutyrate-hydroxyvalerate (PHB-HV) was extracted from *Bacillus thuringiensis B.t.*A102 and used as a potential immunostimulant to enhance the immune system of *Oreochromis mossambicus* by supplementation in the feed (Suguna et al. 2014). Several commercial bacilli probiotics have been or are being used in aquaculture such as Biostart® (Microbial Solutions, Johannesburg, South Africa and

Advanced Microbial Systems, USA), consisting of a mixture of *B. megaterium*, *B. licheniformis*, *Paenibacillus polymyxa* and two strains of *B. subtilis* (Verschuere et al. 2000b), Promarine® (Sino-Aqua company Kaohsiung, Taiwan), containing 4 strains of *B. subtilis* (Urdaci and Pinchuk 2004), and Sanolife probiotics (INVE Aquaculture NV, Belgium) containing a mixture of *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus*. Recently, it has been reported that in shrimp farms (*Penaeus monodon*) in Asia and Latin America probiotics based on *Bacillus subtilis* are recurrently used to prevent the bacterial white spot syndrome (BWSS) (Cruz et al. 2012). The use of bacilli for aquatic food production should, however, always be considered with care. For example, Defoirdt and his colleagues (2011) have recently isolated *Bacillus* strains from N-acyl-homoserine-lactone (AHLs) degradation enrichment cultures originating from whiteleg shrimp and European sea bass. These isolates have been shown to provide protection for *Artemia* nauplii against pathogenic *V. campbellii* (Niu et al. 2014), assumed to result from their quorum sensing interfering activity. Based on 16S r RNA gene analysis, however, these isolates seemed to belong to the *Bacillus cereus* complex. This complex consists of genetically very closely related members that include *B. anthracis, B. thuringiensis* and *B. cereus* (Dwyer et al. 2004). Based on the 16S rRNA gene sequence, however, members of the *B. cereus* group are very difficult to distinguish reliably (EFSA 2007) and although genetically similar this group has different ecological niches. *B. anthracis*, a highly toxic bacterium is the causative agent of anthrax in humans and animals (Ravel et al. 2004). It can cause fatal infection in domestic livestock (DelVecchio et al. 2006). *B. cereus* is a known opportunistic foodborn human pathogen (Granum and Lund 1997; Jackson et al. 1995) while *Bacillus thuringiensis* is known as an insect pathogen and is already used as biopesticide for many years. Because of the large variety in functions that genetically closely related bacilli can have, their identification and characterisation is of primordial importance. For this reason, the European Food Safety Authority (EFSA) has written out specific guidelines that should be fulfilled before they can be approved to be used in food and feed production (EFSA 2014).

1.7 Poly-β-hydroxybutyrate as antimicrobial agent in aquaculture

Disease outbreaks have a negative impact on the development of the aquaculture sector (Subasinghe et al. 2001). Serious losses in the intensive rearing of finfish, mollusks, lobster and shrimp are imputed to luminescent vibrios (Pass et al. 1987; Lavilla-Pitogo et al. 1998; Diggles et al. 2000; Zhang and Austin 2000). Antibiotic treatment, the conventional strategy

to control the bacterial population, has been quite prevalent. However, multiple resistances in several pathogens have appeared when antibiotics are used as a prophylactic approach in aquaculture (Teo et al. 2000; 2002). Alternative strategies based on short chain fatty acids (SCFAs) and poly-β-hydroxybutyrate (PHB) (Defoirdt et al. 2009), are now being tested. A high potential as an alternative antimicrobial agent is bestowed on PHB because it is insoluble in water as opposed to volatile SCFAs, thereby increasing the uptake efficiency (Sui et al. 2012).

1.7.1 The group of polyhydroxyalkanoates

According to Reddy et al. (2003), polyhydroxyalkanoates (PHAs) are synthesized by many Gram-positive and Gram-negative bacteria from at least 75 different genera, under conditions of nutrient limitation and carbon excess (Tian et al. 2009). During periods of carbon shortage in the cells, PHA acts as carbon and energy reserves (Madison and Huisman 1999). Anderson and Dawes (1990) reported that PHAs are accumulated as discrete granules to levels as high as 90% of the cell dry weight. The many different PHAs that have been identified to date, are primarily linear, head-to-tail polyesters composed of β-hydroxy fatty acid monomers (Madison and Huisman 1999). Poly-β-hydroxybutyrate (PHB) (Fig. 1.8) is the simplest and most common member of the group of polyhydroxyalkanoates (Freier et al. 2002). PHB is the most extensively characterized polymer of all PHAs (Lee 1996).

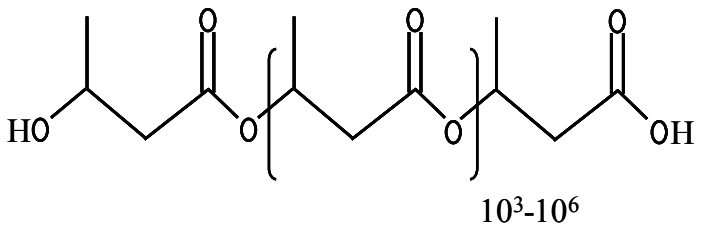


Figure 1.8 Structural formula of poly-β-hydroxybutyrate

1.7.2 The metabolism of polyhydroxyalkanoates

The biosynthetic pathway of poly-3-hydroxybutyrate (PHB) consists of three enzymatic reactions catalyzed by three distinct enzymes (Fig. 1.9). The first enzyme, β-ketothiolase (encoded by *phb*A), is used to promote the condensation of two acetyl coenzyme A (acetyl-CoA) molecules into acetoacetyl-CoA. Next, the second enzyme, acetoacetyl-CoA reductase

(encoded by *phb*B), reduces acetoacetyl-CoA to 3-hydroxybutyryl-CoA. Finally, the third enzyme, P(3HB) polymerase (encoded by *phb*C), synthesizes 3-hydroxybutyryl-CoA monomers into PHB (Huisman et al. 1989; Reddy et al. 2003).

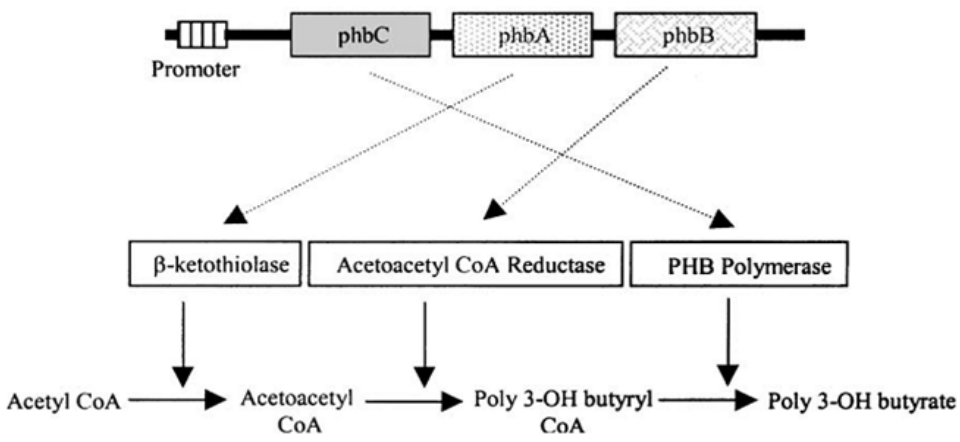


Figure 1.9 Biosynthetic pathway of poly(3-hydroxybutyrate). P(3HB) is synthesized by the successive action of β-ketothiolase (*phb*A), acetoacetyl-CoA reductase (*phb*B) and PHB polymerase (*phb*C) in a three-step pathway. The genes of the *phb*CAB operon encode the three enzymes. The promoter upstream of *phb*C transcribes the complete operon (*phb*CAB) (Madison and Huisman 1999).

According to Doi et al. (1988), in the sequence of reactions of the PHB biosynthetic pathway, β-ketothiolase is the bottle-neck enzyme because it is competitively inhibited under balanced growth conditions by high concentrations of free Coenzyme A (CoASH) that is released when acetyl-CoA enters the Krebs cycle. On the other hand, when limiting conditions for growth are imposed, by providing excess of carbon source and insufficient amounts of other nutrients (mostly nitrogen), production of PHB is stimulated. Under such conditions, the concentration of acetyl-CoA remains high and the concentration of CoASH low (Patnaik 2005), and β-ketothiolase is activated. According to the latter author, this mechanism is the most important point for PHB production based on the C/N ratio. Often a C/N ratio of 20 is considered optimal for producing PHB (Rathore et al. 2014)

According to Rathore (2014) there are two main phases involved in the aerobic process of PHB production: firstly, all necessary nutrients (carbon source, nitrogen source, oxygen) are directed to biomass growth. In a second phase, when nutrient conditions (nitrogen) are becoming limiting, the presence of only adequate amounts of carbon source and oxygen

results in the accumulation of the carbon source in the form of PHB in the bacterial cell. PHB has also been shown to be produced under anaerobic, aerobic and anaerobic/aerobic conditions by activated sludge performing enhanced biological phosphorus removal. However, the maximum PHB content accumulated under anaerobic conditions was only half of that accumulated under aerobic or aerobic/anaerobic conditions (Rodgers and Wu 2009).

1.7.3 The production and cost of polyhydroxyalkanoates

The presence of polyhydroxyalkanoates was first found in *Bacillus megaterium* in 1925 by Lemoigne (Dawes 1988). The function of PHAs in the genus *Bacillus* was known as an intracellular reserve for carbon and energy (Sudesh et al. 2000). Bacteria producing PHAs can be isolated from different environments and include species such as: *Bacillus megaterium* SW1-2 isolated from activated sewage sludge (Berekaa and Al Thawadi 2012), *Ralstonia* spp. isolated from soils (Bonatto et al. 2004) and *Bacillus* spp. isolated from the intestine of various fish species (Kaynar and Beyatli 2009) (Table 1.3). Furthermore, the studies performed on PHA producing mixed microbial cultures in different types of reactor configurations and on recombinant PHA producing strains illustrate the range of possibilities to produce PHAs (De Schryver 2010a) (Table 1.4).

The production costs of PHA are mainly influenced by the PHA yield out of the substrate which is determined by the intrinsic capacity of the bacterium to accumulate PHB, the technological process for PHB production and the type and efficiency of the recovery method (Choi and Lee 1999). Up to 40% of the total PHB production costs are related to the substrate, a main cost contributor in the PHB process (Singh et al. 2013b). The prices of some commercial PHB products are given by Chanprateep (2010) (Table 1.5).

Table 1.3 PHB production (% on cell dry weight) by *Bacillus* species isolated from the intestines of various fish species in nutrient medium (NB – Merck) (Kaynar and Beyatli 2009)

Fish origins	*Bacillus* species	Yield of PHB (%)
Bluefish	*Bacillus megaterium, Bacillus pasteurii*	2.50 - 20.58
Anchovy	*Bacillus megaterium, Bacillus circulans, Bacillus subtilis, Bacillus cereus, Bacillus thuringiensis*	0.81 - 20.57
Horse mackerel	*Bacillus pasteurii, Bacillus coagulans, Bacillus sphaericus, Bacillus circulans, Bacillus pumilus*	1.78 - 13.92
Grey mullet	*Bacillus pasteurii, Bacillus circulans, Bacillus lentus, Bacillus badius, Bacillus brevis*	4.40 - 23.38
Red sea bream	*Bacillus badius, Bacillus licheniformis*	1.76 - 11.34
Whiting	*Bacillus licheniformis, Bacillus thuringiensis, Bacillus thuringiensis*	9.69 - 18.33

Table 1.4 Accumulation of poly-β-hydroxybutyrate (PHB) in different types of bacterial cultures (after De chryver 2010a)

Strain/culture	Carbon source	PHB content (% on cell dry weight)	Yield (g PHB/g substrate)	Production cost (€/kg PHB)	Reference
Pure strains (for commercial PHB production):					
Alcaligenes eutrophus	Glucose	76	0.3	5.6 - 9.2[a]	Choi and Lee 1997
Alcaligenes latus	Sucrose	88	0.42	2.0[a]	Choi and Lee 1999
Methylobacterium organophilum	Methanol	52	0.19	5.3[a]	Choi and Lee 1999
Recombinant *Escherichia coli*	Glucose	77	0.27	3.9[a]	Choi and Lee 1999
Ralstonia eutrophus	Glucose	> 75	[b]	[b]	Chen et al. 2009
Pseudomonas putida	Fatty acids	> 60	[b]	[b]	Chen et al. 2009
Aeromonas hydrophila	Lauric acid	< 50	[b]	[b]	Chen et al. 2009
Bacillus spp.	Sucrose	> 50	[b]	[b]	Chen et al. 2009

[a] *Current production costs can be expected to be different due to changes in substrate costs and process optimization;* [b] *no data available*

Table 1.4 *(continued)*

Strain/culture	Carbon source	PHB content (% on cell dry weight)	Yield (g PHB/g substrate)	Production cost (€/kg PHB)	Reference
Mixed cultures (emphasis on waste valorization):					
Mixed culture	Excess sugar	65 – 70	[b]	2.1	Castilho et al. 2009
Fed batch mixed culture	Acetate	Up to 84	[b]	[b]	Johnson et al. 2010
Activated sludge	Effluent of anaerobically fermented sludge	Up to 57	[b]	[b]	Cai et al. 2009
Enrichment culture	Acetic, lactic and propionic acid	50	0.39 (as COD)	[b]	Dionisi et al. 2005
Activated sludge		21	[b]	[b]	Chua et al. 2003
Sewage bacteria	Municipal wastewater Food waste	Up to 51	0.05 (substrate as COD)		Rhu et al. 2003
Fed-batch culture	Waste glycerol	36	[b]	[b]	Cavalheiro et al. 2012
Fed-batch culture	Biodiesel-glycerol	31	[b]	[b]	Zhu et al. 2010

[a] *Current production costs can be expected to be different due to changes in substrate costs and process optimization;* [b] *no data available.*

Table 1.5 The current large volume manufacturers of polyhydroxyalkanoates (Chanprateep 2010)

Polymer	Trade names	Manufacturers	Market price (in 2010) (€/kg)
PHB	Biogreen®	Mitsubishi Gas Chemical Company Inc. (Japan)	2.5 – 3.0
PHB	Mirel™	Telles (USA)	1.5
PHB	Biocycle®	PHB Industrial Company (Brazil)	n/a
PHBV and PHB	Biomer®	Biomer Inc. (Germany)	3.0 – 5.0
PHBH, PHBV + Ecoflex blend	Enmat®	Tianan Biologic, Ningbo (China)	3.3
PHBH	Nodax™	P&P (USA)	2.5
PHBH	Nodax™	Lianyi Biotech (China)	3.7
PHBH	Kaneka PHBH	Kaneka Corporation (Japan)	n/a
P(3HB-*co*-4HB)	Green Bio	Tianjin Green Bio-Science Co/DSM (The Netherlands)	n/a
PHA from P&G	Meredian	Meredian (USA)	n/a

n/a: price could not be found

The combination between an increase in efficient processing (such as optimization and larger scale production) and the use of cheaper substrates are strongly required to decrease the production cost of PHA. Carbon sources currently under investigation include starch-based materials, (hemi-)cellulolytic materials, oil-, fatty acid-, or glycerol-containing streams, gaseous flows (mixtures of H_2 and CO_2) or the organic matter in waste and wastewater (Castilho et al. 2009). Particularly the use of crude glycerol, a by-product of the biodiesel industry, has been attempted for PHB production by different bacteria strains such as *Paracoccus denitrificans* and *Cupriavidus necator* JMP 134 (Mothes et al. 2007), *Burkholderia cepacia* ATCC 17759 (Zhu et al. 2010) and *Bacillus firmus* NII 0830 (Jincy et

al. 2013). The mechanism of PHB production from crude glycerol is described in Figure 1.10.

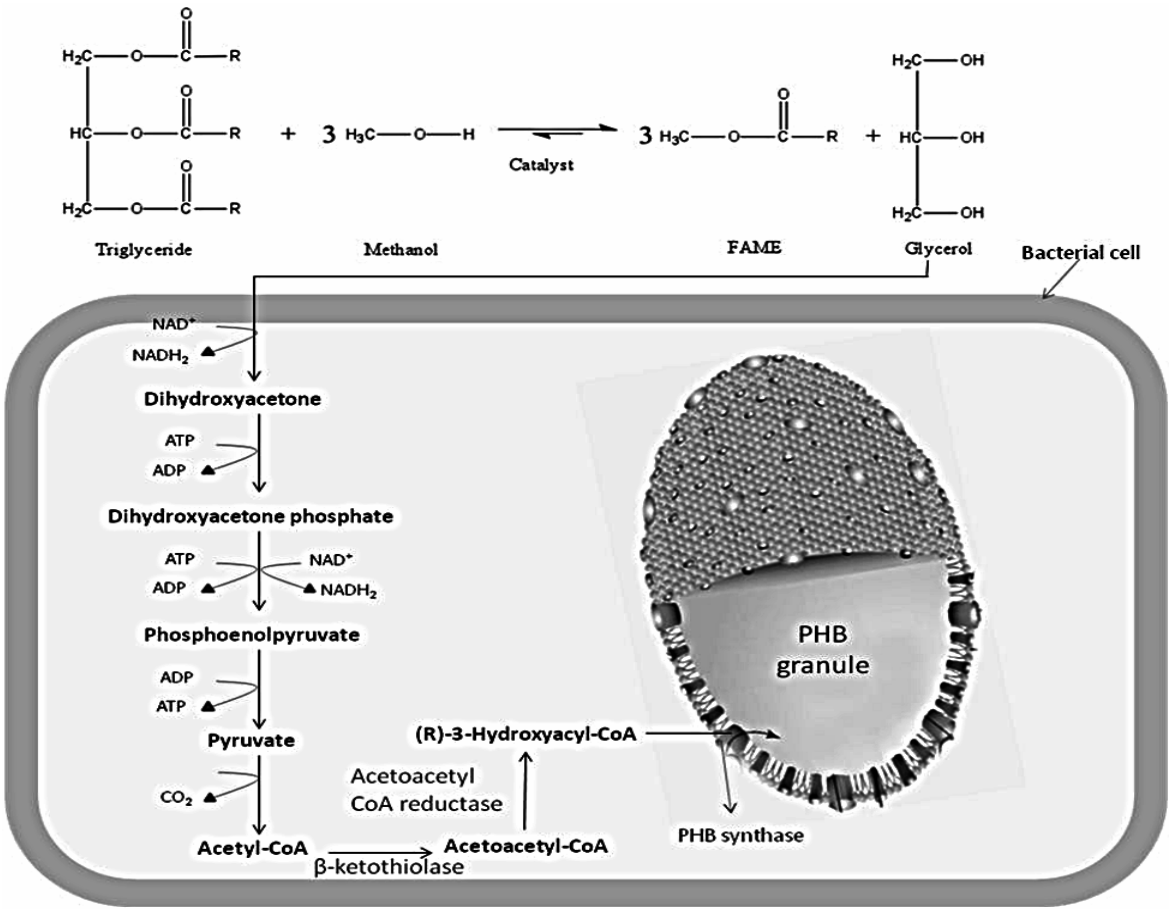


Figure 1.10 Biochemical pathway involved in bacterial production of PHB from glycerol (Jincy et al. 2013).

1.7.4 The degradation of polyhydroxyalkanoates

Microorganisms degrade PHAs by depolymerase enzymes (Jendrossek and Handrick 2002). There are two possible ways by which micro-organisms can use PHB for energy generating purposes: (1) *intracellular degradation* results from the intracellular production of PHA depolymerase enzymes to use PHA that the organism has stored itself (Kadouri et al. 2005; Jendrossek and Handrick 2002) and (2) *extracellular degradation* is the excretion of extracellular PHA depolymerases for the metabolisation of PHA in the environment of the cell after which the degradation products are absorbed through the cell surface (Tokiwa and Calabia 2007). This metabolism can be done by bacteria that do not necessarily accumulate

PHA. The isolation of aerobic and anaerobic microorganisms that are able to degrade extracellular PHAs has been successful from a variety of ecosystems. PHA degrading bacteria, such as *Acidovorax* spp., *Bacillus* spp., *Brevibacillus* spp., *Comamonas testosteroni*, *Pseudomonas* spp., *Rhodoferax* spp., *Terrabacter* spp., and *Variovorax* spp., have been isolated from soils (Mergaert et al. 1994; Suyama et al. 1998), *Nocardiopsis aegyptia*, *Ochrobactrum* spp., *Pseudomonas* spp., and *Spinghomonas* spp. isolated from marine environment (Ohura et al. 1999; Ghanem et al. 2005) and *Corynebacterium aquaticum*, and *Xanthomonas* spp. isolated from activated sludge (Ito et al. 1998; Kim et al. 2000).

1.7.5 The potential of poly-β-hydroxybutyrate as an antimicrobial agent for aquaculture application

Poly-β-hydroxybutyrate (PHB) is the most common and one of the most extensively studied types of PHAs. Its monomer, β-hydroxybutyric acid, is known to exhibit some antimicrobial, insecticidal and antiviral activities (Tokiwa and Ugwu 2007). β-hydroxybutyric acid can pass the cell membrane of bacteria in its undissociated form, turning into the dissociated form in the more alkaline cytoplasm, thereby increasing the intracellular concentration of protons (Kashket 1987; Cherrington at al. 1991). The pathogenic cells then have to spend their energy in order to maintain the intracellular pH at the optimal level and therefore, the growth and virulence of the cells is inhibited (Defoirdt et al. 2007b). Such antimicrobial effect has earlier been shown for the short chain fatty acids (SCFAs) acetate, propionate, butyrate and valerate by Defoirdt et al. (2006b). The antimicrobial efficiency of these compounds seemed to be largely depending on the environmental pH which determines the balance between the dissociated and undissociated form of the acids. Based on these antimicrobial characteristics of SCFAs, the use of PHB in aquaculture was suggested by Defoirdt et al. (2007b). The advantage that PHB has over other organic acid based antimicrobial treatments is that it is insoluble in water, making it very interesting for application in feeds to be thrown in water (Sui et al. 2012). Two kinds of PHB forms can be used in aquaculture: native PHB (amorphous form) still inside cells and with an intact surface layer (such as proteins and phospholipids) and denatured PHB (crystalline form) extracted from the cells (i.e., by solvent extraction) with the polymers crystallized at least

partially (typical degree of crystallinity is 50 – 60%) (Jendrossek and Handrick 2002; Defoirdt et al. 2009).

The potential to apply PHB in the culture of aquatic animals has been investigated for several species. Most research has focused on the use of PHB in particle form due to its ease of application and storage. Defoirdt et al. (2007b) performed a study on axenic *Artemia* nauplii by adding 1000 mg/L crystalline PHB particles in the culture medium. The results showed that this strategy prolonged survival in the case of starved nauplii. This implied that PHB must have been (at least partially) degraded and absorbed during gastrointestinal passage. β-hydroxybutyric acid was reported by Weltzen et al. (2000) as an energy source for *Artemia* nauplii in the form of ketone bodies which is likely the reason for the higher survival of the nauplii. In the same study, Defoirdt and his colleagues found that the supplementation of crystalline PHB particles to *Artemia* nauplii could protect the nauplii against *Vibrio campbellii*. As mentioned previously, this illustrated the potential of PHB as an antimicrobial agent. In another study executed by De Schryver et al. (2010b), the diet of European sea bass (*Dicentrarchus labrax*) juveniles was supplemented with crystalline PHB particles at the level of 2%, 5% and 10%. They suggested that PHB could be degraded and absorbed during gastrointestinal passage in the fish and as such decreased the intestinal pH values from 7.7 to 7.2. A significantly increased growth rate and decreased feed conversion ratio (FCR) was found at PHB levels of 2% and 5% in the diets. Some other studies have been executed to investigate the effects of crystalline PHB specifically for crustacean larviculture. When supplied through live food enrichment, Nhan et al. (2010b) found that PHB treatment significantly increased about 2-fold and 5-fold the survival and the number of postlarvae obtained, respectively, as compared to a control treatment fed non-enriched *Artemia* nauplii after 28 days of culture. In addition, the load of presumed vibrios decreased approximately 10-fold from 13.3 x 10^2 CFU/larva (control treatment) to 1.6 x 10^2 CFU/larva (PHB treatment). This indicated that adding crystalline PHB inhibited the growth of potentially pathogenic microorganism in the gut of the host. Similar results were obtained by Sui et al. (2012) who supplied PHB to larval Chinese mitten crab (*E. sinensis*) through enrichment in *Artemia* nauplii and rotifers.

Fewer studies have been performed on the application of amorphous PHB in aquaculture. Halet et al. (2007) grew a mixed bacterial culture with a PHB content of 32% on cell dry

weight and investigated if this PHB containing biomass could be used as an antimicrobial agent to control luminescent vibriosis. The biomass could indeed significantly enhance the survival of nauplii challenged with pathogenic *V. campbellii* strain LMG21363, a feature which seemed to be dependent on its PHB content. In comparison with the dose of 1000 mg/L of crystalline PHB particles used by Defoirdt et al. (2007b), these authors found that the effective dosage of PHB accumulated in bacteria was only 10 mg/L. The higher efficiency in use of amorphous PHB is probably due to the smaller particle size of PHB particles in the bacterial cells (which are typically 0.2 – 0.5 μm in diameter (Anderson and Dawes 1990)), while crystalline PHB has an average size of 30 μm (Defoirdt et al. 2007b), thereby increasing the susceptibility of cell-stored PHB to breakdown by higher surface/volume ratio. In addition, the *Artemia* nauplii can obtain other nutrients from the bacterial cells, such as protein, carbohydrate, lipid, ash, etc. (Brown et al 1996), because amorphous PHB present in bacterial cells is covered with a surface layer of e.g. proteins and phospholipids (Jendrossek and Handrick 2002).

1.7.6 Obstacles for the application of PHB in aquaculture

As illustrated by the examples given above, PHB has the potential to be applied in aquaculture to control pathogenic bacteria and thus to minimize the amount of antibiotics used. However, the most important issue that hinders the PHB application in aquaculture is the high price of this product. Several solutions have already been proposed to decrease the cost of PHB for use in aquaculture. The crystalline PHB production generally contains several steps, including bacterial selection, culture process, separation of biomass from the broth, biomass drying, PHB extraction, PHB drying and packing of PHB (Chen 2010). For that reason, the use of amorphous PHB rather than crystalline PHB is strongly suggested in aquaculture. The price for amorphous PHB production is undoubtedly less than for crystalline PHB since it does not require the extraction step in the process. However, the price of amorphous PHB can still be decreased further because it is known that in PHB production, the high production cost is accounted for by the raw material, and thus, the use of cheap carbon sources or carbon-rich waste water sources offers high potential for further decreasing PHB production costs (Singh et al. 2013b).

1.8 Thesis objectives and outline

During the last decade, poly-β-hydroxybutyrate (PHB) - a natural compound from bacterial origin - has received a lot of attention of many researchers in the field of aquaculture all over the world. Although its application has been shown to bring benefits to a wide variety of aquaculture animals, its price is prohibiting full scale use in aquaculture. The development of cost-effective strategies to produce PHB and the effective use of PHB produced in that way in larviculture is thus highly required.

General objectives

The general objectives of this thesis were (1) to study the use of amorphous PHB accumulating bacteria in crustacean larviculture and (2) to investigate a novel approach for culturing PHB accumulating bacteria through integrating it with *Artemia* nauplii production (Figure 1.11).

Specific objectives

- **Chapter 2** ***(Effect of*** **Alcaligenes eutrophus** ***carrier of poly-β-hydroxybutyrate for the culture of*** **M. rosenbergii** ***larvae)***:
  - ***Section 1***: The feeding of a lyophilized form of *A. eutrophus* – containing two levels of PHB (10 & 80% PHB on cell dry weight (CDW) was investigated for *M. rosenbergii* larviculture with the aim to verify its effect on growth performance of prawn larvae and disease resistance upon challenge with *Vibrio harveyi* BB120.
  - ***Section 2***: A freshly grown form of PHB accumulating *A. eutrophus* was cultured in standard nutrient medium (LB - 12g/L salinity). The efficiency to protect *Artemia* nauplii and *M. rosenbergii* larvae against the pathogenic bacteria *Vibrio campbellii* LMG21363 and *V. harveyi* BB120 was investigated by adding it into the culture water or by enriching the live food, respectively.

- **Chapter 3** ***(Changes in carbon and nitrogen patterns in the hatching medium of decapsulated*** **Artemia franciscana** ***cysts)***: In this chapter the change in glycerol, glycogen, trehalose, total organic carbon (TOC) and total nitrogen (TN) in the hatching medium of axenic decapsulated *Artemia* cysts throughout the incubation period was

measured. In addition, it was attempted to use the carbon and nitrogen released into the hatching medium as a substrate for growing PHB accumulating bacteria.

- ***Chapter 4 (Protection of gnotobiotic* Artemia *nauplii from pathogenic* Vibrio campbellii *by* Bacillus *sp. LT12 cultured in axenic hatching medium of* Artemia franciscana*)***: The objective of this chapter was (1) to select *Bacillus* strains isolated from the intestine of shrimp and fish based on two criteria, namely their suitability as food for *Artemia* nauplii and their capacity to produce PHB and (2) to investigate whether PHB is accumulated in these *Bacillus* strains when cultured in axenic hatching medium of *A. franciscana* and whether this offers protection for *Artemia* nauplii against pathogenic *V. campbellii* LMG21363 upon addition of these bacilli to the culture medium.

- ***Chapter 5 (PHB-accumulating* Bacillus *sp. cultured in the axenic hatching medium of* Artemia franciscana *can protect* Macrobrachium *larvae against* Vibrio harveyi*)***: The objective of this chapter was to assess the effect of *Bacillus* strain LT12 cultured in axenic hatching medium of *Artemia* (AHMA) and enriched into *Artemia* nauplii on *Macrobrachium* larvae challenged with *Vibrio harveyi* BB120. The *Bacillus* strain LT12 was grown in *Artemia* hatching medium in two different ways: the *Bacillus* was either (1) grown in the axenic hatching medium of *Artemia* separated from the hatching *Artemia* nauplii after 16, 20 or 24 hours of incubation. Subsequently *Artemia* nauplii were enriched for 2 h in a suspension of the *Bacillus*, or (2) directly co-cultured in the axenic hatching medium of *Artemia* during the 26 hour hatching process. In the second case, addition of supplemental glycerol at the start of incubation was tested as a second factor.

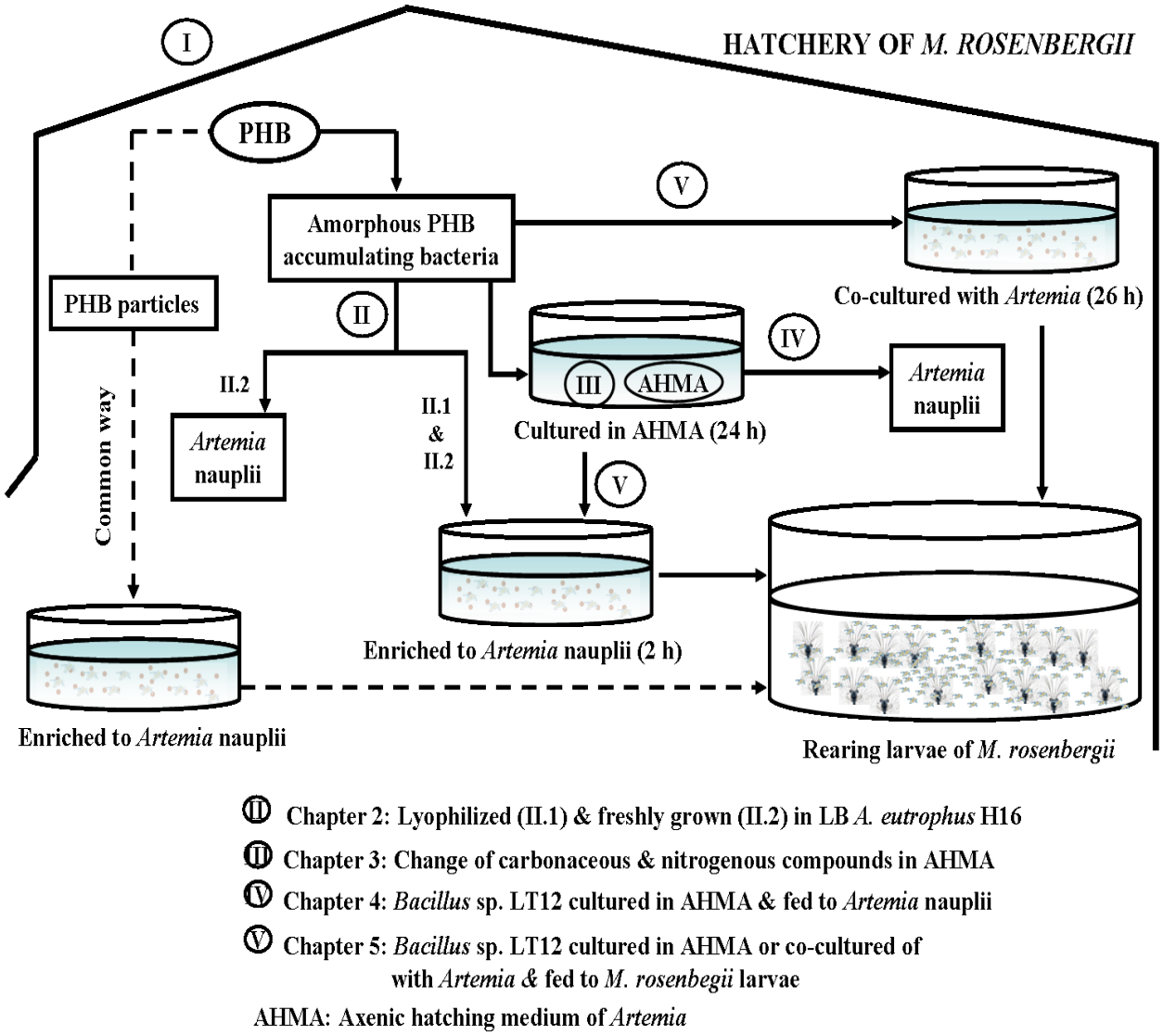


Figure 1.11 Setting of the different chapters in this research with PHB as a common denominator

CHAPTER 2

Section 1

Poly-β-hydroxybutyrate content and dose of the bacterial carrier for *Artemia* enrichment determine the performance of giant freshwater prawn larvae

[1] Redrafted after Thai TQ, Wille M, Garcia-Gonzalez L, Sorgeloos P, Bossier P, De Schryver P (2014) Poly-β-hydroxybutyrate content and dose of the bacterial carrier for *Artemia* enrichment determine the performance of giant freshwater prawn larvae. Appl Microbiol Biotechnol 98:5205-5215.

Abstract

The beneficial effects of poly-β-hydroxybutyrate (PHB) for aquaculture animals have been shown in several studies. The strategy of applying PHB contained in a bacterial carrier has, however, hardly been considered. The effect of administering PHB-accumulated *Alcaligenes eutrophus* containing 10% or 80% PHB on dry weight, named A10 and A80, respectively, through the live food *Artemia* was investigated on the culture performance of larvae of the giant freshwater prawn (*Macrobrachium rosenbergii*). Feeding larvae with *Artemia* nauplii enriched in a medium containing 100 mg/L and 1000 mg/L A80 significantly increased the survival with about 15% and the development of the larvae with a larval stage index of about 1 as compared to feeding non-enriched *Artemia*. The survival of the larvae also significantly increased with about 35% in case of a challenge with *Vibrio harveyi*. The efficiency of these treatments was equal to a control treatment of *Artemia* enriched in a 800 mg/L PHB powder suspension, while *Artemia* enriched in 10 mg/L A80, 100 mg/L A10, and 1000 mg/L A10 did not bring similar effects. From our results, it can be concluded that PHB supplemented in a bacterial carrier (i.e. amorphous PHB) can increase the larviculture efficiency of giant freshwater prawn similar to supplementation of PHB in powdered form (i.e. crystalline PHB). When the level of PHB in the bacterial carrier is high, similar beneficial effects can be achieved as crystalline PHB but at a lower live food enrichment concentration expressed on PHB basis.

2.1.1 Introduction

The occurrence of bacterial infections is a major obstacle in the development of giant freshwater prawn (*Macrobrachium rosenbergii*) production (Sung et al. 2000; Kennedy et al. 2006; Shailender et al. 2012). In larviculture, mass mortality of larvae as well as infections in post-larvae have especially been imputed to opportunistic pathogens (Nhan et al. 2010b). *Vibrio* spp. have been isolated at several occasions from affected *M. rosenbergii* larvae and are considered the main causative agents of disease outbreaks in larviculture of *M. rosenbergii* (Alavandi et al. 2004; Jayaprakash et al. 2006). Besides maintaining optimal environmental conditions in prawn hatcheries, antibiotics have been used as a popular method to control bacterial pathogens. However, the prophylactic use of antibiotics can result in multiple resistances in several pathogens (Teo et al. 2000; 2002), leading to ineffective treatment and an increased risk of resistance transfer to animal and human pathogens (Defoirdt et al. 2007a; Das et al. 2009; Liu et al. 2010). Therefore, new microbial control techniques should be developed to make *M. rosenbergii* culture more sustainable.

Short chain fatty acids (SCFAs) seem good candidates to be used in an alternative strategy as they have been shown to possess bacteriostatic and bactericidal activity depending on the concentration of application (Defoirdt et al. 2009). The mechanism for inhibition of pathogenic growth is based on the un-dissociated form of the acids being able to penetrate the bacterial cell membrane. Once in the neutral cytoplasm, the proton (H^+) is released from the acid resulting in a decreased intracellular pH. The bacterium then has to redirect energy to pumping out the excess in protons in order to maintain a constant intracellular pH. This extra effort in the cell metabolism leads to a lower cell growth and potentially even cell death (Kato et al. 1992; De Schryver et al. 2010b). Applicability of the strategy in an aquaculture context was illustrated by Defoirdt et al. (2006b) who showed that SCFAs significantly enhanced the survival of *Artemia* nauplii in *in vivo* challenge tests with pathogenic *Vibrio campbellii*. Other luminescent vibrios and enteric bacteria (such as *Salmonella* spp.) are also susceptible to SCFAs (Defoirdt et al. 2007b; Najdegerami et al. 2012). The application of SCFAs in aquaculture environments poses, however, a problem as they are highly soluble in water. This results in a low uptake efficiency of SCFAs by aquatic animals and implies the need to use doses much higher than the ones biologically needed. The recently proposed PHB strategy offers a solution in this respect. PHB is synthesized by

a large variety of Gram-positive and Gram-negative bacteria to serve as an intracellular energy and carbon storage (Madison and Huisman 1999; De Schryver et al. 2010b). PHB has the advantage that it is insoluble in water, and that it can be converted into ß-hydroxybutyric acid by biological degradation (Defoirdt et al. 2007a).

The beneficial effects of PHB as a biocontrol compound for sustainable aquaculture production have been illustrated in several studies. Firstly, by direct addition to the culture medium, crystalline PHB (i.e. PHB extracted from bacterial cells) has been reported to protect *Artemia franciscana* nauplii against vibriosis (Defoirdt et al. 2007b). Secondly, by supplementing crystalline PHB in the commercial diet of juveniles of fish species such as European sea bass (*Dicentrarchus labrax*) (De Schryver et al. 2010b) and Siberian sturgeon (*Acipenser baerii*) (Najdegerami et al. 2012) an enhanced weight gain, survival or specific growth rate could be obtained. Finally, by enrichment into *Artemia* nauplii or rotifers crystalline PHB was found to increase the survival and growth performance of larval *M. rosenbergii* (Nhan et al. 2010b) and Chinese mitten crab (*Eriocheir sinensis*) (Sui et al. 2012) and resulted in a lower presence of vibrios in the gut of prawn larvae (Nhan et al. 2010b). A main constraint in the application of PHB is the relatively high price of crystalline PHB particles extracted from PHB-accumulated bacteria. However, PHB in amorphous form (i.e. still contained inside bacteria) has also been shown to induce beneficial effects in the *Artemia* model system (Halet et al. 2007; Cam et al. 2009), while the production costs are lower. Bacteria containing substantial amounts of PHB are thus promising to become a PHB based biocontrol strategy for aquaculture when they can be shown to have a good effect on the culture performance or disease resistance of actual aquaculture animals. Consequently, the goal of the current study was to assess for the first time the beneficial effects of amorphous PHB supplied through the live food *Artemia* on *M. rosenbergii* larvae. A non-challenged growth test and a challenge test using *Vibrio harveyi* were performed. Assessment parameters were survival and growth of the larvae as well as TCBS counts to estimate the number of vibrios in the larval gut in the former test, while survival and TCBS counts in the larval gut were determined in the latter test.

2.1.2 Materials and methods

2.1.2.1 Origin of Macrobrachium *prawn larvae*

Macrobrachium rosenbergii broodstock was imported from Vietnam and housed in a freshwater recirculation system with bio-filter. Daily, approximately 20% of the water in the broodstock tanks was replaced after removing waste and uneaten feed by siphoning. The water quality parameters were maintained in accordance to New (2002) and Nhan et al. (2010b) with NH^+_4-N, NO^-_2-N and NO^-_3-N below 0.2, 0.1 and 10.0 mg/L, respectively. Temperature was maintained at 28 ± 1 °C by a heater. The light had an intensity of 8 µE/(m^2.sec) and was applied in a daily regime of 12 h light/12 h dark. The prawns were fed *ad libitum* two times per day with a commercial pellet feed. A single ovigerous female breeder was selected from the broodstock tank and isolated in a hatching tank (V = 30 x 60 x 40 cm) until egg hatching. The management conditions within this tank were the same as for the broodstock tank with only fifty percent of the water was renewed daily and that salinity of the water was maintained at 6 g/L. Upon hatching, larvae swimming up to the surface of the tank were collected and used for the experiments described below (Cavalli et al. 1999; Nhan et al. 2010b).

2.1.2.2 Experimental feed preparation

2.1.2.2.1 Axenic hatching of Artemia franciscana

Experiments were performed using *Artemia franciscana* as live food. *Artemia* cysts originated from the Great Salt Lake, Utah, USA (EG® Type, INVE Aquaculture, Belgium). Bacteria-free cysts were obtained using the procedures described by Marques et al. (2004a) with the small modification that filtered autoclaved artificial seawater containing 12 g/L of Instant Ocean synthetic sea salt (Aquarium Systems, Sarrebourg, France) (FAASW) was used. The sterilized cysts were resuspended in a bottle containing 1 L of FAASW with 0.22 µm air filter inlet and outlet on the cap to allow continuous sterile aeration. The cysts were hatched for 24 h at 28 °C under constant illumination (approximately 27 µE/(m^2.sec)) for production of sterile nauplii.

2.1.2.2.2 PHB-accumulated Alcaligenes eutrophus *and PHB particles preparation*

Two batches of a PHB-accumulated *A. eutrophus* strain (also known as *Cupriavidus necator*, *Ralstonia eutropha*, and *Wautersia eutropha*), lyophilized and containing 10% PHB and 80% PHB on dry weight (named A10 and A80, respectively) were produced as follows. A stock culture of *A. eutrophus* H16 was stored at -80 °C in 2 mL cryovials containing 0.5 mL of 80% glycerol and 1 mL of a late exponential-phase liquid culture in Luria-Bertani (LB) medium. A 200 µL aliquot of this stock culture was inoculated into 5 mL of LB medium and cultivated in an orbital shaker (Innova 42, Eppendorf, USA) for 24 hours at 30 °C and 200 rpm. Subsequently, 2 mL of this culture was subcultured for 24 hours at 30 °C and 180 rpm in 100 mL of seeding medium in a 500-mL baffled flask. The seed medium contained 10 g/L fructose, 3 g/L $(NH_4)_2SO_4$, 1.5 g/L KH_2PO_4, 4.47 g/L $Na_2HPO_4.2H_2O$, 0.2 g/L $MgSO_4.7H_2O$, and 1 mL/L trace element solution. The trace element solution had the following composition: 10 g/L $FeSO_4.7H_2O$, 2.25 g/L $ZnSO_4.7H_2O$, 1 g/L $CuSO_4.5H_2O$, 0.5 g $MnSO_4.5H_2O$, 2 g/L $CaC1_2.2H_2O$, 0.23 g/L $Na_2B_4O_7.10H_2O$, 0.1 g/L $(NH_4)_6Mo_7O_{24}$, and 35% HC1 10 mL/L and was filter sterilized through a 0.2 µm polyethersulfone filter (Whatman, UK) prior to use. Fructose and $MgSO_4.7H_2O$ were separately autoclaved at 121 °C for 15 minutes. All ingredients were aseptically combined after cooling. Finally, the seed culture (10 mL) was inoculated into 250 mL of cultivation medium in a 1000 mL baffled flask and incubated at 200 rpm and 30 °C. The cultivation medium consisted of 10 g/L fructose, 3 g/L $(NH_4)_2SO_4$, 13.3 g/L KH_2PO_4, 1.2 g/L $MgSO_4.7H_2O$, 1.87 g/L citric acid, and 10 mL/L trace element solution and was prepared similar to the seed medium. The pH of the medium was adjusted to 6.80 with 5 M NaOH. During cultivation, the fructose was manually added on a continuous basis to the flasks to prevent substrate limitation. The fructose concentration in the medium was determined by the phenol-sulfuric acid method using fructose as a standard (Dubois et al. 1956). Simultaneously, the concentration of ammonium (NH_4^+-N) was evaluated colorimetrically with standard Hach Lange cuvette tests (Hach Lange Gmbh, Germany) because when ammonium became limited, the carbon flux was redirected from biomass to PHB synthesis and the cells started to accumulate PHB. Based on the ammonium concentration, samples were therefore collected at the end of the biomass growth phase ($t \approx 16$ h) and at the end of the PHB accumulation phase ($t \approx 30$ h) and analyzed for dry cell mass and PHB concentration. The cell concentration was determined by centrifuging culture broth (15-20 mL) at 7000 x g (SORVALL RC6+ centrifuge, Thermo

Scientific, Clintonpark Keppekouter, Belgium) in pre-weighted screw-cap tubes for 30 min at 4 °C. The cell pellets were washed with distilled water, re-centrifuged, and lyophilized until a constant weight. The cell concentration was determined as the weight difference between tubes containing the cell pellets and empty tubes. For the PHB analysis, dried samples and external PHB standards (Biomer, Germany) were subjected to methanolysis in the presence of 50% (v/v) methanol and 50% (v/v) NaOH. The resulting 3-hydroxybutyric acid was analyzed by HPLC using 0.05% H_3PO_4 as the mobile phase. The PHB content was calculated as the percentage of the ratio of the PHB concentration to the total cell concentration. At the end of the biomass growth phase, the *A. eutrophus* cells had accumulated 10% PHB (hence the term "A10"). The final PHB content reached at the end of the PHB accumulation phase was 80% (hence the term "A80"). The microbial cells used in the study were unviable after lyophilisation as no growth occurred after inoculation into LB medium.

The crystalline PHB particles used in the trials (98% poly-β-hydroxybutyrate – 2% poly-β-hydroxyvalerate, Goodfellow, Huntingdon, England) were ground through a 30 µm sieve prior to use.

2.1.2.2.3 Enrichment of axenic Artemia *nauplii with PHB-accumulated* A. eutrophus *and crystalline PHB particles*

After 24 h of cyst incubation, axenically hatched *Artemia* nauplii (Instar II) were washed with 1 L FAASW. The washed nauplii were enriched with PHB-containing *A. eutrophus* in FAASW as indicated in Table 2.1.1 and 2.1.2. Crystalline PHB particles at a dose of 800 mg/L served as a reference treatment and resembled the amount of PHB in the treatment 1000 mg/L A80, while axenic *Artemia* nauplii treated in the same way as the other treatments but not enriched with PHB served as a negative control. The density of *Artemia* nauplii for enrichment was about 80000 – 100000 individuals per L FAASW. After 2 h of enrichment, the *Artemia* nauplii were washed with clean freshwater before feeding to *M. rosenbergii* larvae *ad libitum*.

Table 2.1.1: Overview of the treatments in experiment 1 – survival and growth test (each treatment was performed in quintuplet)

Treatment name	Live food for *M. rosenbergii* larvae	Enrichment material for *Artemia* nauplii	Concentration enrichment material (mg/L)	PHB concentration during enrichment (mg/L)
Control	Non-enriched (axenic) *Artemia* nauplii	/	0	0
PHB-800 mg/L	Enriched *Artemia* nauplii	Crystalline PHB particles	800	800
A10-100 mg/L	Enriched *Artemia* nauplii	*A. eutrophus* (10% PHB)	100	10
A10-1000 mg/L	Enriched *Artemia* nauplii	*A. eutrophus* (10% PHB)	1000	100
A80-10 mg/L	Enriched *Artemia* nauplii	*A. eutrophus* (80% PHB)	10	8
A80-100 mg/L	Enriched *Artemia* nauplii	*A. eutrophus* (80% PHB)	100	80
A80-1000 mg/L	Enriched *Artemia* nauplii	*A. eutrophus* (80% PHB)	1000	800

Table 2.1.2 Overview of the treatments in experiment 2 – challenge test (each treatment was performed in quintuplet)

Treatment name	Challenge	Live food for *M. rosenbergii* larvae	Enrichment material	Concentration enrichment material (mg/L)
Control	/	Non-enriched (axenic) *Artemia* nauplii	/	0
Control + BB120	+ *V. harveyi*	Non-enriched (axenic) *Artemia* nauplii	/	0
PHB-800 mg/L +BB120	+ *V. harveyi*	Enriched *Artemia* nauplii	Crystalline PHB particles	800
A10-100 mg/L + BB120	+ *V. harveyi*	Enriched *Artemia* nauplii	*A. eutrophus* (10% PHB)	100
A10-1000 mg/L + BB120	+ *V. harveyi*	Enriched *Artemia* nauplii	*A. eutrophus* (10% PHB)	1000
A80-10 mg/L + BB120	+ *V. harveyi*	Enriched *Artemia* nauplii	*A. eutrophus* (80% PHB)	10
A80-100 mg/L + BB120	+ *V. harveyi*	Enriched *Artemia* nauplii	*A. eutrophus* (80% PHB)	100
A80-1000 mg/L + BB120	+ *V. harveyi*	Enriched *Artemia* nauplii	*A. eutrophus* (80% PHB)	1000

2.1.2.3 Experimental design

The experimental larval rearing containers consisted of large (experiment 1) or small (experiment 2) glass cones that were placed in a water bath with thermostatic heater ensuring a temperature of 28 ± 1 °C. The cones were supplied of gentle aeration to ensure a dissolved oxygen level of more than 5 mg/L. A lamp system was installed above the cones to provide around 12 -14 µE/(m^2.sec) at the water surface for 12 h/day.

For experiment 1 (survival and growth test), 35 large glass larval rearing cones containing 800 mL of 0.2 µm filtered brackish water (12 g/L salinity) were stocked with 60 *M. rosenbergii* larvae of 1 day after hatching (DAH). The larvae were fed *ad libitum* twice a day (9:00 and 17:00) for 20 days with enriched *Artemia* according to treatment (Table 2.1.1). Every three days, the non-eaten *Artemia* and waste in the cones were removed by siphoning, and 50% of the culturing water was replaced by new filtered brackish water. This operation was performed with great care to avoid loss of larvae.

For experiment 2 (challenge test), 40 small glass cones containing 100 ml FAASW were stocked with 25 *M. rosenbergii* larvae of DAH 1. The larvae were fed *ad libitum* twice a day (9:00 and 17:00) for 9 days with enriched *Artemia* according to treatment (Table 2.1.2) and were challenged with *Vibrio harveyi* BB120 on day 2. Prior to use, *Vibrio harveyi* BB120 was stored in 40% glycerol at -80 °C. Ten µl was inoculated on a LB agar plate at 12 g/L salinity. The agar plate was put into an incubator for 24 h. Two colonies from the BB120 agar plate were grown in 25 mL of fresh LB medium (12 g/L salinity) for 24 h at 28 °C under constant agitation (120 rpm). The optical cell density of the culture was determined spectrophotometrically at 550 nm, and the pathogenic bacteria were directly added to the *M. rosenbergii* larval culture medium at a density of approximately 10^7 colony forming units (CFU) per mL. No water was exchanged during this experiment.

2.1.2.4 Analyses

2.1.2.4.1 Measurement of PHB content in enriched Artemia *nauplii*

Artemia nauplii enriched as live food for experiments 1 and 2 were carefully rinsed several times with de-ionized water and dried at 103 °C for 4 h. A mortar was used to grind the dried

Artemia nauplii into powder. Five mg of dried *Artemia* powder was incubated at 60 ^{o}C for 1 h with 0.5 mL sodium hypochlorite (5%) to break the cell walls of the dried *Artemia* nauplii (Aslim et al. 1998). The samples were centrifuged at 6,000 x g for 20 min and then washed twice with 2 mL of an acetone and ethanol mixture (1:1) before washing again with de-ionized water. Five mL chloroform was added to the pellet to extract the PHB and the samples were left overnight at 28 °C on an orbital shaker at 250 rpm. After the extracts were evaporated to dryness at 40 °C, concentrated sulfuric acid (4 mL) was added to the dried sample and maintained at 100 °C in a water bath for 20 min. After cooling the samples to room temperature, the absorbance (235 nm) was measured with a spectrophotometer (Kaynar and Beyatli 2009). The concentration of PHB in the sulfuric acid aliquots was determined based on a standard curve constructed of external PHB standards.

The percentage of PHB in the samples was calculated as:
PHB (%) = (PHB (mg) x 100 %) / CWD (mg)

2.1.2.4.2 Measurement of PHB content in enriched and purged Artemia *nauplii*

The effect of intestinal purging of enriched *Artemia* nauplii on the PHB measurement data was assessed. Two purging methods, cellulose purging for 2 h and starvation for 12 h, were applied as follows. After external washing of the PHB enriched *Artemia* nauplii with FAASW, the *Artemia* were transferred to 1.5 g/L cellulose in FAASW for 2 h (Niu et al. 2012), or to FAASW for 12 h. After the purging period, the *Artemia* nauplii were washed with FAASW and the PHB content was measured as mentioned above.

2.1.2.4.3 M. rosenbergii *larval survival*

The *M. rosenbergii* larval survival was determined at days 10 and 20 in experiment 1, and at day 9 in experiment 2. To check the survival of prawn larvae, the glass cone that contained the prawn larvae was gently poured into a white-coloured tray. Prawn larvae were then manually collected and counted using a 1 mL pipette of which the tip was cut and transferred into a beaker with light aeration. When all larvae were counted, the water and larvae from the beaker were transferred back into the cone. The manipulation for checking survival of the larvae was performed with great care to avoid stressing the larvae.

2.1.2.4.4 Larval stage index (LSI)

At days 10 and 20 in experiment 1, sixty larvae from each treatment (12 per replicate) were collected to measure the development of the larvae. After measurement, the larvae were placed back in the cones. Larval stages were determined based on morphological characteristics as described by New (2002). The larval stage indexes were calculated according to Mallasen and Valenti (2006) and Baruah et al. (2009) as follows:

$$\text{LSI} = \sum(S_i \text{ x } n_i)/N$$

Where S_i is larval stage (i = 1 – 12); n_i is the number of larvae in stage S_i; and N is the total number of larvae observed.

2.1.2.4.5 Bacteria in the gut of M. rosenbergii *larvae*

Triplicate samples of 12 prawn larvae were collected randomly at the start and on the last day of the experiments for analysis of the bacteria in the gut of the larval prawn. A surface disinfection of the collected larvae was applied (Huys et al. 2001) to remove externally attached bacteria. The surface disinfected larvae were transferred into sterile plastic bags containing 10 ml of nine salt solution (NSS) (Olsson et al. 1992) and were homogenized with a stomacher blender (400SN, Seward Medical, London, United Kingdom) for 6 minutes. Serial dilutions of the homogenized sample were prepared in NSS. For enumeration of the heterotrophic bacteria and to estimate the number of vibrios, 50 µL from each dilution was plated on Marine Agar (MA, Difco Laboratories, Detroit, USA), and on Thiosulphate-Citrate-Bile Salt-Sucrose agar (TCBS, Biokar Diagnostics, France), respectively. After incubation at 28 °C for 48 h, the number of colonies was counted (Nhan et al. 2010a; 2010b).

2.1.2.4.6 Statistics

The software SPSS version 17.0 was applied for statistical analyses. Larval stage index, larval survival, bacterial numbers and percentage of PHB in enriched *Artemia* nauplii were analyzed by one-way analysis of variance (ANOVA), followed by a Tukey-test for post-hoc comparison ($P<0.05$). The correlation between the measured PHB content in the *Artemia* nauplii used as live food and the survival of *M. rosenbergii* larvae fed with these *Artemia*

was analyzed by bivariate correlation analysis (Pearson coefficient) at the 0.01 significance level. All the percentage data were normalized by arcsine transformation for statistical analysis, but only non-transformed means are presented.

2.1.3 Results

2.1.3.1 PHB content in Artemia *nauplii*

The measurement of the PHB content in *Artemia* nauplii enriched for 2 h with PHB-containing *A. eutrophus* or with PHB particles resulted in values ranging between 9.8 ± 0.8% and 17.4 ± 1.3%, while 8.9 ± 0.9% PHB was measured in the non-enriched *Artemia* nauplii (Table 2.1.3). The PHB content of the *Artemia* nauplii enriched with 800 mg/L PHB particles (17.4 ± 1.3%) was significantly higher than that of the other treatments. The PHB content of the *Artemia* from the treatments A80-100 mg/L and A80-1000 mg/L was significantly higher than that of the control treatment and the A10-100 mg/L, A10-1000 mg/L, and A80-10 mg/L treatments.

Table 2.1.3 PHB content in non-enriched axenic *Artemia* nauplii (control) and axenic *Artemia* nauplii after 2 h enrichment with *A. eutrophus* containing 10% PHB (A10), *A. eutrophus* containing 80% PHB (A80) or PHB particles (PHB).

Treatment	PHB in *Artemia* nauplii (% on dry weight)		
	2 h enrichment	2 h enrichment followed by 2 h cellulose purging	2 h enrichment followed by 12 h starvation
Control	8.9 ± 0.9 ^{a,1}	9.4 ± 1.1 ^{a,1}	9.4 ± 0.2 ^{a,1}
A10-100 mg/L	10.5 ± 1.2 ^{a,1}	9.5 ± 0.5 ^{a,1}	9.5 ± 1.5 ^{a,1}
A10-1000 mg/L	10.3 ± 1.4 ^{a,1}	10.0 ± 0.8 ^{ab,1}	9.6 ± 0.3 ^{a,1}
A80-10 mg/L	9.8 ± 0.8 ^{a,1}	9.5 ± 1.3 ^{a,1}	9.5 ± 1.3 ^{a,1}
A80-100 mg/L	13.4 ± 1.0 ^{b,1}	10.1 ± 1.3 ^{ab,2}	10.8 ± 1.8 ^{ab,2}
A80-1000 mg/L	14.7 ± 0.4 ^{b,1}	11.0 ± 0.7 ^{ab,2}	11.0 ± 1.5 ^{ab,2}
PHB-800 mg/L	17.4 ± 1.3 ^{c,1}	13.2 ± 1.4 ^{b,2}	13.0 ± 0.5 ^{b,2}

Values prior to and after 2 h cellulose purging or 12 h starvation are given. Values represent means ± SD of three *Artemia* nauplii sampled on three random days during the experiment.

Values within the same column not sharing the same superscript letter are significantly different ($P<0.05$). Values within the same row with a different superscript number are significantly different ($P<0.05$).

After purging with cellulose for 2 h or starvation for 12 h, the PHB content in the *Artemia* nauplli from the control, A10-100 mg/L, A10-1000 mg/L and A80-10 mg/L did not change significantly as compared to newly enriched *Artemia* nauplii. For the PHB-800 mg/L treatment, the PHB content value decreased significantly with 4.2 ± 1.9% (cellulose purging) and 4.38 ± 1.34% (starvation), while significant decreases of 3.7 ± 0.9% (cellulose purging) and 3.7 ± 1.8% (starvation), and 3.3 ± 1.6% (cellulose purging) and 2.6 ± 1.1% (starvation) for the A80-1000 mg/L and A80-100 mg/L treatment, respectively, were observed.

2.1.3.2 Experiment 1: survival and growth test

2.1.3.2.1 Effect of feeding PHB enriched Artemia *nauplii on survival and growth of* M. rosenbergii *larvae*

The larval survival and growth of the *M. rosenbergii* larvae was determined at day 10 and at day 20. At day 10, no significant differences could be observed in terms of survival between the different treatments, with values of around 80% for all treatments (Fig. 2.1.1). At day 20, however, the larvae from the A80-100 mg/L, A80-1000 mg/L and the PHB-800 mg/L treatments showed a significantly higher survival as compared to the control treatment and the A10-100 mg/L treatment, but showed no significant differences among each other. The survival of the larvae from the A10-100 mg/L, A10-1000 mg/L and A80-10 mg/L treatments was not significantly different from the control treatment.

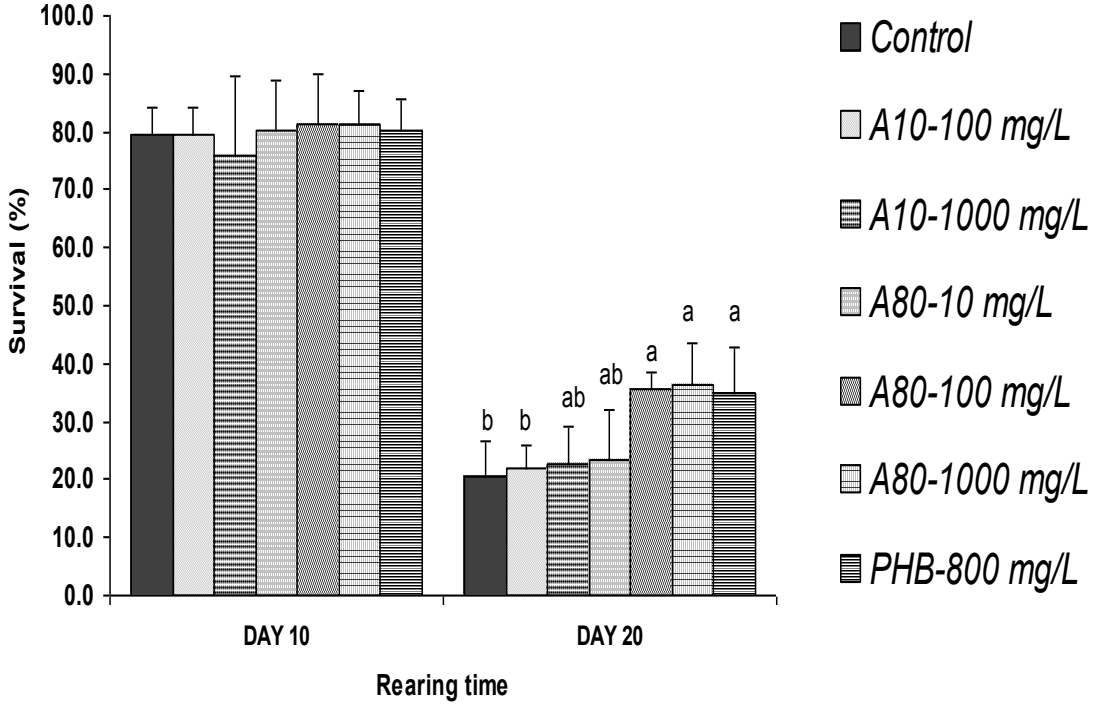


Figure 2.1.1 Survival *of M. rosenbergii* larvae (mean ± SD of five replicates) at day 10 and day 20 of rearing with differentially enriched *Artemia* nauplii as feed. Treatments not sharing the same superscript letter on day 20 are significantly different ($P < 0.05$).

The LSI at day 10 was significantly higher for the A80-100 mg/L, A80-1000 mg/L and the PHB-800 mg/L treatments as compared to the control treatment and the A10-1000 mg/L treatment (Fig. 2.1.2). At day 20, a similar pattern was observed as for the survival on day 20.

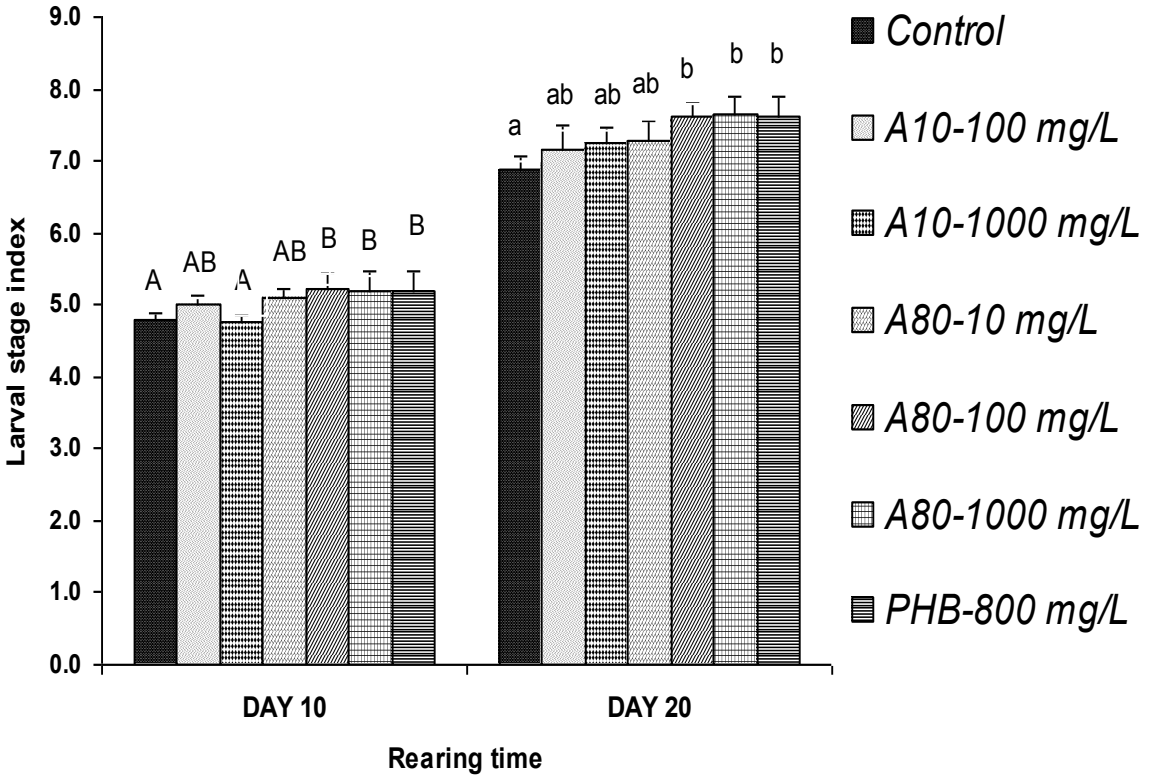


Figure 2.1.2 Larval stage index of *M. rosenbergii* larvae (mean ± SD of five replicates) at day 10 and day 20 of rearing with differentially enriched *Artemia* nauplii as feed. Treatments not sharing the same superscript letter within a day are significantly different ($P < 0.05$).

2.1.3.2.2 Effect of feeding PHB enriched Artemia *nauplii on the gut microbiota of* M. rosenbergii *larvae*

The total number of culturable heterotrophic bacteria increased with about a factor 100 between the start and the end of the experiment for all treatments (Table 2.1.4). At the end of the experiment, the larvae from the control treatment contained a significantly higher number of total heterotrophic bacteria than the other treatments. The treatments A80-1000 mg/L and PHB 800 mg/L showed significantly lower number of total heterotrophic bacteria as compared to the other PHB treatments except for A10-1000 mg/L treatment. No presence of colonies on the TCBS plates was observed at the start of the experiment. At day 20, the number of TCBS counts in the gut of the larvae was significantly higher in the control treatment as compared to the other treatments except for A10-100 mg/L. The treatments

A10-100 mg/L and A10-1000 mg/L showed significantly more colonies than the other PHB treatments, where no colonies were recorded on the TCBS plates.

Table 2.1.4 Number of culturable micro-organisms in the gut of *M. rosenbergii* larvae (mean ± SD of three replicates) reared for 20 days with differentially enriched *Artemia* nauplii as feed. Values within the same column with a different superscript letter are significantly different ($P<0.05$)

Treatment	Total heterotrophic bacteria (log CFU/larva)		TCBS counts (log CFU/larva)	
	Initial	Final	Initial	Final
Control		4.4 ± 0.1^{a}		3.0 ± 0.1^{a}
A10-100 mg/L		4.0 ± 0.0^{d}		2.6 ± 0.1^{ab}
A10-1000 mg/L		4.1 ± 0.0^{bc}		2.5 ± 0.3^{b}
A80-10 mg/L	0.0	4.0 ± 0.0^{cd}	0.0	n.d.^{c}
A80-100 mg/L		4.0 ± 0.0^{d}		n.d.^{c}
A80-1000 mg/L		4.2 ± 0.0^{b}		n.d.^{c}
PHB-800 mg/L		4.2 ± 0.0^{b}		n.d.^{c}

n.d. = non-detectable

2.1.3.3 Experiment 2: Challenge test

2.1.3.3.1 Effect of feeding PHB enriched Artemia *nauplii on the survival of* M. rosenbergii *larvae challenged with* V. harveyi *BB120*

The challenged *M. rosenbergii* larvae from the A10-1000 mg/L, A80-100 mg/L, A80-1000 mg/L and the PHB-800 mg/L treatments showed a significantly higher survival than the challenged larvae fed non-enriched *Artemia* nauplii (control + BB120) and a not significantly different survival as compared to the larvae from the unchallenged control treatment (Fig. 2.1.3). The other PHB treatments were not significantly different from the challenged control treatment, but significantly lower than the non-challenged control treatment.

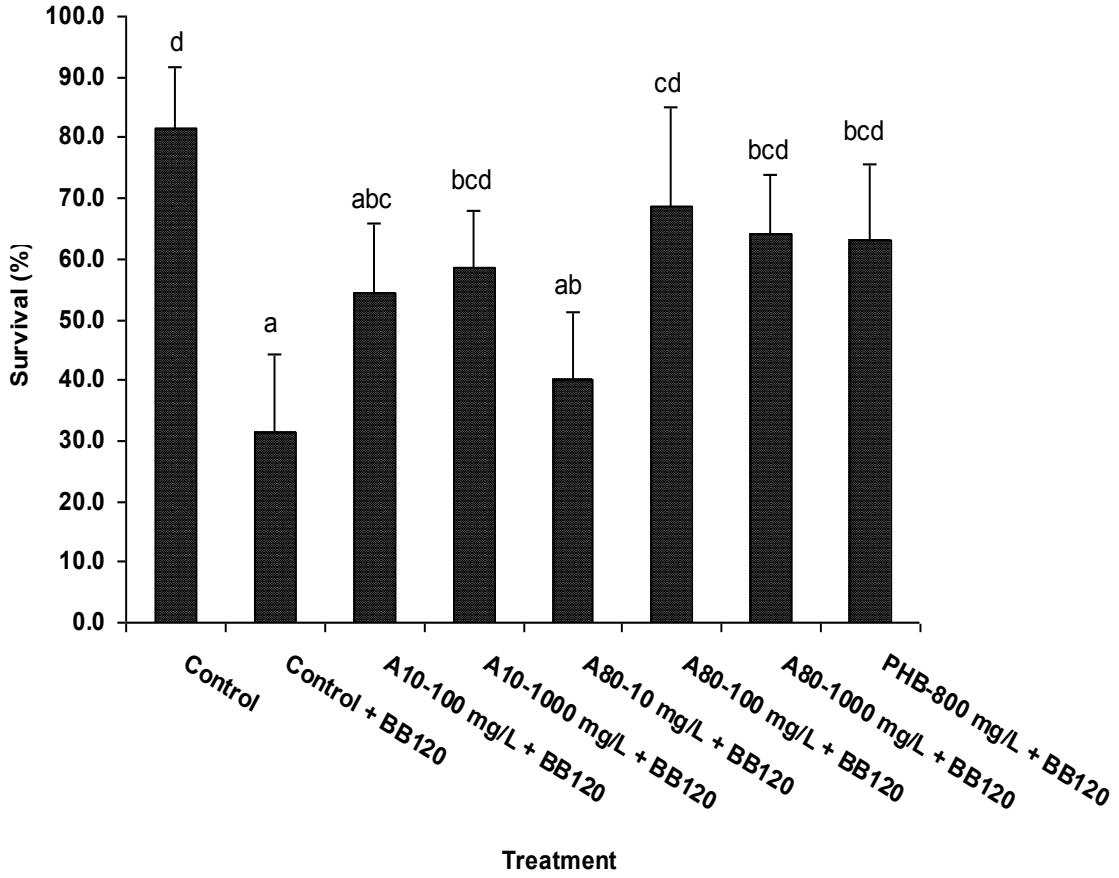


Figure 2.1.3 Survival of *M. rosenbergii* larvae (mean ± SD of five replicates) in a challenge test with *Vibrio harveyi* BB120 during 9 days of culture. Treatments not sharing the same superscript letter are significantly different ($P<0.05$).

2.1.3.3.2 Effect of feeding PHB enriched Artemia *nauplii on the number of vibrios in the gut of* M. rosenbergii *larvae challenged with* V. harveyi *BB120*

Similar to experiment 1, TCBS agar was used for estimating the number of vibrios in the gut of challenged *M. rosenbergii* larvae. At day 9 of the challenge experiment, the treatments with supplementation of PHB to the larvae showed significantly lower numbers of TCBS colonies (a factor 10 – 100) as compared to the challenged control treatment (Table 2.1.5). The use of PHB significantly lowered the TCBS counts as compared to the challenged control. However, only the treatments A80-100 mg/L and A80-1000 mg/L could lower it to a level that was even lower than the unchallenged control.

Table 2.1.5 TCBS counts in the gut of *M. rosenbergii* larvae challenged with *Vibrio harveyi* BB120 (mean ± SD of three replicates) and reared for 9 days with differentially enriched *Artemia* nauplii as feed. Values with a different superscript letter are significantly different (*P<0.05*)

Treatment	Challenge	TCBS counts (log CFU/larva)
Control	/	2.8 ± 0.0 ^{d}
Control	+ *V. harveyi*	4.2 ± 0.0 ^{a}
A10-100 mg/L	+ *V. harveyi*	3.1 ± 0.1 ^{b}
A10-1000 mg/L	+ *V. harveyi*	2.9 ± 0.1 ^{cd}
A80-10 mg/L	+ *V. harveyi*	3.1 ± 0.1 ^{bc}
A80-100 mg/L	+ *V. harveyi*	2.2 ± 0.1 ^{e}
A80-1000 mg/L	+ *V. harveyi*	2.5 ± 0.1 ^{f}
PHB-800 mg/L	+ *V. harveyi*	3.0 ± 0.1 ^{bcd}

2.1.4 Discussion

Halet et al. (2007) suggested the use of bacteria containing amorphous PHB as an alternative to the application of crystalline PHB extracted from bacterial cells. These authors were able to show that the efficiency of the amorphous PHB strategy for the model organism *Artemia franciscana* was up to a factor 100 times more efficient than the crystalline PHB strategy applied by Defoirdt et al. (2007b), independently of whether the PHB containing microbial biomass was viable, frozen and thawed, or pasteurized. This indicated that the smaller dimension of the amorphous PHB of less than 1 µm (Anderson and Dawes 1990) in comparison to the larger crystalline PHB particles (on average 30 µm) was likely to be a primary reason of the higher treatment efficiency. So far, the amorphous PHB strategy was not verified for actual aquaculture animals. In the current study, it was shown that the strategy of supplying PHB-accumulated bacteria through live food to *M. rosenbergii* larvae resulted in positive effects in terms of growth and survival as well as in terms of protection against pathogenic infections. A prerequisite of the strategy seems to be, however, that bacteria with a high content in PHB are used at an optimized enrichment concentration to

provide sufficient amounts of PHB to the larvae while avoiding excessive use of the enrichment material.

Artemia nauplii, being a live food for larvae (Sorgeloos and Léger 1992) have been used for delivering PHB by enrichment in several studies (Defoirdt et al. 2007b; Nhan et al. 2010b). In our case, *Artemia* nauplii Instar II were used to deliver lyophilized PHB-accumulated *A. eutrophus* H16 or PHB particles to *M. rosenbergii* larvae. The enrichment of the nauplii with bacteria containing different levels of PHB (10% or 80% on dry weight) at different enrichment concentrations resulted in clear differences in the *Artemia* PHB content. The *Artemia* nauplii that were not enriched in PHB also showed a substantial value for the PHB content. This may be explained by the natural presence of PHB or 3-hydroxybutyrate (3-HB) in the body of *Artemia*. Short-chain PHB has been shown to be present in eukaryotic cells although its function is not well understood (Elustondo et al. 2013). The levels of short-chain PHB in the tissues of higher organisms are low, with reported values in the order of 0.01% on dry weight in the tissues of for example rats (Reusch et al., 2003). Unfortunately, the natural levels of PHB in *Artemia*, or in crustaceans in general, have not been reported. More information is available on PHB's monomer 3-HB, as it is quantitatively the most important of the ketone bodies in the *Artemia* body, used as fuel for development and growth. It is found back in starved *Artemia* nauplii at a concentration of 1.0 – 1.5 nmol/nauplius (Weltzien et al. 2000). With an average dry matter body weight of about 2.1 µg/nauplius, this corresponds to a 3-HB content of 5.0 - 7.4 % on dry body weight, which closely corresponds to the PHB content value of 8.9 ± 0.9% that was observed in the current study. As 3-HB is the monomer of PHB this compound also yields positive results in the PHB measurement assay (Seebach and Fritz 1999). It is thus likely that the presence of 3-HB in the tissue of the untreated *Artemia* resulted in the relatively high PHB values, although experimental proof of this statement needs to be provided. To verify whether the significantly higher PHB contents in the *Artemia* nauplii after enrichment for the A80-100 mg/L, A80-1000 mg/L, and PHB-800 mg/L treatments as compared to the control treatment were due to gastrointestinal presence of PHB only, or from an increase in body PHB and/or 3-HB as well, the purging of the *Artemia* nauplii intestine by cellulose or by starvation was applied. The differences in PHB content before and after purging were 4.2 ± 1.9 – 4.4 ± 1.3% PHB for the PHB-800 mg/L, 3.7 ± 1.8 – 3.7 ± 0.9% PHB for the A80-1000 mg/L treatment, and of 2.6 ± 1.1 – 3.3 ± 1.6% PHB for the A80-100 mg/L treatment. These decreases represent the actual PHB content in the nauplii resulting from enrichment and

show that the PHB content in the *Artemia* nauplii guts from these 3 treatments after enrichment was similar but varied slightly. When complete gut filling is assumed for these treatments, the slightly higher values for the crystalline PHB treatment than for the amorphous PHB treatments can be explained by the fact that 20% of the microbial enrichment material was not PHB. The significantly higher PHB content value in the nauplii from the PHB-800 mg/L treatment after purging (13.0 ± 0.5 - 13.2 ± 1.4%) as compared to the other PHB treatments after purging suggests that the PHB or 3-HB content in the body of the *Artemia* from this treatment increased during the short enrichment period of 2 h.

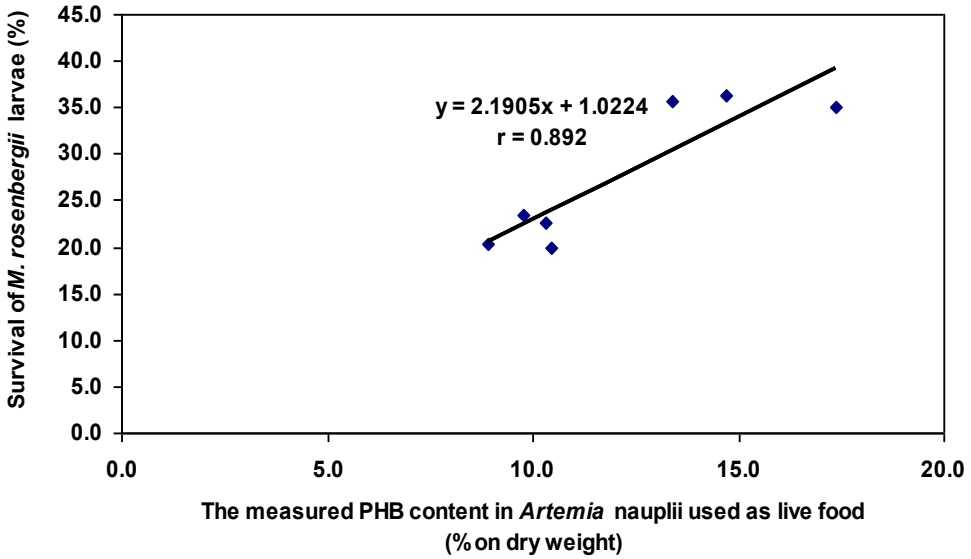


Figure 2.1.4 Correlation between the measured PHB content in the *Artemia* nauplii used as live food and the survival of *M. rosenbergii* larvae fed with these *Artemia* during a 20 days rearing period.

The PHB content in the gastrointestinal tract of the *Artemia* nauplii used as live food was significantly correlated to the survival of the *M. rosenbergii* larvae with a Pearson product moment correlation coefficient (r) of 0.892 ($P<0.01$) (Fig. 2.1.4.). The development of the larvae in the A80-100 mg/L, A80-1000 mg/L and PHB-800 mg/L treatments was also significantly improved. These results are in accordance with the findings of Nhan et al. (2010b) who showed beneficial effects of crystalline PHB on the survival and growth of *M. rosenbergii* larvae. The increased growth and survival in the current study may result from the antimicrobial activity of the PHB, as illustrated by the decrease in total heterotrophic bacteria but especially in the number of TCBS counts, being an approximation of the number

of vibrios (López-Torres and Lizárraga-Partida 2001). Several *Vibrio* spp. are opportunistic pathogens and were suggested to hamper the growth and survival of *M. rosenbergii* larvae in the control treatments during previous experiments (Nhan et al. 2010b; Baruah et al. 2009). Alternatively, the PHB may have aided by the provision of extra energy to the *M. rosenbergii* larvae resulting in increased development (Defoirdt et al. 2007b). It should also be noted that *A. eutrophus* may also have contributed to the survival and growth of the prawn larvae by serving as single cell protein (SCP) or other nutrients (Kunasundari et al. 2013). Schulz and Oslage (1976) have found that the crude protein and essential amino acids are one of the main compositions in SCP of bacteria.

The challenge tests implying *V. harveyi* BB120 infection of the *M. rosenbergii* larvae showed that the amorphous PHB contained within the bacteria could protect the larvae to the same degree as the crystalline PHB. As for growth and development, a trend of higher protection at higher PHB contents in the *Artemia* seemed to exist although no significant differences were observed among PHB treatments. These results are in accordance with the findings reported by Defoirdt et al. (2007b) and Sui et al. (2012). The former showed that *Artemia* nauplii were significantly protected against pathogenic *Vibrio campbellii* by crystalline PHB particles in suspension at a concentration of 100 and 1000 mg/L, while the latter found that crystalline PHB supplied through live food protected Chinese mitten crab from *Vibrio anguillarum* infection. The significantly lower presence of TCBS counts in the gut of the PHB treated *M. rosenbergii* larvae suggests that an antimicrobial effect towards the applied pathogen was causal to the current observations. The antimicrobial effect of PHB so far has been ascribed to the gastrointestinal conversion of PHB into its monomer, the short-chain fatty acid 3-hydroxybutyrate, that subsequently acts like other SCFAs as described earlier (Defoirdt et al. 2009). Recent research suggests, however, that the antimicrobial effect of PHB may be the result of immunostimulation of the treated host as well (Suguna et al. 2014; Baruah et al. 2015).

In the current study, it was shown that feeding *Artemia* nauplli enriched in a suspension of 800 mg/L crystalline PHB resulted in similar performance (growth, survival, disease resistance) of the *M. rosenbergii* larvae as when *Artemia* enriched in a suspension of 100 or 1000 mg/L of *A. eutrophus* containing 80% PHB were fed. The latter two corresponded to a PHB enrichment concentration of 80 mg/L and 800 mg/L, respectively, in the enrichment

medium. The similar performances can be explained by the fact that despite the differences in PHB concentrations in the enrichment medium, the *Artemia* nauplii from these treatments showed a similar PHB content as verified by the purging method. *Artemia* is a filter feeder and filters particles from suspension until complete gut filling (Sorgeloos et al. 1986). The concentrations of 100 and 1000 mg/L bacteria thus likely resulted in equal filling of the gut with *A. eutrophus* containing 80% PHB. The enrichment concentration of 10 mg/L was then hypothesized not to result in a complete gut filling in 2 h resulting in a lower PHB content. This could be confirmed by stereomicroscopic comparison of the gut filling of *Artemia* nauplli enriched in a 10 mg/L and 1000 mg/L A80 suspension (Fig. 2.1.5). The lower PHB contents observed for the *Artemia* nauplii from the A10-100 mg/L, and A10-1000 mg/L can then be explained by the lower PHB content in the bacterial cells despite the gut of the *Artemia* nauplii being completely filled with the bacteria. It thus seems that a combination of PHB content in the PHB-containing bacteria and the applied concentration of enrichment of the live food determine the efficiency of the amorphous PHB strategy.

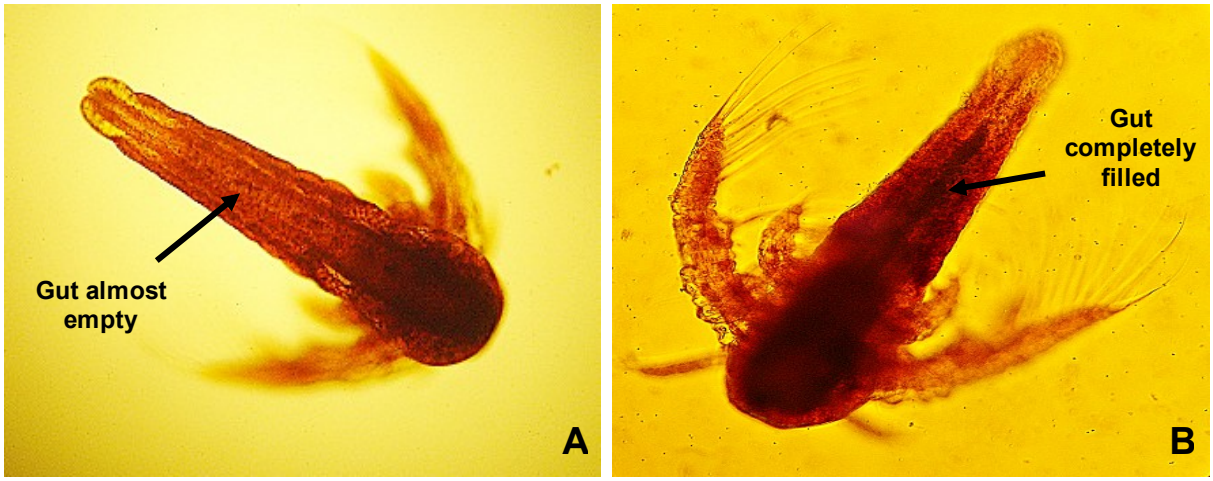


Figure 2.1.5 Stereomicrosopic visualization of gut filling of *Artemia* nauplii after 2 h enrichment with *A. eutrophus* containing 80% PHB at an enrichment concentration of 10 mg/L (A) and 1000 mg/L (B)

In conclusion, our study has provided the first practical information on the application of amorphous PHB supplied through live food for crustacean larviculture. The strategy resulted in a significantly enhanced survival, development and disease protection of the larvae of the giant freshwater prawn *M. rosenbergii*. The strategy requires the application of bacteria that

contain a high content of PHB to allow applying lower *Artemia* nauplii enrichment concentrations than has so far been applied for crystalline PHB. It should be ensured, however, that complete gut filling of the live food used to deliver PHB can be achieved during enrichment.

Acknowledgements

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CHAPTER 2

Section 2

Alcaligenes eutrophus as a live carrier of poly-β-hydrobutyrate for the culture of giant freshwater prawn (*Macrobrachium rosenbergii*) larvae

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Abstract

Using poly-β-hydroxybutyrate (PHB) supplemented in a bacterial carrier (i.e., amorphous PHB) has been shown to work positively for the culture of fresh water prawn (*Macrobrachium rosenbergii*) larvae (Chapter 2-1). Similar effects could be obtained as compared to the use of crystalline PHB, be it at a lower PHB enrichment level. In this study, the use of a freshly cultured bacterium *Alcaligenes eutrophus* – known for its PHB accumulating capacity - was investigated as a biocontrol agent for disease in *Artemia* and *M. rosenbergii* larvae culture. The application of amorphous PHB in a living bacterial carrier could shown to significantly protect *Artemia* nauplii from *V. campbellii* at 10^7 CFU/mL, while *M. rosenbergii* larvae were protected from *V. harveyi* that was enriched in *Artemia* nauplii at 10^8 CFU/mL. Furthermore, feeding prawn larvae with amorphous PHB enriched in *Artemia* nauplii completely inhibited the growth of *Vibrio* spp. in the gut of larvae. From our results obtained, it can be concluded that the strategy of growing PHB accumulating bacteria and supplying these alive rather than lyophilized can increase the survival of *Artemia* nauplii and *M. rosenbergii* larvae when challenged with pathogenic bacteria.

2.2.1 Introduction

Poly-β-hydroxybutyrate (PHB) is a polymer that can be produced by a wide variety of Gram-negative and Gram-positive bacteria as an intracellular energy and carbon storage compound (Madison and Huisman 1999) resulting from unbalanced growth conditions (Madison and Huisman 1999; Bonartseva et al. 2002). After ingestion of PHB by aquatic animals, it is hypothesized that β-hydroxybutyric acid is one of the products released by the enzymatic and/or microbial activity in the gastrointestinal tract (Defoirdt et al. 2007b; Cam et al. 2009; Sui et al. 2012; Najdegerami et al. 2013). β-hydroxybutyric acid potentially inhibits the growth of pathogenic bacteria by decreasing their intracellular pH after passage through the cell membrane, a mechanism that has been described for other short chain fatty acids (Cherrington et al. 1991). As a result, the cell must redirect energy use to the regulation of the intracellular pH thus having less opportunity for other metabolic processes such as virulence expression (Defoirdt et al. 2007b).

Although the cost of PHB production is decreasing, the report by Xu et al. (2014) has shown that the production cost of PHB is still about US$ 4 per kg. It seems that it is still too high to become a cost effective application in aquaculture. In recent studies on PHB application in aquaculture, crystalline PHB particles (i.e., extracted PHB from bacterial cells) and amorphous PHB (i.e., still contained inside bacterial cells) are the two forms of PHB that have been used. Use of PHB in amorphous form is more advantageous than the crystalline form from an economical point of view because of its lower production costs as no extraction from the bacterial cell is required. The beneficial effects of amorphous PHB have been reported by Halet et al. (2007) using the *Artemia franciscana* research model and in Chapter 2.1 for larval *Macrobrachium rosenbergii* in challenge tests with *Vibrio harveyi*.

Practically, *Artemia* nauplii are being used as a live food for shrimp and fish larvae (Sorgeloos and Léger 1992). Verdonck et al. (1994) suggested that live food is an important source of potentially pathogenic bacteria which are easily transferred through the food chain to the predator larvae in commercial hatcheries. During the hatching of *Artemia* cysts, *Vibrio spp.* can become dominant in the microbial community of the hatching medium by using secreted organic substances such as glycerol (Sorgeloos et al. 1986). *Artemia* nauplii can thus be vectors of *Vibrio* spp. in aquaculture activities (López-Torres and Lizárraga-Partida

2001). Particularly when cultured larvae are undergoing stress or are reared under unfavorable conditions, vibrios transferred with the *Artemia* nauplii can constitute a substantial burden to the cultured larvae (New et al. 2010). Consequently, the aim of the current study was primarily to investigate the effect of feeding amorphous PHB contained in *A. eutrophus* (when cultured in the standard nutrient rich medium LB) on the performance of *Artemia* nauplii and giant freshwater prawn (*Macrobrachium rosenbergii*) larvae and secondly to establish a dose response relationship for protecting *Artemia* nauplii and *M. rosenbergii* larvae (fed *Artemia* nauplii enriched) using freshly grown PHB containing *A. eutrophus* in a challenge test with *Vibrio campbellii* LMG21363 and *Vibrio harveyi* BB120, respectively.

2.2.2 Materials and methods

2.2.2.1 Origin of Macrobrachium *prawn larvae*

Macrobrachium rosenbergii broodstock originated from Vietnam and was housed in a freshwater recirculation system with bio-filter. The prawn larvae for experiments were collected according to the procedures presented in Chapter 2.1.2.1.

2.2.2.2 Axenic hatching of Artemia franciscana

Axenic hatched *Artemia* nauplii were used as a food for *M. rosenbergii* larvae. The process for hatching *Artemia* cysts in axenic condition was described in Chapter 2.1.2.2.1.

2.2.2.3 Seed preparation of Alcaligenes eutrophus

In this study, *A. eutrophus* was used. The stock culture of *A. eutrophus* was stored in 20% glycerol at -80 °C. A 10 µL aliquot of this stock culture was inoculated on an agar plate of Luria-Bertani (LB) with 12 g/L salinity. The agar plate was incubated for 24 h at 28 °C. One colony of *A. eutrophus* from the agar plate was cultured in 25 mL of fresh LB (12 g/L) medium for 24 h at 28 $^{\circ}$C under constant agitation (120 rpm), and used as seed culture in the rest of the study. Bacterial cell numbers were determined by measuring their optical density at 550 nm with a spectrophotometer (Thermo Spectronic, USA) according to the McFarland standard, assuming that an optical density of 1.0 corresponds to 1.2 x 10^9 cells/mL.

2.2.2.4 **A. eutrophus** *H16 preparation for feeding and PHB measurement*

A. eutrophus H16 to be used as feed for *Artemia* or *M. rosenbergii* larvae was inoculated in 25 mL of fresh LB (12 g/L salinity) at an initial density by 10^7 CFU/mL and incubated for 24 h at 28 °C under constant agitation (120 rpm). Then, the bacterial medium was centrifuged at 5.900 rpm for 15 min and washed with FAASW (12 g/L salinity). The pellets were again re-suspended with FAASW and the cell density was determined by a spectrophotometer at 550 nm. The bacterial suspension was stored at 4 °C until used as feed for *Artemia* (Experiment 1) or until used to enrich *Artemia* nauplii as feed for *M. rosenbergii* larvae (Experiment 2).

To determine the PHB content of freshly cultured *A. eutrophus,* a seed culture of *A. eutrophus* H16 was inoculated at 10^7 CFU/mL in each of 6 sterile erlenmeyer flasks (100 mL) containing 25 mL of fresh LB (12 g/L salinity) medium and incubated at 28 °C under constant agitation (120 rpm). The cell dry weight (CDW) of the bacterial culture was determined and PHB content measured at 24 h and 48 h as described further (three replicates at each time point).

2.2.2.5 Enrichment of axenic **Artemia nauplii** *with* **A. eutrophus** *as feed for* **M. rosenbergii** *larvae*

Axenically hatched *Artemia* nauplii (Instar II) were washed with 1 L FAASW (12 g/L salinity). The washed nauplii were enriched with *A. eutrophus* H16 at a concentration of 10^7 CFU/mL and 10^8 CFU/mL in FAASW, while axenic *Artemia* nauplii treated in the same way as above but not enriched with PHB bacteria served as a negative control. The density of *Artemia* nauplii for enrichment was about 80,000 – 100,000 individuals per L FAASW (12 g/L salinity). After 2 h of enrichment, the *Artemia* nauplii were washed with 1 L clean freshwater before being feeding to *M. rosenbergii* larvae *ad libitum*.

2.2.2.6 Experimental design

Two experiments were set up in this study to assess the effect of *A. eutrophus* H16 for PHB-accumulating *A. eutrophus* H16 in *Artemia* nauplii (Experiment 1) and *M. rosenbergii* larvae (Experiment 2) challenge tests.

For experiment 1 on *Artemia* nauplii, 18 glass rearing tubes containing 20 mL FAASW (12 g/L salinity) were used. The *Artemia* nauplii challenge tests were performed according to Defoirdt et al. (2005) with slight modifications. Groups of 25 sterile nauplii were transferred to each tube. These glass tubes were put on a rotor (4 rpm) in a room with constant temperature (28 ± 1 °C). *A. eutrophus* was fed daily to the *Artemia* nauplii as a sole source of food at two feeding concentrations 10^6 and 10^7 CFU/mL/day. The *Artemia* nauplii in the control treatment did not receive any feed. This manipulation was carried out in a laminar flow hood to avoid contamination. As a challenge, *Vibrio campbellii* LMG21363 was prepared as described by Defoirdt et al. (2005) and added at 10^7 CFU/mL to the *Artemia* nauplii after 2 days of culturing with or without *A. eutrophus*. Non-challenged *Artemia* served as control treatments, resulting in a total of 6 treatments each performed in triplicate.

For experiment 2 on *M. rosenbergii* larvae, 20 small glass cones containing 100 mL FAASW (12 g/L salinity) were stocked with 25 *M. rosenbergii* larvae of 1 day after hatching (DAH 1). The environmental parameters and light regime were managed according to method presented in the Chapter 2.1.2.3. The larvae were fed *ad libitum* twice a day (9:00 and 17:00) for 8 days with *Artemia* nauplii enriched or non-enriched in a 10^7 and 10^8 CFU/mL *A. eutrophus* suspension as described above. Larval prawns were challenged with *Vibrio harveyi* BB120 as described above on day 2. Non-challenged larvae served as control treatments. No water was exchanged during this experiment and each treatment was performed in quintuplet.

2.2.2.7 Analyses

2.2.2.7.1 Cell dry weight (CDW)

According to the method described by Cam et al. (2009) with slight modifications, four mL of *A. eutrophus* culture was centrifuged at 6,000 x g for 20 min. The pellets were resuspended and washed two times in 1 mL of autoclaved deionized water. At the second wash, the resuspended samples were transferred to pre-weighed eppendorf tubes and centrifuged as described above. The pellets were weighted after drying the pellets at 80 $^{\circ}$C until constant weight.

2.2.2.7.2 Measurement of PHB content in A. eutrophus *and enriched* Artemia *nauplii*

The PHB content in *A. eutrophus* and enriched *Artemia* nauplii was measured following the procedures described in Chapter 2.1.2.4.1. The preparation of samples of *A. eutrophus* H16 to measure PHB consisted of centrifuging four mL of *A. eutrophus* suspension at 6,000 x g for 20 min and washing two times with autoclaved de-ionized water. The bacterial pellet was used for determining the amount of PHB.

2.2.2.7.3 Artemia *nauplii and* M. rosenbergii *larval survival*

The survival of the *Artemia* nauplii (Experiment 1) and of the *M. rosenbergii* larvae (Experiment 2) was determined after 30 h of challenge with *V. campbellii* LMG21363 and after 8 days of challenge with *V. harveyi* BB120, respectively.

2.2.2.7.4 Bacteria in the gut of M. rosenbergii *larvae*

At the end of experiment 2, triplicate samples of 12 prawn larvae were collected randomly from all replicates to determine the bacteria in the gut according to method presented in the Chapter 2.1.2.4

2.2.2.8 Statistics

The software SPSS version 17.0 was applied for statistical analyses. Larval survival, bacterial numbers and percentage of PHB in enriched *Artemia* nauplii were analyzed by One-way analysis of variance (ANOVA), followed by a Tukey-test for post-hoc comparison ($P<0.05$). Only the percentage of PHB in *A. eutrophus* at 24 h and 48 h was analyzed by Independent-sample T-test ($P<0.05$). All the percentage data were normalized by arcsine transformation before statistical analysis, but only non-transformed means are presented.

2.2.3 Results

Experiment 1: The effect of PHB-containing live *A. eutrophus* H16 on *Artemia* nauplii

LB medium (12 g/L salinity) was used to culture *A. eutrophus* and the percentage of PHB in the biomass was determined at 24 h and 48 h of culture (Fig. 2.2.1). A significant difference

in PHB content was found between the two time points with the highest content of 52.4 ± 4.4% being observed at 24 h. At 48 h of culture, it decreased to 41.0 ± 4.8%.

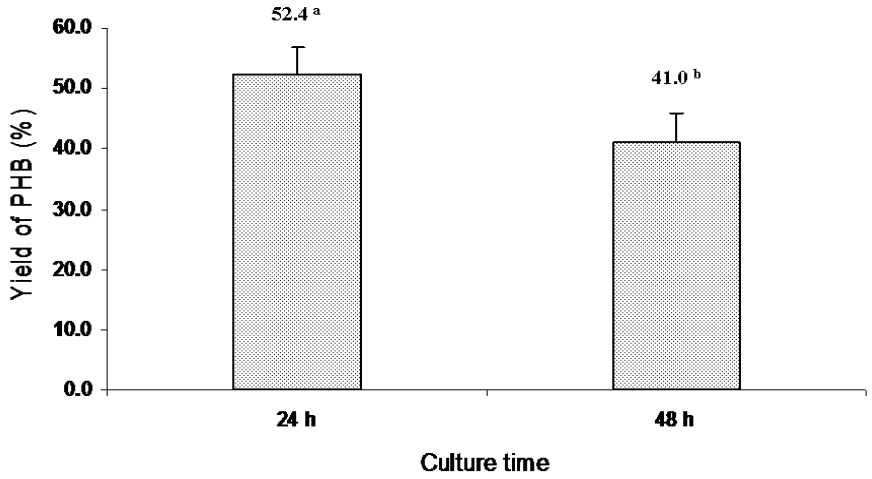

Figure 2.2.1 PHB content in *A. eutrophus* H16 biomass (mean ± SD of three replicates) at 24 h and 48 h of culturing in LB. Different superscript letters indicate significant differences ($P<0.05$).

The survival of *Artemia* nauplii after 30 h of challenge with *V. campbellii* LMG21363 is given in Table 2.2.1 The survival of starved control *Artemia* nauplii decreased significantly upon challenge with *V. campbellii*. The supplementation of *A. eutrophus* at 10^6 CFU/mL did not change the effect of the pathogen on the *Artemia*. However, supplementation with 10^7 CFU/mL significantly increased the survival as compared to the challenged control. No significant differences in survival were recorded for the *Artemia* nauplii of all non-challenged treatments.

Table 2.2.1 Survival of *Artemia* nauplii (mean ± SD of three replicates) supplemented with and without PHB accumulating *A. eutrophus* in a challenge test with *V. campbellii* LMG21363. Values with a different superscript letter are significantly different ($P< 0.05$)

Treatment name	Daily dose of *A. eutrophus* (CFU/mL)	Challenge	Estimated daily addition of PHB * (mg/L)	Survival (%)
Control	0	/	0	89 ± 8^{bc}
Challenge	0	+ *V. campbellii*	0	12 ± 4^{a}
A-10^6	10^6	/	0.5	93 ± 2^{c}
A-10^6	10^6	+ *V. campbellii*	0.5	16 ± 4^{a}
A-10^7	10^7	/	5.4	96 ± 4^{c}
A-10^7	10^7	+ *V. campbellii*	5.4	77 ± 5^{b}

* *Estimated concentration of PHB based on the PHB content in* A. eutrophus *(52.4% on CDW; see figure 2.2.1) and the assumption of* 10^{12} *CFU = 1 g CDW.*

Experiment 2: The effect of PHB accumulating *A. eutrophus* on *M. rosenbergii* larvae

The PHB content in the axenic *Artemia* nauplii enriched with *A. eutrophus* was significantly higher than the non-enriched *Artemia* nauplii (control) (Table 2.2.2). With a PHB content of 13.4 ± 0.3%, the PHB enrichment was the highest for *Artemia* nauplii enriched with *A. eutrophus* H16 at 10^8 CFU/mL.

The challenge of *M. rosenbergii* larvae with *V. harveyi* BB120 resulted in a significant decrease in survival as compared to the unchallenged larvae (Table 2.2.2). The supplementation of *Artemia* nauplii enriched with *A. eutrophus* at 10^7 CFU/mL did not result in a significant increase as compared to the challenged control treatment. This was the case for the *M. rosenbergii* larvae supplemented with *Artemia* nauplli enriched at 10^8 CFU/mL. The increased survival did not, however, reach the level of the non-challenged control larvae.

Table 2.2.2 PHB content in *Artemia* nauplii enriched with *A. eutrophus* at two concentrations (mean ± SD of three replicates) and survival of *M. rosenbergii* larvae (mean ± SD of five replicates) supplemented with these *Artemia* nauplii in a challenge test with *V. harveyi* BB120. Values within the same column not sharing the same superscript letter are significantly different ($P<0.05$).

Treatment	Enrichment dose of *A. eutrophus* for nauplii (CFU/mL)	PHB content in enriched *Artemia* (% on CDW)	Estimated enrichment dose of PHB * (mg/L)	Challenge	Survival of *M. rosenbergii* larvae (%)
Control	0	8.9 ± 0.3^{c}	0	/	83 ± 10^{a}
Challenge	0	8.9 ± 0.3^{c}	0	+ *V. harveyi*	27 ± 3^{c}
A-10^7	10^7	11.1 ± 0.4^{b}	5.4	+ *V. harveyi*	38 ± 9^{c}
A-10^8	10^8	13.3 ± 0.3^{a}	54.2	+ *V. harveyi*	61 ± 5^{b}

** Estimated concentration of PHB based on the PHB content in* A. eutrophus *(52.4% on CDW; see figure 2.2.1) and the assumption of 10^{12} CFU = 1 g CDW.*

TCBS agar was used to enumerate the presumptive number of vibrios in the gut of the challenged *M. rosenbergii* larvae at day 8 of the experiment. In the challenged control larvae, the number of TCBS colonies was a 100-fold higher as compared to the non-challenged control larvae (Table 2.2.3). Although the number of colonies in the treatment with 10^7 CFU/mL *A. eutrophus* was significantly lower as compared to the challenged control, it was still a factor 10 higher than the unchallenged control. When *Artemia* enriched in 10^8 CFU/mL *A. eutrophus* H16 suspension were fed to the larvae, the TCBS count was still significantly higher as compared to the unchallenged control treatment.

Table 2.2.3 TCBS counts in the gut of *M. rosenbergii* larvae challenged with *V. harveyi* BB120 (mean ± SD of three replicates) and reared for 8 days with non-enriched *Artemia* nauplii or *Artemia* nauplii enriched with *A. eutrophus* at two concentrations. Values with a different superscript letter are significantly different ($P<0.05$)

Treatment	Enrichment dose of *A. eutrophus* for nauplii (CFU/mL)	Challenge	TCBS counts (log CFU/larva)
Control	0	/	2.9 ± 0.0^{a}
Challenge	0	+ *V. harveyi*	4.2 ± 0.0^{d}
A-10^7 CFU/mL	10^7	+ *V. harveyi*	4.0 ± 0.0^{c}
A-10^8 CFU/mL	10^8	+ *V. harveyi*	3.0 ± 0.0^{b}

2.2.4 Discussion

The concept of using PHB-containing bacteria as a biocontrol strategy in aquaculture has been suggested by Halet et al. (2007). The selection of bacterial strains with a high PHB production capacity using straightforward culture conditions will be important for the practical application of the strategy. In this study, it was shown that *A. eutrophus* bacteria can accumulate PHB to more than 50% on cell dry weight in a standard rich culture medium and that their use allowed for an effective protection of *Artemia* nauplii and *M. rosenbergii* larvae against *V. campbellii* LMG21363 and *V. harveyi* BB120, respectively. The supplementation of freshly grown *A. eutrophus* with 52.4% PHB on CDW at 10^6, 10^7 and 10^8 CFU/mL was estimated to represent a PHB concentration of 0.5, 5.2 and 52.4 mg/L, respectively. Defoirdt et al. (2007b) and Halet et al. (2007) reported that supplementation of PHB to the culture medium of *Artemia franciscana* nauplii resulted in an increased resistance of the animals against pathogenic *V. campbellii* infection. In Chapter 2.1 the effects of crystalline and amorphous PHB in the culture of *M. rosenbergii* larvae were compared of which the amorphous form was most effective. The use of PHB still contained within bacteria has the advantage that it avoids the need for the expensive extraction of PHB from the cells (Heinrich et al. 2012). In the experiments of Chapter 2.1, however, lyophilized bacterial biomass of the strain *A. eutrophus* produced according to a laborious and relatively

complicated protocol was used. The production method of PHB thus still presents a substantial bottleneck for its application as there is a need for cheap and easy accessible sources of PHB. Therefore, we attempted in this study to easy culture *A. eutrophus* in a standard rich medium. The PHB accumulation under these standard conditions and its effect on the disease resistance of *Artemia* nauplii and *M. rosenbergii* larvae was assessed. Even without specific methods to boost PHB production, the *A. eutrophus* cultured in LB (12 g/L salinity) accumulated 52.4% PHB on cell dry biomass at 24 h of culture, while after 48 h it had significantly decreased again to about 41%. Normally during the stationary phase of growth when the cells become limited for an essential nutrient, but have an excess of carbon source, the synthesis of PHB is induced (Page 1989). In this experiment, the optimal time for harvest of the *A. eutrophus* for enrichment in *Artemia* to be used as live food was at 24 h of culture. The subsequent enrichment period (in this study 2 h) should be kept short to avoid loss of PHB content in bacterial cells..

Experiment 1 showed that the addition of *A. eutrophus* to the culture medium of *Artemia* nauplii could protect the animals against pathogenic *V. campbellii*. The degree of protection depended, however, on the concentration of *A. eutrophus* added. While a concentration of 10^7 CFU/mL (5.2 mg/L PHB) almost completely protected the *Artemia* nauplii from *V. campbellii* disease, the opposite was true for the lower concentration of 10^6 CFU/mL (0.5 mg/L PHB). These dose-response effects of PHB are in accordance with the findings of Halet et al. (2007) and Chapter 2.1. Similar observations as for the *Artemia* nauplii were done for *M. rosenbergii* in experiment 2. The results showed that feeding *Artemia* nauplii enriched with PHB-containing *A. eutrophus* to *M. rosenbergii* larvae enhanced the survival of the prawn. The concentration of *A. eutrophus* in the enrichment medium of the *Artemia*, however, also here determined the efficiency of the treatment. This resulted in a higher PHB content of the *Artemia* enriched at 10^8 CFU/mL (52.4 mg/L PHB) as compared to the *Artemia* enriched at 10^7 CFU/mL (5.2 mg/L PHB) or the non-enriched *Artemia* nauplii. The antimicrobial activity of the PHB, as indicated by the decrease in TCBS counts, is likely to be at the basis for the protective effect of the compound. The obtained results are supported by the findings of Chapter 2.1 where it was found that *M. rosenbergii* larvae were significantly protected against pathogenic *V. harveyi* by feeding *Artemia* nauplii enriched in lyophilized PHB-accumulated *A. eutrophus*. In that study, the performance of the larvae could also be explained by the content of PHB in the *Artemia* nauplii after enrichment, and

the concurrent decrease in TCBS counts in the *Macrobrachium* larvae. The transformation of PHB in the gut into its monomer (3-hydroxybutyrate) resulting in an antimicrobial activity has been hypothesized by Defoirdt et al. (2009) as a mode of action. However, immunostimulation in the treated host may be another explanation for the reduced TCBS counts (Suguna et al. 2014).

The fact that amorphous PHB has an antimicrobial activity for both the *Artemia* nauplii and the *M. rosenbergii* larvae is an interesting feature. Gómez-Gil et al. (1994), Muroga et al. (1994), Pector et al. (1994), López-Torres and Lizárraga-Partida (2001) also reported that *Artemia* - a common live food for larval aquaculture animals - is a vector for pathogenic bacteria, especially *Vibrio* spp., to fish and shrimp larvae. The bacterial contamination in the hatching medium of *Artemia* can reach numbers of more than 10^7 CFU/mL (Van Stappen 1996). The results thus seem promising in terms of controlling the transmission of *Vibrio* spp. from *Artemia* nauplii to *M. rosenbergii* larvae by the use of amorphous PHB. It seems that the concentration of *A. eutrophus* was 10 times lower to be effective in *Artemia* as compared to *M. rosenbergii* larvae. The *Artemia* to be used as food and enriched with *A. eutrophus* at the dose required for *M. rosenbergii* larvae can thus be considered to be sanitized prior to consumption by the larvae.

In conclusion, our study has shown the effectiveness of conventionally cultured *A. eutrophus* H16 in a standard nutrient rich medium as a protective agent against pathogenic infection in *Artemia* nauplii and *M. rosenbergii* larvae. Besides significantly enhancing the survival of the target animals, the PHB contained within *A. eutrophus* also decreased the number of vibrios in the gut of the *Artemia* nauplii and the *M. rosenbergii* larvae.

Acknowledgements

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CHAPTER 3

Changes in carbon and nitrogen patterns in the hatching medium of decapsulated *Artemia franciscana* cysts

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Abstract

During hatching, a carbon metabolism drives the release of nauplii from the cysts of the zooplankton brine shrimp *Artemia*. In this study, the change in carbonaceous compounds (trehalose, glycerol, glycogen and total organic carbon) and total nitrogen in the axenic hatching medium of decapsulated *Artemia fransicana* cysts was determined throughout the hatching process. Three different salinities (5, 12 and 35 g/L) of the incubation medium were applied. Trehalose appeared in the medium in small quantities (maximally 2.6 mg C/g incubated dry cysts) as compared to glycerol and glycogen (maximally 28.5 ± 1.2 and 13.8 ± 1.0 mg C/g incubated dry cysts, respectively). This is consistent with the conversion of trehalose within the cyst into glycogen for energy delivery during respiration and into glycerol for hatching of the nauplius out of the shell. Salinity only seemed to substantially affect glycerol release which was significantly higher at 12 g/L and 35 g/L than at 5 g/L for most of the incubation period. The maximum total organic carbon (TOC) levels were 38.6 ± 9.0, 55.2 ± 6.0 and 58.7 ± 7.7 mg/g incubated dry cysts and the maximum total nitrogen (TN) levels were 3.0 ± 0.4, 4.6 ± 0.3 and 6.9 ± 0.4 mg/g incubated dry cysts for the 5 g/L, 12 g/L and 35 g/L treatment, respectively. The resulting C/N ratio was 10 or more throughout most of the cyst incubation period in each treatment and showed adequate to support bacterial growth of the probiotic bacterium *Bacillus* sp. LT12. The observations from this study can form the basis of further studies on the integration of *Artemia* live food production and bacterial culture to apply in larviculture.

3.1 Introduction

The brine shrimp *Artemia* is used as a live food for the larval culture of almost all fish and crustacean species due to its high protein content and ability to produce encysted embryos for long term storage (Léger et al. 1987; Sorgeloos et al. 1998; Interaminense et al. 2014). *Artemia* can also be raised easily to produce nauplii, larvae and adults (Bagshaw 1989; Hajirostamloo 2008). According to FAO (2011), *Artemia* can be found globally in hypersaline habitats such as inland salt lakes, coastal salt pans and man-managed saltworks. Over 90 percent of the world's cyst market originates from the Great Salt Lake (Utah, USA), with annual harvests in the order of 2,500 – 3,000 tonnes of finished product. The activity of shrimp hatcheries consumes about 80 to 85 percent of the total sales of *Artemia* cysts, the remainder being used in marine fish culture in Europe and East Asia and for the pet fish market (Chapter 1).

Artemia can reproduce by two modes depending on the prevailing environmental conditions. When living conditions are favorable, the fertilized eggs in the brood pouch of the female develop into free-swimming nauplii (ovoviviparous reproduction). When embryonic development is held back at the gastrula stage because of unfavorable environmental conditions such as high salinity, low oxygen levels, temperature stress, food depletion, etc (Lavens and Sorgeloos 1987), cysts are produced inside the uterus (oviparous reproduction). In the processes of cyst production and later hatching of nauplii from the cyst, three carbon compounds - trehalose, glycogen and glycerol - are of primordial importance. During the process of embryos entering into a cryptobiotic state, trehalose accumulates in the dormant cysts at the expense of glycogen (Clegg 1965). The presence of trehalose prevents the denaturation of proteins and retains cellular integrity in the cysts (Jain and Roy 2008) as it is particularly effective in stabilizing dry membranes, phospholipid bilayers and proteins (Crowe et al. 1987). It is not maternally derived but is synthesized by the embryo itself (Clegg 1965) and accumulates up to 15% of the dry weight of a dormant cyst which weighs about 2.5 μg (~ 1.05 μg trehalose-C) (Clegg 1962). Glycogen and glycerol are two other forms of carbon present in dormant cysts. Clegg (1962) reported that the glycogen remaining after conversion into trehalose represents about 1% on cyst dry weight. Glycerol is suggested to serve as a cryoprotectant to prevent low temperature damage to the embryos (Cheng et al. 2014). It is mainly produced out of trehalose at the moment the metabolism in the cyst

resumes. Nonetheless, it is present in dormant cysts in small quantities of approximately 2 - 5% on dry weight (Clegg 1962). Together, trehalose, glycogen and glycerol have been estimated to make up to 98% of the total carbohydrate content in dormant cysts (Dutrieu 1960; Carpenter and Hand 1986).

During aerobic incubation of *Artemia* cysts under favorable environmental conditions, the embryonic metabolism resumes and conversion of trehalose into glycogen and glycerol sets in (Dutrieu 1960; Muramatsu 1960; Emerson 1963; Clegg 1964; Carpenter and Hand 1986; Lavens and Sorgeloos 1987). The oxidation of trehalose during the trehalose-glycogen conversion acts as an energy supply for respiration (Clegg 1965), while the glycerol accumulates between the embryo and the shell as a hygroscopic compound necessary for the embryo to break out of the shell and hatch (Clegg 1964). The changes in biochemical components in the dormant cyst during embryonic development have been considered in several studies. It was found that by the moment of hatching when trehalose within the cyst (at 15 g/L salinity) had decreased with about 51.3 mg C/g (from 68.6 mg C/g to 17.3 mg C/g) after 14 h incubation, while glycogen and glycerol increased with 29.9 mg C/g cyst dry weight and 8.8 mg C/g cyst dry weight, respectively (Clegg 1964). It has also been estimated that upon hatching about 3.1 mg of glycerol-C is released per gram cyst dry weight into the medium (Clegg 1964). The above data are valid for non-decapsulated cysts while information for decapsulated cysts is currently not available.

The presence of trehalose and glycogen in the *Artemia* cyst hatching medium has not been determined so far. Likewise, the occurrence of nitrogen compounds inside *Artemia* cysts and their release upon hatching has not yet been considered. The objective of this study was therefore to investigate the changes in carbonaceous compounds (trehalose, glycerol, glycogen and total organic carbon) and total nitrogen in the hatching medium of axenic decapsulated *Artemia* cysts throughout the incubation period up to hatching. As the incubation salinity seems to affect the amount of glycerol that needs to be build up inside the cyst to reach the critical osmotic pressure necessary for hatching (Lavens and Sorgeloos 1987), three different salinity concentrations (5, 12 and 35 g/L) were applied in the medium to assess their effect on each of the measured parameters. Based on this knowledge, bacterial growth was quantified in the *Artemia* hatching medium as this may be a very interesting feature for the use of beneficial bacteria in aquaculture.

3.2 Materials and methods

3.2.1 Axenic cysts of Artemia franciscana

Experiments were performed with high quality hatching *Artemia franciscana* cysts originating from the Great Salt Lake, Utah, USA (EG® Type, INVE Aquaculture, Belgium). Axenic cysts were obtained according to the procedure described in Chapter 2.1.2.2.1.

3.2.2 Bacillus *sp. LT12 preparation*

Bacillus sp. LT12 is a probiotic Gram-positive and spore-forming rod-shaped bacterium that was previously isolated from a bacterial enrichment culture (EC5) obtained from the digestive tract of *Litopenaeus vannamei* (Tinh et al. 2007). A ten µl aliquot of the original culture of *Bacillus* sp. LT12 stored in 40% glycerol at -80 °C was inoculated on a Luria-Bertani (LB) agar plate at 12 g/L salinity. The agar plate was put into an incubator at 28 °C for 24 h. One colony from the *Bacillus* sp. LT12 agar plate was grown in 25 mL of fresh LB medium (12 g/L salinity) for 24 h at 28 °C under constant agitation (120 rpm). The optical cell density of the bacterial culture was determined by a spectrophotometer (Therme Spectronic, USA) at 550 nm, washed with FAASW (12 g/L salinity) and set at a value of 1 (i.e. 1.2 x 10^9 cells/mL).

3.2.3 Experimental design

In a first experiment, the changes in carbon and nitrogen patterns during the incubation of axenic and decapsulated *Artemia* cysts were determined. In a second experiment, the growth of *Bacillus* sp. LT12 in the hatching medium of axenic and decapsulated *Artemia franciscana* cysts was assessed.

For experiment 1, glass Schott bottles containing 1 L of FAASW prepared by adding Instant Ocean synthetic sea salt at three different salinities (5, 12 and 35 g/L) were used to incubate 2 g axenic and decapsulated *Artemia* cysts (n = 3 for each salinity). To maintain axenic conditions during the incubation period, each bottle was supplied of a screw cap with 0.22 µm air filters on the inlet and outlet to provide continuous strong aeration. The cysts were incubated for 22 h at 28 °C under constant illumination of approximately 27 µE/(m^2.sec). At

2 h time intervals (from 4 h to 22 h relative to the start of incubation), 100 mL axenic hatching medium of *Artemia* (AHMA) was collected under sterile conditions in a laminar flow after thorough mixing of each bottle for measurement of glycerol, glycogen, trehalose, TOC, TN and cyst hatching success.

In experiment 2, 6 Schott bottles were filled with 1 L FAASW at 12 g/L and supplemented with 2 g axenic and decapsulated cysts (This medium is called axenic hatching medium of *Artemia* – AHMA). *Bacillus* sp. LT12 was added in three bottles at a concentration of 10^7 cells/mL (LT12-AHMA). The 3 bottles without addition of *Bacillus* sp. LT12 served as control treatments (Control-AHMA). *Bacillus* sp. LT12 was also added at 10^7 cells/mL in 3 Schott bottles containing 250 mL LB broth at 12 g/L (LT12-LB). Each bottle was supplied with a screw cap with 0.22 µm air filters on the inlet and outlet to provide continuous strong aeration and to avoid bacterial contamination. After 26 h of incubation at 28 ^oC under constant illumination of approximately 27 µE/(m^2.sec), the incubation medium was separated from the *Artemia* cysts, the hatched nauplii and other small particles by filtering over a sterile 30 µm sieve for analysis of the concentration of bacterial cell dry weight (CDW). The samples were stored at -80 °C until analysis.

3.2.4 Hatching success of **Artemia** *cysts in experiment 1*

The abundances of umbrella and nauplii of *Artemia* in the AHMA samples from experiment 1 were determined in accordance with the method presented by Brendan and Schwarz (2009). One mL of AHMA collected from each well-mixed experimental bottle was diluted 10-folds. A Sedgwick-Rafter slide was used to load 1 mL of the diluted sample and supplemented with one or two drops Lugol's solution to immobilize the *Artemia* nauplii. Using a stereomicroscope (Nikon, Model Alphaphot YS), the number of intact umbrella and nauplii of *Artemia* was counted. This procedure was repeated 3 times for each bottle to determine an average. The averages were multiplied by the dilution factor to obtain the number of umbrella and nauplii of *Artemia* in the experimental bottles.

3.2.5 Carbon and nitrogen analyses of AHMA samples from experiment 1

The liquid phase in the AHMA samples form each sampling time in experiment 1 was separated from the *Artemia* cysts, already hatched nauplii and other small particles by

subsequent filtering over a sterile 30 µm sieve and a sterile 0.22 µm filter (VWR® Syringe Filter, USA). The filtered AHMA samples were stored at -20 °C until analysis.

3.2.5.1 Glycerol

A glycerol assay kit (Megazyme International, Bray, Ireland) was used to measure the glycerol concentration in the filtered AHMA samples according to the manufacturer's protocol.

3.2.5.2 Glycogen

A glycogen colorimetric assay kit (BioVision, Inc., Milpitas, California, USA) was used to measure the glycogen concentration in the filtered AHMA samples according to the manufacturer's protocol.

3.2.5.3 Trehalose

A trehalose assay kit (Megazyme International, Bray, Ireland) was used to measure the trehalose concentration in the filtered AHMA samples according to the manufacturer's protocol.

3.2.5.4 Total organic carbon (TOC)

The TOC in the AHMA samples was determined according to the ISO 10694:1995 standard protocol "Determination of organic and total carbon after dry combustion (elementary analysis)" using a Shimadzu TOC-V analyzer with IR detection following thermal oxidation Shimadzu Solid Sample Module (SSM-5000A). In brief, the principle of total organic carbon analysis was as follows: firstly, the AHMA sample was introduced into the combustion tube for total carbon (TC) analysis. Then, it was filled with an oxidation catalyst and heated to 680 °C. The sample was burned in the combustion tube and as a result the carbon in the sample was converted into carbon dioxide (CO_2). The peak area of the CO_2 is proportional to the TC concentration in the sample. Secondly, the inorganic carbon (IC) was measured as the carbon contained in carbonates and carbon dioxide dissolved in the AHMA sample. By acidifying the sample with a small amount of hydrochloric acid, all the carbonates were

converted to carbon dioxide, after which it was volatilized by a sparging process and detected. Finally, the total organic carbon was determined by the formula: TOC = TC – IC.

3.2.5.5 Total nitrogen (TN)

The TN in the AHMA samples was measured according to the method described by Gross et al. (1999). Briefly, 10 mL of the AHMA sample was pipetted into a 30 mL glass vial. In succession, 5 mL of 0.075 N NaOH and 0.1 g of potassium persulfate were added. All forms of nitrogen compounds were oxidized to nitrate in the persulfate digestion (Ebina et al. 1983; Gross and Boyd 1998). The vial was autoclaved for 30 minutes after capping and mixing. The sample was cooled to room temperature, added and mixed with 1 mL of borate buffer (61.8 g boric acid H_3BO_3 and 8 g NaOH in 1 L of distilled water). Turbid samples were centrifuged for 5 min at 4000 rpm to remove the suspended solids. The total nitrate in the digested sample was analysed by UV-absorption using a spectrophotometer (Perkin Elmer Lambda UV/VIS spectrophotometer) at 220 nm.

3.2.6 OD of **Bacillus** *LT12 in experiment 2*

Triplicate 1 mL samples of the culture media including control-AHMA, LT12-AHMA and LT12-LB for 26 h were collected as OD samples. OD of *Bacillus* sp. LT12 was determined by a spectrophotometer (Thermo Spectronic, USA) at 550 nm. The cell dry weight of *Bacillus* sp. LT12 grown in AHMA for 26h was determined as described in Chapter 2.2.2.7.

3.2.7 Verification of axenity during hatching in experiment 1 and experiment 2

At the end of experiment 1, 100 µL of non-filtered AHMA from each bottle was plated on marine agar (MA) (Becton Dickinson Benelux N.V., Erembodegem, Belgium) in duplicate. After incubation for 5 days at 28 °C, the MA was checked for the presence of bacterial colonies (Gunasekara et al. 2012). The same was done for the control bottles (non-inoculated) from experiment 2. In case contamination was observed, the experiment was repeated.

3.2.8 Statistics

The software SPSS version 17.0 was applied for statistical analyses. Glycerol, glycogen, trehalose, TOC, TN and CDW were analyzed by one-way analysis of variance, followed by a Tukey test for post hoc comparison ($P \leq 0.05$). All the percentage data were normalized by arcsin transformation for statistical analysis, but only non-transformed means are presented.

3.3 Results

3.3.1 Hatching success

At each of the three tested salinities, around 40% of the cysts hatched into umbrella-stage *Artemia* during the 6^{th} to 8^{th} hour of incubation (Fig. 3.1). The number of umbrella stage *Artemia* reached its maximum after 10 h of incubation, with limited presence of *Artemia* nauplii. Between 10 h and 12 h, *Artemia* nauplii started to appear and made up about half of the total number of cysts that hatched. At about 14 h, the number of hatched cysts reached its maximum with the nauplii stage dominating over the umbrella stage and from then onwards remained relatively constant during the incubation period.

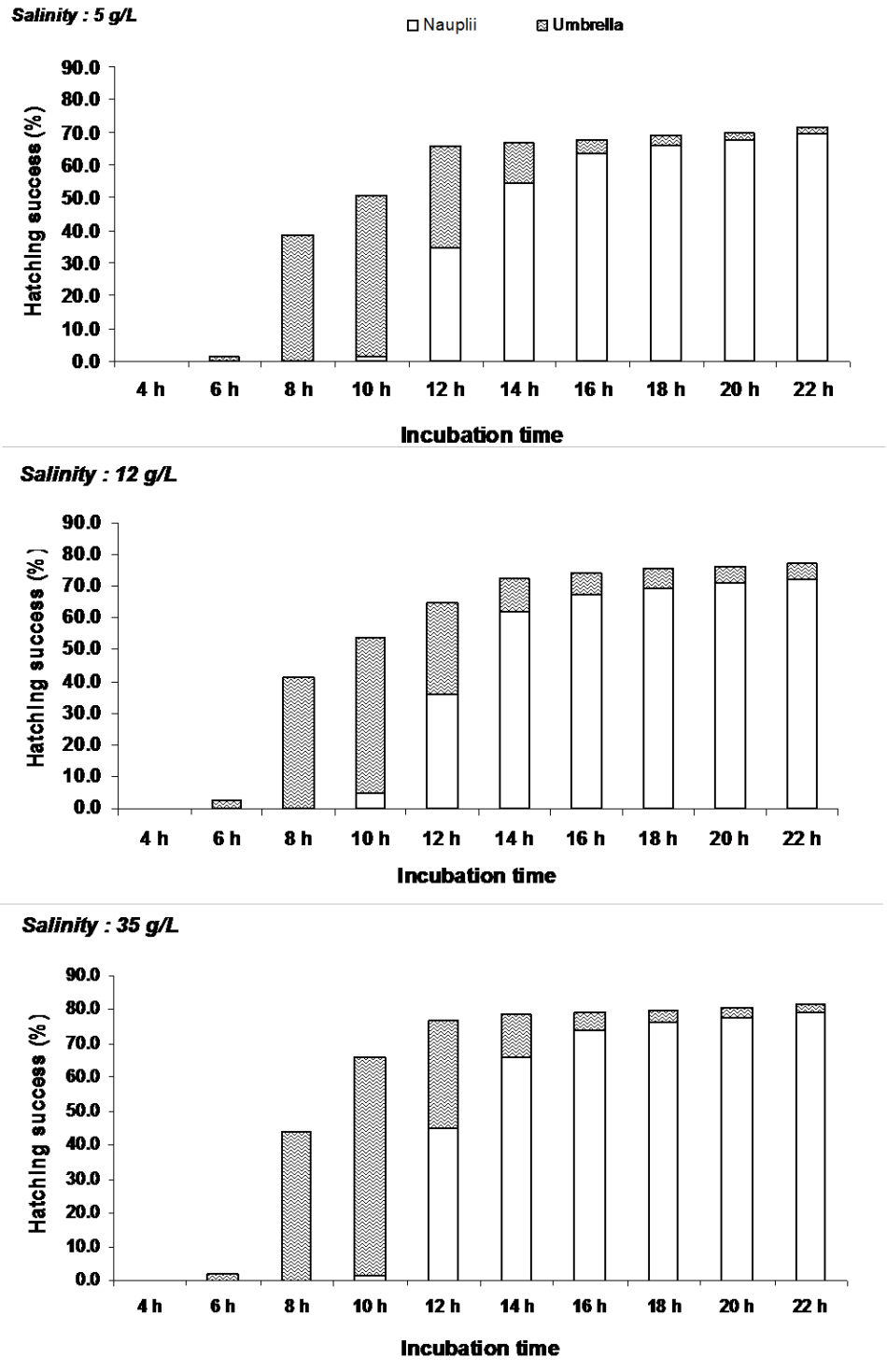

Figure 3.1 Hatching percentage of axenic *Artemia* cysts into the umbrella stage and nauplius stage at different incubation salinities.

3.3.2 Glycerol, glycogen and trehalose content in the AHMA of **Artemia**

The evolution in the concentration of carbonaceous compounds during the 22 h incubation period of axenic *Artemia* cysts in different salinities (5, 12 and 35 g/L) is presented in Fig. 3.2. The amount of glycerol carbon in the hatching medium increased rapidly during the first 8 to 10 h of incubation and reached its highest level at about 16 h for each of the three salinities (Fig. 3.2A). After 16 h the glycerol carbon level showed a decrease in each treatment. From 10 h up to 16 h the glycerol carbon level in the hatching medium was significantly higher at 12 and 35 g/L salinity as compared to 5 g/L. After 16 h, there were no significant differences anymore until 22 h. The amount of glycogen carbon released in the hatching medium steadily increased up to 14 h in each treatment, after which it showed a slight decrease (Fig. 3.2B). The concentration of glycogen carbon in the medium was highest at 35 g/L salinity, followed by 12 g/L and 5 g/L salinity although there were no significant differences between the different treatments. Trehalose carbon appeared in the hatching medium of each treatment in low amounts (< 2.6 mg/g cysts) (Fig. 3.2C). The concentration increased during the first 8 to 10 h, after which it either remained relatively constant (5 g/L and 12 g/L) or decreased again to the original level (35 g/L). The amount of trehalose carbon released was significantly lower in the 35 g/L treatment as compared to the other two treatments from 14 h up to 20 h.

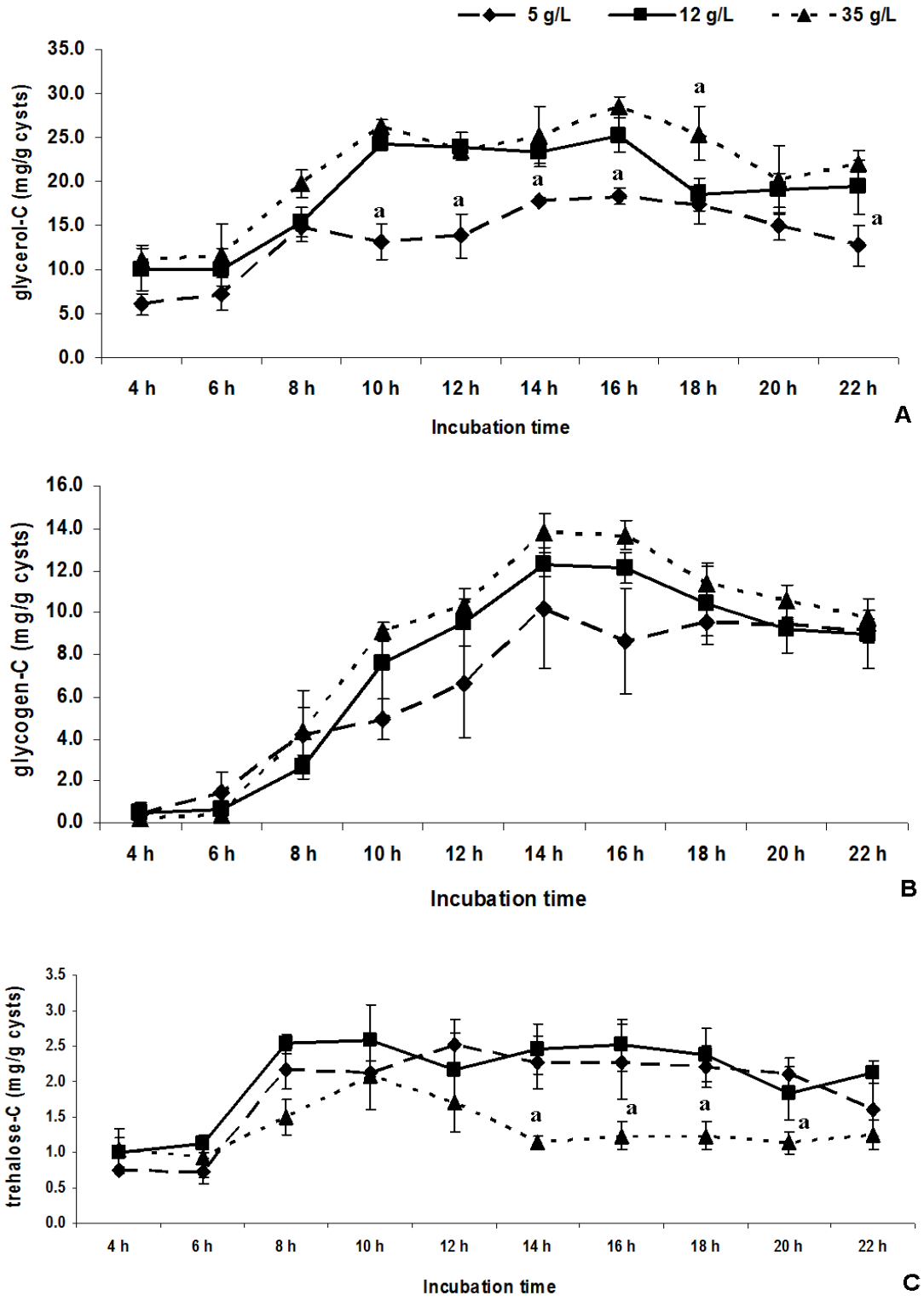

Figure 3.2 Glycerol-C (A), glycogen-C (B), and trehalose-C (C) release in the hatching medium of *Artemia* cysts at different incubation salinities. Values indicated with a letter are significantly different from the other values at that specific time point ($P<0.05$)

3.3.3 Total organic carbon content in the hatching medium of Artemia

The amount of TOC in the hatching medium during the incubation of the *Artemia* cysts increased progressively for each treatment up to 16 h. At this point, a slight decrease in the TOC concentration set in (Fig. 3.3). The TOC released was higher in the 35 g/L than in the 12 g/L treatment, and higher in the 12 g/L than in the 5 g/L. The difference, however, was only significant between the 35 g/L and the 5 g/L from 4 h to 22 h (except at time point 18 h). The sum of glycerol, glycogen and trehalose carbon in general was lower than the TOC for the 35 g/L treatment. The differences were, however, only significant at 6 h. For the 5 g/L and 12 g/L treatment, the TOC values were more or less similar to the sum of the different C compounds until 14 h. From 16 h onwards, the TOC values were higher. At none of the time points were the differences between the 5 g/L and the 12 g/L treatment significant.

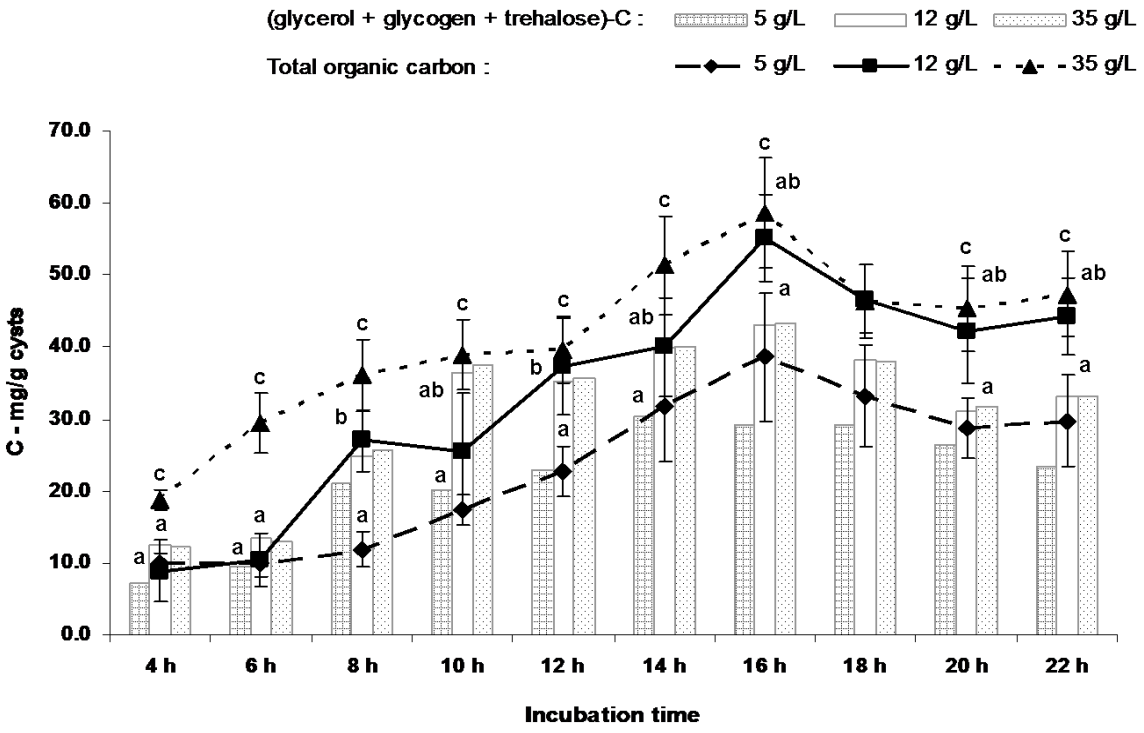

Figure 3.3 Total organic carbon release and sum of glycerol-C, glycogen-C and trehalose-C (standard deviation not shown) release in the hatching medium of *Artemia* cysts at different incubation salinities. Values at a specific time point indicated with a different letters are significantly different ($P<0.05$)

3.3.4 Total nitrogen content in the hatching medium of Artemia

The level of TN in the hatching medium gradually rose in each treatment from the beginning of incubation of the *Artemia* cysts until the end of the experiment (Fig. 3.4). The influence of salinity on the total nitrogen level in the hatching medium became obvious from 8 to 10 h of incubation onwards. At that moment, the total nitrogen concentration in the different treatments started to differentiate with the highest amount being released in the 35 g/L treatment, followed by the 12 g/L and the 5 g/L treatment. However, significant differences in TN concentration in the hatching medium appeared only from 14 h onwards.

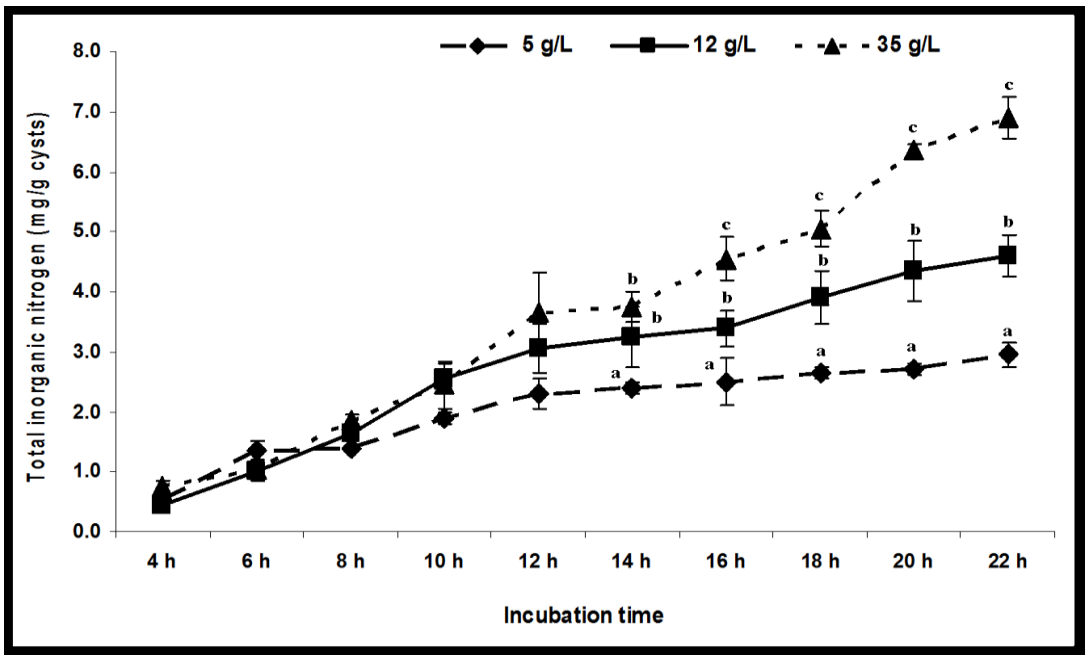

Figure 3.4 Total nitrogen release in the hatching medium of axenic *Artemia* cysts at different incubation salinities. Values at a specific time point indicated with a different number of letters are significantly different ($P<0.05$)

3.3.5 Growth of Bacillus *sp. LT12 in axenic hatching medium of* Artemia *(Experiment 2)*

Bacillus sp. LT12 grew significantly in AHMA and LB medium (12 g/L salinity) (Fig. 3.5). The OD of *Bacillus* sp. LT12 was significantly higher when cultured in LB medium (about 3.3) as compared to culturing in AHMA (about 1.5). The OD of Bacillus sp. LT12 in AHMA corresponded to a density of 0.53 g CDW/L. No bacterial cells were detected in the control treatment without inoculation of *Bacillus* sp. LT12.

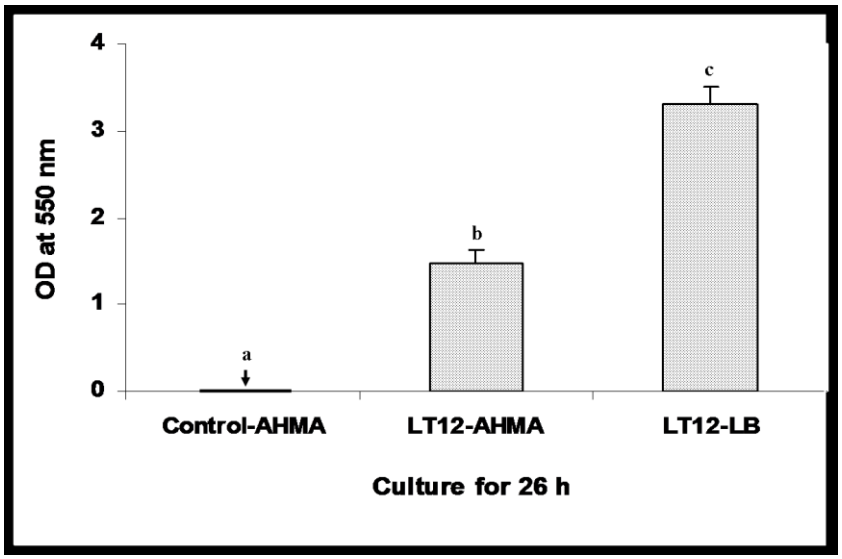

Figure 3.5 The OD of *Bacillus* sp. LT12 after 26 h in non-inoculated AHMA (control-AHMA) or in AHMA and LB medium inoculated with 10^7 cells/mL of *Bacillus* sp. LT12 (LT12-AHMA and LT12-LB, respectively). Bars indicated with different letters are significantly different.

3.4 Discussion

In dormant embryos of *Artemia*, trehalose is the main carbohydrate (Clegg 1962; Ewing and Clegg 1969; Nambu et al. 1997). This compound not only enables dormant embryos to survive stress conditions, it also serves as an energy supply for embryonic development and leads to hatching of the embryo from the cysts under suitable environmental conditions by the formation of glycogen and glycerol, respectively (Clegg 1964; Cam et al. 2009; Yang et al. 2013). Previous researches on the trehalose metabolism have mainly focused on the biochemical changes within the cyst during different development stages (Muramatsu 1960; Clegg 1962; 1964; Boulton and Huggins 1977). However, very few information is available on the release of metabolic compounds to the cyst hatching medium. In this study, the changes in carbon and nitrogen concentrations in the hatching medium of axenic decapsulated *Artemia* cysts were therefore documented.

According to Clegg (1964), glycerol contained within the cyst is located both in the undeveloped embryo and between the outer membrane and the cyst shell. During embryonic development trehalose is converted into glycerol that accumulates in the extra-embryonic space as a hygroscopic compound. Upon rupture of the cell, this glycerol is released to the

medium. In our study, the glycerol carbon concentration in the medium at 5, 12 and 35 g/L salinity increased rapidly up to 8 - 10 h and then only slightly increased further to attain its maximum after 16 h of incubation (18.3 ± 0.9, 25.3 ± 2.0 and 28.5 ± 1.2 mg C/g cysts, respectively). The time point of maximal glycerol release corresponded to the time point of maximal *Artemia* cyst breaking, while the slow increase until 16 h was concomitant with a lower novel appearance of *Artemia* umbrella from the cysts and the increased presence of new nauplii (Fig. 3.6). This supports the concept that glycerol is accumulated as a hygroscopic compound required for hatching (Van Stappen 1996). Clegg (1964) found that 3.1 mg of glycerol-C was released into the medium per gram dry cysts upon hatching. Higher values were found for the release of glycerol from the cysts in this study for all salinity treatments (18.3 mg C/g cysts, 25.3 mg C/g cysts, 28.5 mg C/g cysts for the 5 g/L, 12 g/L and 35 g/L salinity treatment, respectively). Normally, one would expect that the difference in pretreatment of the *Artemia* cysts between the experiment of Clegg (1964) and the current experiment may explain for this discrepancy. The former experiment made use of non-decapsulated cysts while for the current experiment decapsulated cysts were incubated. Because the outer layer (chorion) of *Artemia* cysts is removed during the decapsulation process, the breaking of the embryo's outer cuticular membrane requires less osmotic pressure hence a lower production of glycerol would be expected (Sorgeloos et al. 1977; Bruggeman et al. 1980; Ribeiro and Jones 1998). Differences in cyst origin or even cyst strain, differences in incubation conditions (e.g. temperature and/or light conditions) might account for this. Secondly, in the study of Clegg (1964) antibiotics were used to control the microbiota, while the current study was executed under axenic conditions. It might be that in the former study there was still a certain level of microbial activity which could have resulted in an underestimation of glycerol levels. The release of glycerol also depended on the salinity of the axenic hatching medium. Clegg (1964) and Crowe (2008) stated that at higher external osmotic pressures, more trehalose is converted into glycerol which was confirmed by the results from the current experiment.

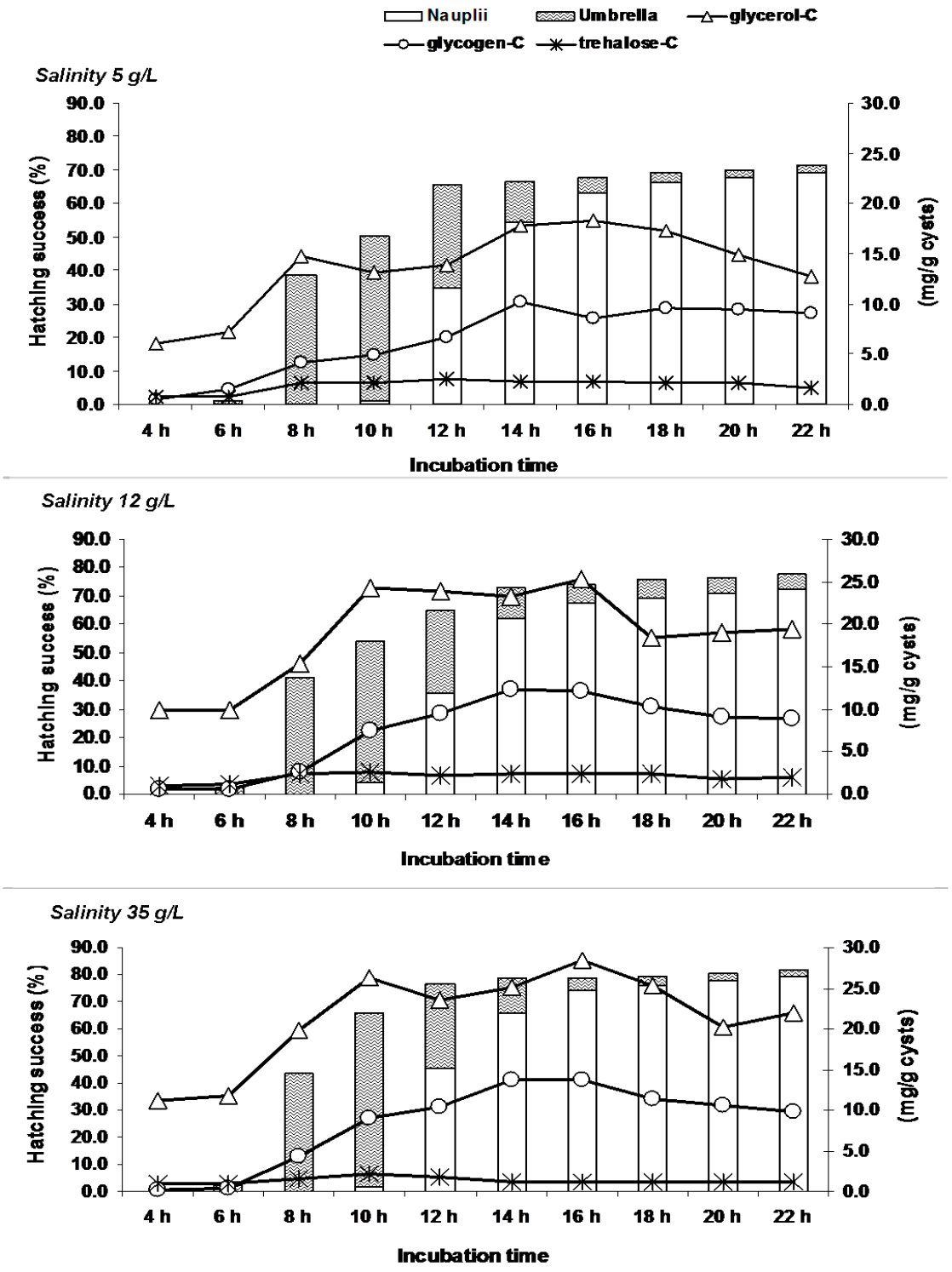

Figure 3.6 The relationship between glycerol, glycogen and trehalose release in the hatching medium with hatching percentage of axenic *Artemia* cysts into the umbrella stage (grey area in bars) and nauplius stage (white area in bars) at different incubation salinities.

The organic carbon released into the incubation medium of the *Artemia* cysts consisted not only of glycerol but also of trehalose and glycogen which have hardly been considered before. The low release of less than 2.6 mg C/g cysts of trehalose was indicative for the trehalose-glycerol osmoregulatory system and the trehalose-glycogen conversion during embryonic development prior to hatching (Lavens and Sorgeloos 1987). This study is the first that describes the occurrence of glycogen in the hatching medium of *Artemia* cysts. So far, glycogen has only been considered within the cysts and various results have been recorded by several authors. Boulton and Huggins (1977) reported that the glycogen-C content in *Artemia salina* cysts after 12 h of hydration was 2.6 mg/g cyst dry weight, while Clegg (1964) reported a value of 37 mg/g cyst dry weight at 14 h incubation. In this study, the highest amount of glycogen present in the hatching medium was 10.2 ± 2.9 mg C/g cysts (5 g/L salinity), 12.3 ± 0.6 mg C/g cysts (12 g/L salinity) and 13.8 ± 1.0 mg C/g cysts (35 g/L salinity). No significant effect of salinity was detected on the presence of glycogen in the AHMA. The steady increase in glycogen concentration up to 14 h, the moment at which the number of *Artemia* nauplii stabilized, indicates that glycogen in the incubation medium can be explained by its release from the *Artemia* cysts at breaking stage. Since trehalose and glycogen are normally not expected in the hatching medium, it is likely that part of the organic carbon that appears in the hatching medium of *Artemia* can be ascribed to the use cysts that were broken or destroyed during the decapsulation process but nevertheless incubated in the hatching bottle. The presence of such cysts in the hatching bottles is illustrated by the 25% unhatched cysts in this study.

The TOC attained maximum values of 38.6 ± 9.0, 55.2 ± 6.0 and 58.7 ± 7.7 mg C/g cysts at 5, 12 and 35 g/L salinity, respectively. The sum of carbon from glycerol, glycogen and trehalose reached on average 93.9% (at 5 g/L salinity), 91.6% (at 12 g/L salinity) and 75.4% (at 35 g/L salinity) of these TOC results. It thus appears that other carbon compounds besides glycerol, glycogen and trehalose may be released during cyst hatching. In this respect, Boulton and Huggins (1977) stated that glucose can also be present be it in very low amounts. In addition, some crustaceans were also found to excrete urea (Weihrauch et al. 2008), which, if this is also the case for *Artemia*, could also contribute to the observed difference. As for any other organism, nitrogen is one of the most important elements in the *Artemia* tissue with a content that was determined at 7.8% on dry weight in any size of *Artemia* nauplii (Nimura and Miah 1991). Nitrogen waste is constantly produced by *Artemia*

nauplii as an excretion product during morphogenesis. The metabolic activity has been determined before and it seems that ammonia nitrogen is excreted by *Artemia* nauplii throughout the different stages of larval development (Hernandorena and Kaushik 1981). This may explain the substantial increase in TN in the *Artemia* hatching medium from 10 h onwards. However, in this study nitrogen was also observed in the hatching medium before *Artemia* cysts reached the hatching stage (before 10 h). It can be hypothesized that this results from metabolic activity of the embryo inside the cyst that created nitrogenous waste that then diffuses through the membrane into the medium. Alternatively, part may also originate from cysts that were broken during the decapsulation process as explained above. The concentration of TN released in the hatching medium was positively related to and significantly affected by the salinity used to incubate the *Artemia* cysts, and the highest TN concentrations were reached at 22 h of incubation with 3.0 ± 0.2, 4.6 ± 0.4 and 6.9 ± 0.3 mg N/g cysts at 5, 12 and 35 g/L salinity, respectively.

As there already is a substantial body of established literature describing the carbon metabolism in (developing) *Artemia* cysts, the novelty in the current research lies in that it considers the combined release of carbon and nitrogen in the hatching medium of *Artemia* for a practical purpose. *Artemia* nauplii are routinely used as live food for the larvae of fish and shrimp, and on a daily basis several kilograms of cysts can be incubated within a larviculture unit. While the hatching medium of *Artemia* cysts is currently considered as a waste, it may be used as a growth source for the culture of beneficial bacteria to be used in aquaculture. Examples are bacterial probionts with homoserine lactone-degrading or/and poly-β-hydroxybutyrate-accumulating characteristics (Cam et al. 2009). Based on the results of TOC and TN, the evolution of the C/N ratio in the hatching medium could be established (Fig. 3.7) which is an important criterium when one wants to culture bacteria. For the largest part of the incubation period, the C/N ratio is about 10 or more in the hatching medium of the *Artemia* which is a good value to achieve bacterial growth as can be deducted from Avnimelech (1999). The growth of the probiotic bacterium *Bacillus* sp. LT12 in AHMA illustrates the potential to use AHMA as a bacteria culture medium. Although the yield of LT12 was not as high as in a traditional nutrient rich medium such as LB, about 0.5 g of dry weight biomass could be produced in each liter of hatching medium. This is sufficient for the dose that is suggested for commercially available probiotic *Artemia* enrichment mixtures such as Sano-Life® MIC-F, INVE Aquaculture (0.5 g/L for bio-encapsulating in live food

with a minimum 10^{10} CFU/g bacterial concentration). This makes the feature that the beneficial bacteria are cultured in the same medium as the live food extra interesting. It should be noted, however, that the amount of TOC measured in the hatching medium (110 mg C/L) is stoichiometrically not enough to support the growth of 0.5 g CDW/L of LT12. Thus, it seems that the bacteria use some other carbon sources in the hatching medium that do not originate from hatched cysts. Most plausible, the presence of unhatched (broken or destroyed) cysts and dead *Artemia* in the incubation medium delivered this carbon. The unhatched cysts represented about 25% of all incubated cysts, or about 500 mg dry weight/L or 250 mg C/L. As *Artemia* nauplii start filter feeding at about 24 h after start incubation (Vanhaecke et al. 1983), they would enrich themselves with these freshly grown beneficial bacteria and make up the perfect delivery medium of these bacteria for the cultured larvae of fish and shrimp. This would make an additional enrichment step unnecessary. However, further research is needed to verify that biosafety can be guaranteed in this way.

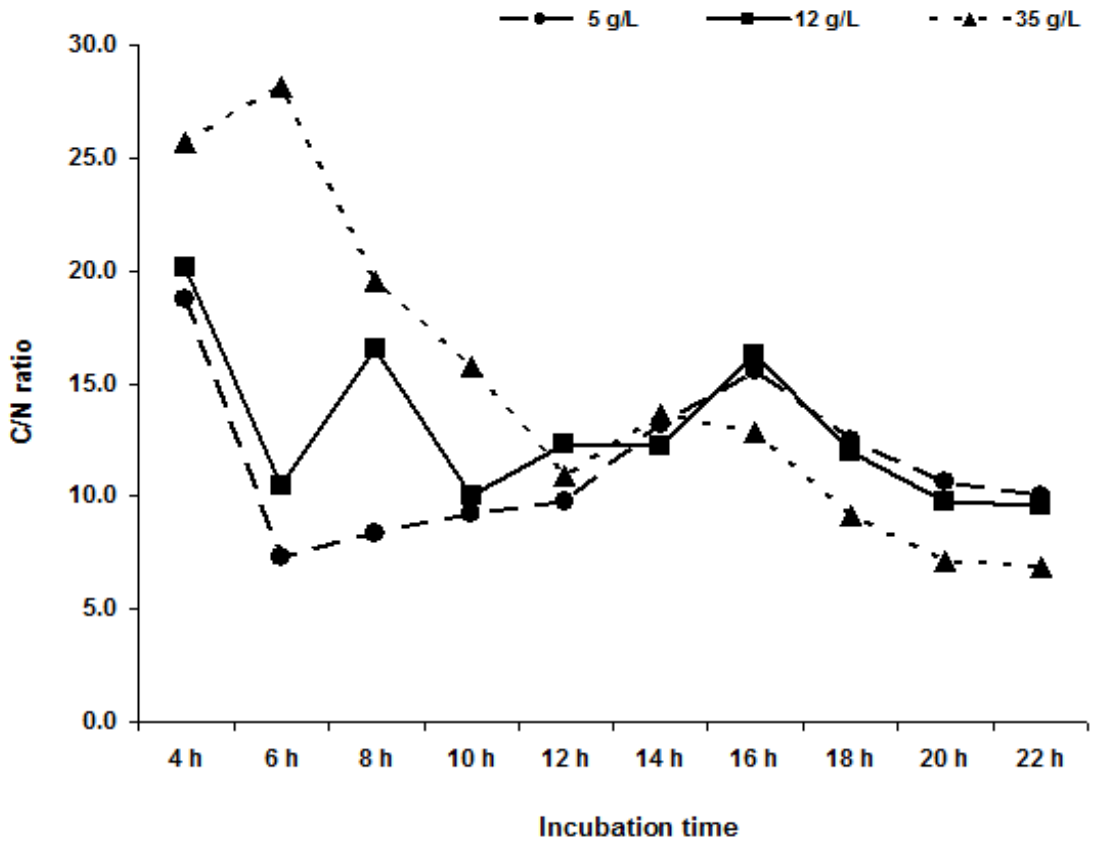

Figure 3.7 The C/N ratio in the hatching medium of axenic *Artemia* cysts at different incubation salinities

In conclusion, we reported for the first time the change in total organic carbon and total nitrogen concentrations in the hatching medium of decapsulated *Artemia* cysts during incubation at different salt concentrations. The carbon compounds glycerol and glycogen are released at substantial amounts from the cysts at the moment of shell rupture while nitrogen waste is secreted by *Artemia* continuously set free during incubation. The knowledge that is obtained concerning the C/N ratio in the hatching medium is of fundamental importance for the reuse of the *Artemia* hatching medium as a growth source for beneficial bacteria and will form the basis of future studies on the integration of *Artemia* culture and bacteria culture to apply in larviculture.

Acknowledgements

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CHAPTER 4

Protection of gnotobiotic *Artemia* nauplii from pathogenic *Vibrio campbellii* by *Bacillus* sp. LT12 cultured in axenic hatching medium of *Artemia franciscana*

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Abstract

In this study, five *Bacillus* strains were compared for their effect on survival and total length of *Artemia* nauplii when fed as a sole food. Their ability to produce poly-β-hydroxybutyrate (PHB) was assessed as well. The most promising strain, *Bacillus* sp. LT12, was cultured in axenic hatching medium of *Artemia* collected after 16 and 20 h of incubation (AHMA16 and AHMA20, respectively) and investigated for its ability to produce amorphous PHB and for its protective effect on *Artemia* nauplii challenged with *Vibrio campbellii* LMG21363. The PHB content in *Bacillus* sp. LT12 cultured for 24h in AHMA16 and AHMA20 increased over two times as compared to culture in Luria-Bertani (12 g/L salinity) medium. Upon challenge with *V. campbellii*, axenic *Artemia* nauplii were completely protected against this pathogen when *Bacillus* sp. LT12 was added at 5 x 10^7 CFU/mL into the culture medium. From these results, it can be concluded that the PHB production capacity of *Bacillus* sp. LT12 can be increased by growing it in axenic hatching medium of *Artemia* and application of these bacteria can improve the survival of *Artemia* nauplii challenged with *Vibrio campbellii*.

4.1 Introduction

The beneficial effects of PHB as a biocontrol compound for sustainable aquaculture production have been reported in recent studies (Defoirdt et al. 2009; Chapter 2.1; Chapter 2.2). In these, two common forms of PHB - crystalline PHB particles (extracted PHB from bacterial cells) and amorphous PHB (still contained inside bacterial cells) – are used. The former has been shown to protect *Artemia franciscana* nauplii against pathogenic bacteria through directly adding it to the culture medium (Defoirdt et al. 2007b), to enhance weight gain, survival or specific growth rate of juvenile fish such as European sea bass (*Dicentrarchus labrax*) (De Schryver et al. 2010b) through supplementing it in the formulated diet and to increase survival and development of larval giant freshwater prawn (*Macrobrachium rosenbergii*) (Nhan et al. 2010b) and Chinese mitten crab (*Eriocheir sinensis*) (Sui et al. 2012) through enrichment into the live food. Similarly, for the amorphous form beneficial effects have been documented both in the *Artemia* model system (Halet et al. 2007; Cam et al. 2009) and in larval *M. rosenbergii*, both in non-challenge conditions and when challenged with *Vibrio* spp. (Chapter 2.1).

The application of PHB in industrial aquaculture in general and larviculture in particular is still constrained with the high cost of commercial PHB products (Nhan et al., 2010b; De Schryver et al., 2010b). Selection of PHB-accumulating bacterial strains and sourcing cheap carbon sources has been proposed for reducing the cost of PHB production (Kim 2000). With respect to strain selection, it is known that PHB can be produced by a large variety of Gram-negative and Gram-positive bacteria as an intracellular energy and carbon storage compound (Madison and Huisman 1999; De Schryver et al. 2010b), including *Bacillus* strains (Wu et al., 2001). Kaynar and Beyatli (2009) isolated thirty *Bacillus* strains from various fresh water fish species with PHB production ranging from 0.81 to 23.38 %. Recently Defoirdt et al. (2011) isolated five *Bacillus* strains from enrichment cultures (EC) that were collected from healthy shrimp and fish by Tinh et al. (2007) and Cam et al. (2009). According to Cam et al. (2009), the PHB content in the EC collected from European sea bass (*Dicentrarchus labrax*) was around 9.4% of cell dry weight. It thsu is possible to isolate *Bacillus* strains capable of PHB accumulation from the gastrointestinal tract of aquaculture animals. Sourcing cheaper carbon sources is a second possibility to decrease the high production cost of PHB. Singh et al. (2013b) stated that the raw material cost accounts for as

much as 40% of the total cost of PHB production. In hatcheries, the hatching medium of *Artemia* is considered a waste once the *Artemia* nauplii are collected to be fed to fish and shrimp larvae. It should, however, be considered that during incubation of *Artemia* cysts large amounts of organic carbon (mainly glycerol) are released in the hatching medium when the *Artemia* embryo develops to the emerging stage (Clegg 1964; Chapter 3). Glycerol is released in the conversion process of trehalose when the dehydrated *Artemia* cysts are re-hydrated and aerated upon incubation (Clegg, 1962; Nambu, 1997). In this study, *Artemia* hatching medium was utilized as a nutrient medium for culturing *Bacillus* spp. with PHB production capacity to apply in aquaculture. Hatching medium of *Artemia* was previously tested as a medium for culturing mixed intestinal bacteria with homoserine lactone-degrading and PHB-accumulating properties by Cam et al. (2009). To our knowledge however, there are no studies to assess the effect of *Artemia* hatching medium on PHB production of single *Bacillus* strains and their ability to control pathogenic bacteria in the host organism. The aims of this study were (1) to select *Bacillus* strains isolated from the intestine of shrimp and fish based on two criteria, namely their suitability as food for *Artemia* nauplii and their capacity to produce PHB and (2) to investigate whether the PHB content of the *Bacillus* strains is increased when it is cultured in axenic hatching medium of *A. franciscana* and whether it offers protection of *Artemia* nauplii against pathogenic *V. campbellii* when these bacilli are added to the culture medium. The combination of selecting *Bacillus* strains with PHB accumulating characteristics and its culture in hatching medium of *Artemia* to offer protection against the pathogens, might be an interesting novel disease control strategy in aquaculture. Apart from increased cost-efficiency through using hatching medium of *Artemia* for producing amorphous PHB, it may also reduce the environmental impact of hatchery operations through recycling nutrients.

4.2 Materials and methods

4.2.1 Source of Bacillus *strains and growth conditions*

The five *Bacillus* strains tested in this study, namely *Bacillus* sp. LT3, LT6, LT8, LT12 and LCDR16, were previously isolated by Defoirdt et al. (2011). Four strains were isolated from an enrichment culture, previously collected from the intestinal tract of whiteleg shrimp (*Litopenaeus vanamei*) (Tinh et al. 2007). The fifth strain was isolated from an enrichment

culture, previously collected from the intestinal tract of European seabass (*Dicentrarchus labrax*) (Cam et al. 2009). The genus *Bacillus* of the five strains was confirmed with 16S rRNA sequence as shown in Table 4.1. (Defoirdt et al. 2011).

Table 4.1 The GenBank sequence accession numbers of partial 16S rRNA gene sequences of the isolated *Bacillus* strains and related strains sharing 100% sequence identity with the sequences of the isolates (based on NCBI BLAST).

Isolate	Accession no.	Related strains[a] (GenBank accession no.)
LT3	HQ235052	*Ba* JMC-UBL 06 (HM451437.1), *Bt* JMC-UBL 03 (HM451439.1)
LT6	HQ235053	*Ba* B (HQ200405.1), *Bc* HMT6 (HQ156459.1), *Bs* ov2004-03268-01 (GU585579.1), *Bt* ODPY (HM770098.1)
LT8	HQ235054	*Ba* JMC-UBL 06 (HM451437.1), *Bc* HMT6 (HQ156459.1), *Bt* JMC-UBL 03 (HM451439.1)
LT12	HQ235055	*Ba* JMC-UBL 06 (HM451437.1), *Bt* JMC-UBL 03 (HM451439.1)
LCDR16	HQ235056	*Ba* JMC-UBL 06 (HM451437.1), *Bt* JMC-UBL 03 (HM451439.1)

[a] *Ba: Bacillus anthracis; Bc: Bacillus cereus; Bs: Bacillus subtilis, and Bt: Bacillus thuringiensis*

Ten µL of the *Bacillus* strains, which were stored in 20% glycerol at -80 ^{o}C, were inoculated in 25 mL fresh Luria-Bertani (LB) medium with 12 g/L salinity for 24 h at 28 ^{o}C under constant agitation (120 rpm). These cultures were used as stock culture for the experiments in this study.

4.2.2 Axenic hatching medium and sterile nauplii of Artemia franciscana

Both axenic hatching medium and sterile nauplii *of A. franciscana* cysts were used in this study. *Artemia* cysts from the Great Salt Lake, Utah – USA (EG® Type, INVE Aquaculture, Belgium) were decapsulated and incubated under axenic condition following the method described in Chapter 2.1.2.2.1.

Axenic hatching medium of *Artemia* (AHMA) was collected after 16 or 20 h incubation by removing un-hatched *Artemia* cysts and nauplii with an autoclaved sieve (30 µm size) under a laminar flow hood. The axenic hatching medium of *Artemia* collected after 16 h and 20 h incubation is hereafter called AHMA16 and AHMA20, respectively. The *Artemia* hatching media collected as described above was subsequently used as a nutrient medium to culture *Bacillus* sp. LT12 in experiment 2.

To obtain sterile nauplii of *Artemia*, sterile cysts were incubed for 24 h in axenic condition, and the resulting sterile nauplii harvested as described above for further use in experiment 1 and 2.

4.2.3 Preparation of Bacillus *strains for the experiments*

For experiment 1: an aliquot of the *Bacillus* stock was inoculated in fresh LB (12 g/L salinity) at an initial density of 10^7 CFU/mL and incubated at 28 ^oC under constant agitation (120 rpm). After 24 h (all five strains) and 48 h of culture (only strains LT3 and LT12), samples of the bacterial cultures were centrifuged and washed two times with FAASW (12 g/L salinity) at 5,900 rpm for 15 min. The resulting pellets were re-suspended in FAASW, the cell density was set at an optical density of 1 using a spectrophotometer (Thermo Spectronic, USA) at 550 nm corresponding to 1.2 x 10^9 cells/mL, and kept in the fridge at 4 ^oC until use.

For experiment 2: initial density, growth conditions and harvesting of *Bacillus* sp. LT12 was similar as in experiment 1 except that apart from LB (12 g/L salinity), also AHMA16 and AHMA20 were used as culture medium. After 24 h of culture, samples of the bacterial cultures were centrifuged and washed two times with FAASW (12 g/L salinity) at 5,900 rpm for 15 min. The resulting pellets were re-suspended in FAASW. The cell density of the bacterial samples was measured by a spectrophotometer before feeding to *Artemia* nauplii.

4.2.4 Experimental design

For experiment 1 (*Selecting* Bacillus *strains*), the experimental setup consisted of 24 glass rearing tubes containing 25 *Artemia* nauplii. The different *Bacillus* strains were fed as a sole food at 0.5 x 10^7 CFU mL/day for 7 days to quadruplicate groups of *Artemia* nauplii.

Artemia nauplii in the control treatment were starved. All manipulations were carried out in a laminar flow hood to avoid contamination.

For experiment 2 (*In vivo challenge tests*), in total 42 glass tubes containing 25 *Artemia* nauplii were used to set up the different treatments. Two different concentrations of *Bacillus* sp. LT12 (1 x 10^7 and 5 x 10^7 CFU/mL), cultured in three different media, namely LB (12 g/L salinity), AHMA16 and AHMA20 were fed daily to the *Artemia* nauplii. Each treatment was performed in triplicate. *Vibrio campbellii* LMG21363 was used to challenge *Artemia* nauplii following the method presented in the Chapter 2.2.2.6.

4.2.5 Analysis

4.2.5.1 Survival and total length of *Artemia* nauplii

At the end of the experiments, the number of swimming nauplii was determined and survival percentage was calculated. The total length was determined according to Marques et al. (2004a):

Total length (mm per glass tube) = number of survivors x mean individual length

Individual length was measured by using a dissecting microscope equipped with a drawing mirror, a digital plan measure and the software *Artemia* 1.0® (Courtesy Marnix Van Damme).

4.2.5.2 Cell dry weight (CDW) of *Bacillus* sp. LT12

Four mL of resuspended bacteria in FAASW was collected for determining the cell dry weight following the method described in Chapter 2.2.2.7.1.

4.2.5.3 Measurement of PHB content in *Bacillus* sp. LT12

Four mL of resuspended bacteria in FAASW was centrifuged and washed two times with autoclaved de-ionized water at 6,000 x g for 20 min to obtain a pellet. This pellet was used for determining the PHB content. The PHB in bacteria was determined according to the procedures presented in Chapter 2.1.2.4.1.

4.2.5.4 Statistics

Means of survival and total length of *Artemia* nauplii and PHB content in bacterial strains were analyzed by one-way analysis of variance, followed by a Tukey test for post hoc comparison ($P<0.05$) using the software SPSS version 17.0. All percentage data were normalized by arcsine transformation for statistical analysis, but only non-transformed means are presented.

4.3 Results

4.3.1 Experiment 1 (Selecting Bacillus *strains)*

4.3.1.1 Survival of *Artemia* nauplii fed the different *Bacillus* strains as a sole food

The effect of feeding the different *Bacillus* strains as a sole food for 7 days on *Artemia* survival is presented in Table 4.2. No significant differences were noted in *Artemia* survival from day 2 until day 4 between the different *Bacillus* treatments. Nauplii in the control/starvation treatment could survive until day 4, however survival was low (14 ± 8%). From day 5 onwards, a significantly lower survival of nauplii fed LCDR16 was observed as compared to the other *Bacillus* strains. From day 6 onwards, the survival percentage of *Artemia* nauplii fed *Bacillus* LT3, LT8 and LT12 was significantly higher than those fed LCDR16 or LT6. At the last day of the experiment, the nauplii fed LT3 and LT12 demonstrated a significantly higher survival (79 ± 5% and 85 ± 6%, respectively) as compared to the other treatments.

Table 4.2 Survival of *Artemia* nauplii (mean ± SD of 4 replicates) fed with different *Bacillus* strains as a sole food for 7 days. The control treatment was starved. Treatments not sharing the same superscript letter in the same column are significantly different (*P<0.05*).

Treatment	Survival (%)					
	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
LT3	98 ± 3 ^{a}	96 ± 5 ^{a}	91 ± 5 ^{a}	90 ± 7 ^{a}	84 ± 8 ^{a}	79 ± 5 ^{a}
LT6	98 ± 3 ^{a}	96 ± 5 ^{a}	95 ± 5 ^{a}	88 ± 7 ^{a}	64 ± 9 ^{b}	26 ± 3 ^{c}
LT8	99 ± 3 ^{a}	95 ± 4 ^{a}	93 ± 3 ^{a}	88 ± 3 ^{a}	83 ± 7 ^{a}	66 ± 9 ^{b}
LT12	99 ± 3 ^{a}	96 ± 5 ^{a}	95 ± 3 ^{a}	93 ± 3 ^{a}	90 ± 4 ^{a}	85 ± 6 ^{a}
LCDR16	96 ± 5 ^{a}	95 ± 4 ^{a}	90 ± 8 ^{a}	71 ± 9 ^{b}	54 ± 6 ^{b}	10 ± 6 ^{d}
Control	88 ± 3 ^{b}	73 ± 7 ^{b}	14 ± 8 ^{b}	0 ^{c}	0 ^{c}	0 ^{d}

4.3.1.2 Total length (TL) of *Artemia* nauplii

The total length of *Artemia* nauplii fed different *Bacillus* strains as a sole food for 7 days is presented in Table 4.3. A significantly higher total length was observed for *Artemia* nauplii fed with LT3, LT8 and LT12. The treatment LT12 showed the highest total length (18.0 ± 1.8 mm); this was however not significantly different as compared to the LT3 (15.3 ± 3.3 mm) and LT8 (14.2 ± 3.3 mm) treatments.

Table 4.3 Total length of *Artemia* nauplii per treatment (mean ± SD of 4 replicates) fed different *Bacillus* strains as a sole food for 7 days. Control *Artemia* were starved. Treatments not sharing the same superscript letter are significantly different (*P<0.05*).

Treatment	Total length (mm)
LT3	15.3 ± 3.3 ^{a}
LT6	6.0 ± 1.5 ^{b}
LT8	14.2 ± 3.3 ^{a}
LT12	18.0 ± 1.8 ^{a}
LCDR16	2.2 ± 1.5 ^{bc}
Control	0.0 ^{c}

4.3.1.3 PHB content of *Bacillus* sp. LT3 and LT12 cultured in LB (12 g/L salinity)

Fig. 4.1 shows the PHB content in *Bacillus sp.* LT3 and LT12 after 24 h and 48 h culture in LB (12 salinity) with an initial inoculating density of 10^7 CFU/mL. A higher PHB content was recorded for *Bacillus* sp. LT12 than for *Bacillus* sp. LT3 after both 24 and 48 hours of culture. However this difference in statistic was only significance at 48 h, but not at 24 h.

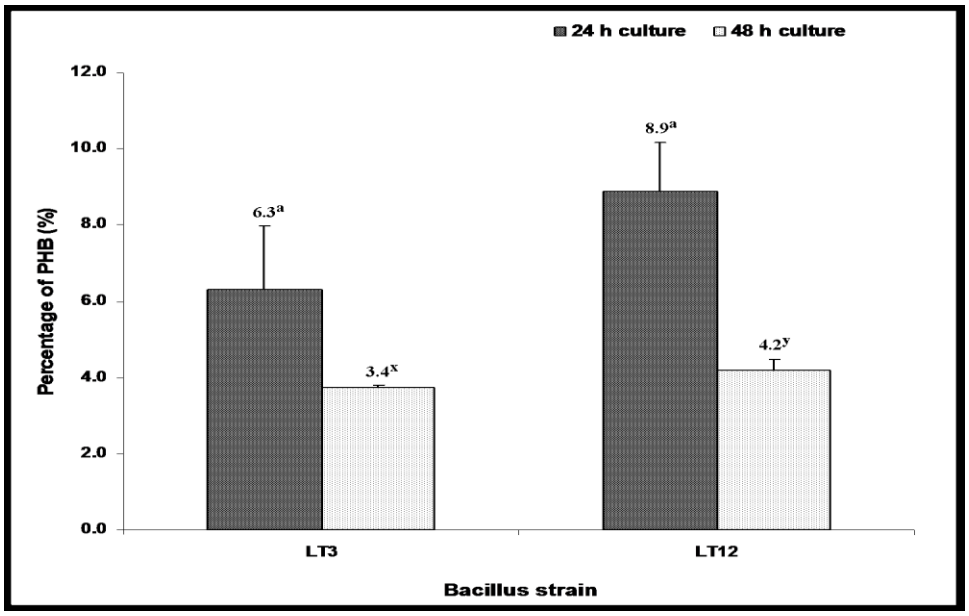

Figure 4.1 Percentage PHB in *Bacillus* sp. LT3 and LT12 (mean ± SD of 3 replicates) after 24 h and 48 h culture in LB medium (12 g/L salinity). Treatments not sharing the same superscript letter within the same time point are significantly different ($P<0.05$).

4.3.2 Experiment 2 (In vivo challenge tests)

4.3.2.1 PHB content of *Bacillus* sp. LT12 cultured in LB, AHMA16 and AHMA20 medium

The PHB content in *Bacillus* sp. LT12 cultured in LB, AHMA16 and AHMA20 after 24 and 48 h is presented in Fig. 4.2. A significantly higher percentage of PHB was recorded when LT12 was grown in AHMA16 and AHMA20 as compared to LB medium (12 g/L salinity), both after 24 and 48 hours. On the other hand, there was no significant difference in PHB content of *Bacillus* sp. LT12 grown in AHMA16 and AHMA20 for both culture periods. For all media, the PHB content in *Bacillus* sp. LT12 was lower at 48 h than at 24 h.

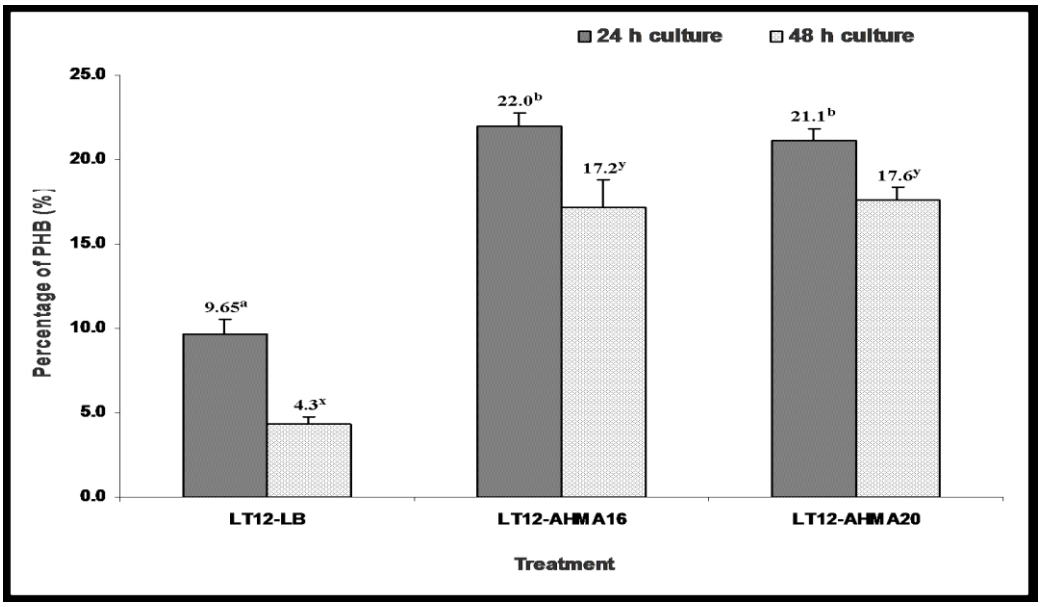

Figure 4.2 Percentage of PHB in *Bacillus* sp. LT12 (mean ± SD of 3 replicates) after 24 h and 48 h of culture in LB, AHMA16 and AHMA20. Treatments not sharing the same superscript letter within the same time point are significantly different ($P<0.05$)

4.3.2.2 Effect of feeding *Bacillus* sp. LT12 cultured in LB, AHMA16 or AHMA20 medium on the survival of *Artemia* nauplii in *in vivo* challenge test with *Vibrio campbellii* LMG21363

Bacillus sp. LT12 was cultured in either LB, AHMA16 or AHMA20 medium and fed at two different concentrations to *Artemia* nauplii on a daily basis. The survival of the *Artemia* nauplii in all treatments was determined at 30 h after challenge with *V. campbellii* (Table 4.4). Of the treatments that were not challenged, survival in all treatments fed *Bacillus* sp. LT12, except two treatments (LT12-LB at 1 x 10^7 CFU/mL and LT12-AHMA16 at 1 x 10^7 CFU/mL), was significantly higher as compared to the (starved) control treatment. Of the treatments that were challenged, the survival of *Artemia* larvae fed 5 x 10^7 CFU/mL *Bacillus* sp. LT12 was significantly higher than the treatments fed only 1 x 10^7 CFU/mL *Bacillus* sp. LT12 (irrespective of the medium) and the control treatment. However, a significantly lower survival was observed in the LT12-LB treatment compared to LT12-AHMA16 and LT12-AHMA20 treatments at a feeding level of 5 x 10^7 CFU/mL. No significant difference was found between the challenged treatments LT12-AHMA16 at 5 x 10^7 CFU/mL and LT12-AHMA20 at 5 x 10^7 CFU/mL and all unchallenged treatments, except for the unchallenged control treatment.

Table 4.4 Survival of *Artemia* nauplii (mean ± SE of 3 replicates) fed different concentrations of *Bacillus* sp. LT12 grown in different media after challenge with *Vibrio campbellii* LMG21363 for 30 h. Treatments not sharing the same superscript letter are significantly different ($P<0.05$).

Treatment*	Daily addition of *Bacillus* sp. LT12 (CFU/mL)	Challenge	Estimated daily addition of PHB ** (mg/L)	Survival (%)
Control	0	/	0	83 ± 6^{d}
Challenge	0	+ *V. campbellii*	0	15 ± 3^{a}
LT12-LB	1×10^{7}	/	1.0	92 ± 3^{de}
LT12-LB	1×10^{7}	+ *V. campbellii*	1.0	19 ± 1^{ab}
LT12-LB	5×10^{7}	/	4.9	96 ± 2^{e}
LT12-LB	5×10^{7}	+ *V. campbellii*	4.9	66 ± 2^{c}
LT12-AHMA16	1×10^{7}	/	2.2	92 ± 3^{de}
LT12-AHMA16	1×10^{7}	+ *V. campbellii*	2.2	25 ± 3^{b}
LT12-AHMA16	5×10^{7}	/	11.0	96 ± 2^{e}
LT12-AHMA16	5×10^{7}	+ *V. campbellii*	11.0	92 ± 2^{de}
LT12-AHMA20	1×10^{7}	/	2.1	95 ± 1^{e}
LT12-AHMA20	1×10^{7}	+ *V. campbellii*	2.1	22 ± 2^{ab}
LT12-AHMA20	5×10^{7}	/	10.6	98 ± 1^{e}
LT12-AHMA20	5×10^{7}	+ *V. campbellii*	10.6	94 ± 7^{e}

** Treatments: LB/AHMA16/AHMA20 denotes the culture medium of* Bacillus *sp. LT12.*

*** Estimated concentration of PHB based on the PHB content in* Bacillus *sp. LT12 (in LB: 9.7% on CDW; AHAM16: 22.0% on CDW and AHAM20: 21.1% on CDW) and the assumption of* 10^{12} *CFU = 1 g CDW.*

Artemia *nauplii were fed 3 times with* Bacillus *sp. LT12 in this experiment.* V. campbellii *LMG21363 was challenged at day 2 of this experiment.*

4.4 Discussion

A selection of *Bacillus* strain based on PHB production capacity has been done in some previous studies. In these studies *Bacillus* strains were isolated from different sources such as molasses contaminated soil (Wu et al. 2001), soil (Singh et al. 2013a; 2013b) and intestine of various fish species (Kaynar and Beyatli 2009). However, when selecting *Bacillus* strains for application in aquaculture, one may not only consider the PHB production capacity but also other benefits to the live food and host animals. For selecting *Bacillus* strains in this study, five *Bacillus* strains isolated from the gut of shrimp and fish were tested as a food source for *Artemia* nauplii, being a widely used live food for larvae of various aquatic animals (Sorgeloos and Léger 1992). The results of survival and total length showed that only two of the *Bacillus* strains tested, namely LT3 and LT12, were a suitable food for *Artemia* nauplii during 7 days of culture, resulting in a survival rate of 79 ± 5 and 85 ± 6% and a total length per treatment of 15.3 ± 3.3 and 18.0 ± 1.8 mm, respectively. The PHB production of these *Bacillus* strains was checked to select the best bacteria strain to use in the next experiment. Finally, *Bacillus* sp. LT12 was chosen as it gave a higher PHB content than LT3 despite the fact that no significant difference was observed after 24 h culture in LB (8.9 ± 1.3 and 6.3 ± 1.7% PHB of cell dry weight, respectively). Kaynar and Beyatli (2009) isolated thirty *Bacillus* species from the intestine of various fish species. Within these isolates, these authors found the highest PHB production in a *Bacillus* species isolated from Gray mullet (*B. lentus)* (strain P18 – 23.38% PHB) and the lowest in a strain isolated from Anchovy (*B. megaterium)* (strain P4 – 0.84% PHB). The above demonstrates the potential of selecting *Bacillus* strains from aquatic animals for their PHB production capacity. PHB production capacity however not only depends on the species or strain, but also on culture conditions.

Using AHMA16 and AHMA20 as bacterial nutrient medium to culture *Bacillus* sp. LT12 increased the PHB content over two times as compared to using LB medium (22.0 ± 0.8; 21.1 ± 0.7 and 9.7 ± 0.9% PHB in cell dry weight, respectively after 24 h culture). The different results in percentage PHB may be explained by the different compositions in terms of nitrogen and carbon in the different culture media. Clegg (1964) and Cam et al. (2009) have shown that the glycerol released in the hatching medium during incubation of *Artemia* is converted from trehalose. Trehalose is the main respiratory substrate for the developing

embryos and may constitute up to 17% of the cyst dry weight. During development of the *Artemia* embryo, the amount of trehalose decreases from 163 µg to 41 µg from 0 to 14h during incubation (Chapter 3). The main nitrogen source in the hatching medium comes from the excretion process of *Artemia* during development. Hernandorena and Kaushik (1981) have shown in their experiment that ammonia is excreted by *Artemia* nauplii, but ammonia during cyst incubation was not measured. Furthermore, the role of urea excretion in aquatic crustacean is still uncertain (Weihrauch et al. 2008). In this respect the carbon and nitrogen sources of *Artemia* hatching medium are quite different compared to LB medium (12 g/L salinity), which is mainly composed of tryptone and yeast extract. Using crude glycerol medium to culture *Bacillus sphaericus* NII 0838 a PHB production of about 31% was reported by Sindhu et al. (2011). Differences in culture medium composition strongly affect the synthesis of PHB as was demonstrated by Mercan et al. (2002) who found that the PHB content in the cell dry weight of two strains of *Rhizobium* sp. was much lower when cultured in yeast extract mannitol broth medium (40% for *Rhizobium* sp. 2426 and 9.09% for *Rhizobium* sp. 640) as compared to a medium with L-cysteine (70% for *Rhizobium* sp. 2426) or L-glycine (56.67% PHB for *Rhizobium* sp. 640) as carbon and nitrogen source. Another study showed that the PHB production of *B. pasteurii* P2 and *B. lentus* P18 isolated from the gut of bluefish and gray mullet increased more than 25% and more than 30%, respectively, when these strains were cultured in lactose and L-glycine and glucose and DL-tryptophan as compared to culturing in nutrient broth medium (Kaynar and Beyatli 2009). Yüksekdag et al. (2004) stated that *Bacillus* strains should be selected for PHB synthesis in function of the carbon and nitrogen sources to be utilized. In that study, two *Bacillus* strains were investigated for the effect of the carbon and nitrogen sources in the culture medium on PHB production. An approximately ten times lower PHB content in the *Bacillus* strains was obtained in nutrient broth medium as compared to a culture medium with protease peptone (*B. subtilis* 25: 7.98 - 78.69% and *B. megaterium* 12: 6.55 - 77% on cell dry weight, respectively). Also the carbon to nitrogen (C/N) ratio is very important when culturing bacteria for PHB production. A study performed by Wei et al. (2011) showed that the optimal C/N in terms of PHB production of *C. taiwanensis* 184 was 8/1 and the resulting PHB content and PHB production were 58.81% and 2.44 g/L, respectively. Concerning to C/N ratio in the hatching medium of *Artemia*, the results in Chapter 3 showed that the C/N of AHMA16 and AHMA20 is approximately 16 and 10, respectively. These C/N ratios should be good for the bacteria to synthesize PHB during the culturing period. It should

further be verified if the C/N ratio of LB medium is highly different from that of the hatching medium of *Artemia*. The latter may also contain other kinds of compounds released from the cysts such as proteins, amino acids, vitamins. Therefore, it would be interesting to determine the exact composition of AHMA as well, and compare this to the composition of LB medium.

In the *in vivo* experiments, *Artemia* nauplii were fed two different concentrations of *Bacillus* sp. LT12 that were cultured in either LB, AHMA16 or AHMA20. In the subsequent challenge test with *V. campbellii* LMG21363, it was recognized that the survival of *Artemia* nauplii was directly positively correlated to the feeding concentration of *Bacillus* sp. LT12. Feeding 5 x 10^7 CFU/mL of *Bacillus* sp. LT12 significantly enhanced the survival of *Artemia* nauplii as compared with feeding only 1 x 10^7 CFU/mL. A higher concentration of *Bacillus* sp. LT12 may result in more bacteria being taken up by the nauplii. This is supported by the results of Chapter 2.1, which showed that the gut of *Artemia* nauplii was almost empty after 2 h enrichment with 10 mg/L *Alcaligenes eutrophus* as compared to enrichment with 1,000 mg/L in which the gut was found completely filled upon stereomicroscopic visualization. As a result, the amount of PHB contained in the nauplii fed *Bacillus* sp. LT12 at 5 x 10^7 CFU/mL might have been higher than those fed only 1 x 10^7 CFU/mL, resulting in higher survival of the larvae. Apart from the feeding level, the amount of PHB in the *Artemia* nauplii will probably also have been influenced by the percentage of PHB in the *Bacillus* sp. LT12. In this respect, even at the higher feeding level of 5 x 10^7 CFU/mL, the lower PHB level in *Bacillus* sp. LT12 cultured in LB medium resulted in a lower survival of the *Artemia* nauplii (66 ± 2%) as compared to the nauplii fed *Bacillus* sp. 12 grown in AHMA16 (92 ± 2%) and AHMA20 (94 ± 7%). In similar *in vivo* challenge tests, Halet et al. (2007) reported complete protection of *Artemia* nauplii against 10^5 CFU/mL *V. campbellii* when 10^7 CFU/mL bacterial PHB2 (99% sequence similarity to *Brachymonas denitrificans* AS-P1) with 32% PHB content were added into the culture medium. The effect of the PHB content of the *Bacillus* sp. LT12 on the survival of *Artemia* nauplii only became apparent upon challenge with *V. campbellii*, since in the non-challenged groups, there were no significant differences in survival of the *Artemia* nauplii fed different concentrations of *Bacillus* sp. LT12 grown in different culture media. Defoirdt et al. (2007b) suggested that the degradation of PHB contained in *Bacillus* sp. LT12 by *Artemia* nauplii in

axenic conditions is most probably physico-chemical or by the enzyme activity of the brine shrimp. β-hydroxybutyrate, the monomer which results from this degradation process and is released in the *Artemia* gut, may protect the nauplii from the pathogen in two ways, i.e. by providing the nauplii with energy and by inhibiting the growth of the pathogen. Although the role of PHB contained in *Bacillus* sp. LT12 on the disease resistance of *Artemia* is undeniable in this study, *Bacillus* sp. LT12 is also known to have quorum sensing interfering activity by which it can protect *Artemia* from infection with bacterial pathogens (Defoirdt et al. 2011). It is a beneficial feature that may have had an additional contribution to the value of *Bacillus* sp. LT12 as a disease protecting agent.

In conclusion, our results demonstrated that the selected *Bacillus* sp. LT12, which was isolated from the gut of whiteleg shrimp (*Litopenaeus vanamei*), had positive effects on the survival and total length of *Artemia* nauplii when used as a sole feed and also has the ability to synthesize PHB. Furthermore, the current study proved that (1) the hatching medium of *Artemia* can be used to culture the *Bacillus* strain leading to an increase in its PHB content; (2) the *Bacillus* cultured in AHMA can protect *Artemia* nauplii against pathogenic *V. campbellii*. Turning this waste product into a nutrient source may moreover contribute to improve the ecological and economic efficiency of using PHB as a novel disease control strategy. In future experiments, we plan to test the efficiency of this technique in disease resistance of different aquaculture organisms.

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CHAPTER 5

PHB-accumulating *Bacillus* sp. cultured in the axenic hatching medium of *Artemia franciscana* can protect *Macrobrachium* larvae against *Vibrio harveyi*

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Abstract

In this study, we investigated the effect of feeding *Artemia* nauplii enriched with the poly-β-hydroxybutyrate (PHB) accumulating *Bacillus* strain LT12, on the survival of larval prawn. In experiment 1, the *Bacillus* strain was grown in hatching medium of *Artemia* that was harvested by separating the *Artemia* from the axenic hatching medium after respectively 16, 20 or 24 hours of incubation. Subsequently *Artemia* nauplii were enriched with this suspension for 2 hours. In a second experiment, the *Bacillus* strain was directly co-cultured in the axenic *Artemia* hatching medium during the 26 hours *Artemia* incubation. In the latter case, addition of supplemental glycerol at the start of incubation was assessed as a way to increase the carbon to nitrogen ratio of the medium and hence PHB yield. The PHB level in *Bacillus* LT12 increased 2-fold when this bacteria was cultured in hatching medium of *Artemia* as compared to culture in Luria Bertani medium (Exp. 1) with a further 50% increase in co-culture through addition of 0.17 and 0.51 g/L glycerol as compared to without supplemental glycerol (Exp. 2). In addition, the disease resistance of *M. rosenbergii* larvae when challenged with *Vibrio harveyi* BB120 was significantly increased when (1) being fed *Artemia* nauplii enriched with 10^9 CFU/mL of *Bacillus* LT12 grown in AHMA16, AHMA20 and AHMA24 (45 ± 5, 56 ± 6 and 46 ± 5% survival, respectively) as compared to 27 ± 3% in the control fed non-enriched nauplii (Experiment 1) and (2) being fed *Artemia* nauplii co-cultured with *Bacillus* LT12 with addition of 0.17 and 0.51 g/L glycerol (63 ± 12 and 60 ± 14 survival, respectively) as compared to 36 ± 8% in the control fed normal *Artemia* nauplii (Experiment 2).

5.1 Introduction

PHB is known to accumulate in the bacterial cell as intracellular energy and carbon storage compound in a wide variety of microorganisms (Kato et al. 1992; Halet et al. 2007). It is not only produced by Gram-negative bacteria, but also by Gram-positive bacteria. Several *Bacillus* spp. has been described as being able to produce PHB (Wu et al. 2001). The accumulation of PHB in bacteria is happening during the stationary phase when the cells become limited for essential nutrients but have excess carbon available (Yüksekdağ et al. 2004). Previous studies have indicated that PHB can be produced relatively easily by *Bacillus* spp. isolated from different sources such as: soil (Aslim et al. 2002; Yilmaz et al. 2005), molasses contaminated soil (Wu et al. 2001) or cassava waste (Mukesh Kumar et al. 2012). In addition, *Bacillus* spp. with the characteristic to produce PHB can also be isolated from the intestine of aquatic animals as Kaynar and Beyatli (2009) isolated 30 strains of the genus *Bacillus* with PHB yields from 0.81 to 23.38% (w/v). Similarly, strains have been isolated from aquaculture animals such as whiteleg shrimp (*Penaeus vannamei*) and European sea bass (*Dicentrarchus labrax*) (Tinh et al. 2007; Cam et al. 2009; Defoirdt et al. 2011), demonstrating the opportunity for using *Bacillus* strains isolated from aquaculture species to produce PHB. The application of PHB in aquaculture, or more specifically in prawn larviculture is limited by the high cost of commercial PHB products (Nhan et al. 2010b; De Schryver et al. 2010b). Currently, the cost of the carbon-source is one of the main obstacles in PHB production because it accounts for 40% of the total expenditure of this process (Singh et al. 2013). There are several ways to reduce the high cost of PHB production, such as: strain optimalisation, improving the culture process and selecting cheap carbon sources (Kim 2000).

Brine shrimp *Artemia* are used very widespread as a live food in fish and shellfish hatcheries since they have a number of convenient characteristics such as high protein content and the ability to produce dormant cysts from which live prey organism can be obtained after merely 24 hours of incubation (Sorgeloos and Léger 1992; Interaminenses et al. 2014). Another special aspect of *Artemia* is that the cysts contain high concentrations of trehalose. It is the respiratory substrate in *Artemia* cysts and constitutes up to 17% of cyst weight. Most of the trehalose present in the dormant cysts is converted to glycogen and glycerol when the dehydrated encysted embryos are re-hydrated and aerated upon incubation (Clegg 1962;

Nambu 1997; Chapter 3). As a result, organic carbon is released in the hatching medium when the embryonic *Artemia* develops and hatches. Within the hatching medium, glycerol, a free organic carbon source is found in substantial concentrations (Clegg 1964). In hatcheries for shrimp or fish, however, the hatching medium of *Artemia* is discarded as waste water after *Artemia* nauplii are collected for feeding the larvae. This hatching medium could however potentially be utilized as a nutrient source to culture beneficial bacteria such as *Bacillus* strains in hatcheries. So far, no studies reported on the use of hatching medium of *Artemia* as a medium to culture PHB-accumulating *Bacillus* spp. for subsequent application in larviculture.

The goal of the present research was to assess the effect of *Artemia* nauplii enriched with *Bacillus* strain LT12 (originally isolated from the digestive tract of *Litopenaeus vannamei*) cultured in the hatching medium of *Artemia* by feeding these to *M. rosenbergii* larvae challenged with *Vibrio harveyi* BB120.

5.2 Materials and methods

5.2.1 Origin of Macrobrachium *prawn larvae and nursing conditions*

Macrobrachium rosenbergii broodstock originated from Vietnam and was housed in a freshwater recirculation system with bio-filter. The prawn larvae for experiments were collected according to the procedures presented in Chapter 2.1.2.1.

5.2.2 Experimental live food preparation

Axenic hatching of A. franciscana

In this study, *Artemia franciscana* cysts were used as live food for *M. rosenbergii* larvae. The sterilized cysts were obtained through the method presented in Chapter 2.1.2.2.1. The cysts were hatched for 24 h (Exp. 1) and 26 h (Exp. 2).

Axenic hatching medium of Artemia *(AHMA)*

In experiment 1, AHMA was collected at 16, 20 and 24 hours after the start of *Artemia* incubation through the procedures presented in Chapter 4.2.2. In what follows, these treatments are referred to as AHMA16, AHMA20 and AHMA24, respectively.

Stock culture of Bacillus *LT12*

In this study, *Bacillus* LT12 was used for its known capacity to accumulate PHB. The origin of this bacterium was presented in the Chapter 4.2.1. Prior to use, a stock culture of *Bacillus* LT12 was stored in 40% glycerol at -80 °C in 1.5 mL eppendorfs. Ten µl aliquot of this stock culture was inoculated on a Luria-Bertani (LB) agar plate at 12 g/L salinity. The agar plate was put into an incubator at 28 ^{o}C for 24 h. One colony from the *Bacillus* LT12 agar plate was grown in 25 mL of fresh LB medium (12 g/L salinity) for 24 h at 28 $^{\circ}$C under constant agitation (120 rpm) and the resulting bacterial culture was used as the start culture. The optical cell density of the bacterial start culture was determined at 550 nm with a spectrophotometer (Thermo Spectronic, USA) according to the McFarland standard assuming that an optical density of 1.000 corresponds to 1.2 x 10^9 cells/mL.

5.2.3 Experimental design

In experiment 1, 500 mL of fresh LB medium, AHMA16, AHMA 20 and AHMA 24 were inoculated with the start culture of *Bacillus* LT12 according to the procedure described in Chapter 5.2.3. Axenically hatched *Artemia* nauplii (Instar II) were collected after 24 h incubation and washed with FAASW 12 g/L. These nauplii were re-suspended in FAASW and enriched with different concentrations of *Bacillus* LT12 (10^8 and 10^9 CFU/mL), which had been cultured either in LB, AHMA16, 20 and 24 for 24 h and were washed twice with FAASW. The density of *Artemia* nauplii for enrichment was about 80,000 – 100,000 individuals/L FAASW. After 2 h of enrichment, the *Artemia* nauplii were washed with clean water before being fed to *M. rosenbergii* larvae. Non-enriched axenically hatched *Artemia* nauplii served as control treatment.

In experiment 2, the start culture of *Bacillus* sp. LT12 was directly co-cultured in a bottle containing 250 mL FAASW (containing 20 mg/L sodium bicarbonate as pH buffer around 7) and *Artemia* cysts for incubation. In some treatments, a pure glycerol solution (Sigma-Aldrich, 98%) was added, namely 0.17 g and 0.51 g per 1 L FAASW containing 2 g cysts of *Artemia*. This corresponds to about 66.3 and 198.9 mg glycerol-C/L FAASW, respectively, as an additional carbon source for growth of the bacteria. The average C/N ratio in the hatching medium of *Artemia* during 22 h incubation was found to be around 10, with a total organic carbon amount of about 110 mg C/L originating from 2 g incubated *Artemia* cysts

(at 12 g/L salinity) (Chapter 3). Hence, through the addition of 0.17 and 0.51 g glycerol/L FAASW containing 2 g *Artemia* cysts, the C/N ratio in the hatching medium was approximately doubled, respectively quadrupled to 20 and 40 in this experiment. After 26 hours of incubation, the *Artemia* nauplii (mostly Instar II stage) enriched with *Bacillus* LT12 were harvested and washed with clean water before being fed to *M. rosenbergii* larvae. Similar as experiment 1, non-enriched axenic *Artemia* nauplii served as control treatment.

5.2.4 Larval rearing procedure and challenge test

50 (Exp. 1) and 25 (Exp. 2) glass cones containing 100 ml of 0.2 μm filtered FAASW (12 g/L salinity) were stocked with 25 *M. rosenbergii* larvae of 1 day after hatching (DAH). The environmental parameter, light regime and challenge with *V. harveyi* BB120 were managed according to the procedured described in Chapter 2.1.2.3.

5.2.5 Analyses

5.2.5.1 Cell density of *Bacillus* LT12

Triplicate 1 mL samples of the *Bacillus* LT12 cultured for 24 h in LB, AHMA16, AHMA20 and AHMA24 (Exp. 1) and co-cultured for 26 h during *Artemia* incubation (Exp. 2) were collected as cell density samples. Cell density of *Bacillus* sp. LT12 was determined by a spectrophotometer (Thermo Spectronic, USA) at 550 nm, with optical density value of corresponding to 1.2 x 10^9 cells/mL.

5.2.5.2 Cell dry weight (CDW) of *Bacillus* LT12

Four mL of resuspended bacteria in FAASW (Exp.1) and bacterial culture (Exp. 2) was collected for determining the cell dry weight following the method described in Chapter 2.2.2.7.1.

5.2.5.3 Measurement of PHB content in *Bacillus* LT12 and *Artemia* nauplii

The PHB content in *Bacillus* LT12 and enriched *Artemia* nauplii was measured following the method described in Chapter 2.1.2.4.1. The preparation of the samples of *Bacillus* LT12 was done by centrifuging four mL of *Bacillus* LT12 suspension at 6,000 x g for 20 min and

washing the pellet two times with autoclaved de-ionized water. The bacterial pellet was used for determining the amount of PHB.

5.2.5.4 *M. rosenbergii* larval survival

The *M. rosenbergii* larval survival was determined at day 8 in both experiments. For this the number of surviving larvae in each cone was counted manually.

5.2.5.5 TCBS plate counts of bacteria in the gut of *M. rosenbergii* larvae

Thiosulphate-Citrate-Bile Salt-Sucrose agar (TCBS, Biokar Diagnostics, France), a type of selective agar culture plate that is used in microbiology laboratories to isolate *Vibrio* spp., was used to determine the number of TCBS counts in the gut of *M. rosenbergii* larvae. The method followed the steps presented in Chapter 2.1.2.4.5.

5.2.5.6 Statistics

The software SPSS version 17.0 was used for statistical analyses. Larval survival, bacterial numbers, percentage of PHB in bacteria and enriched *Artemia* nauplii were analyzed by one-way analysis of variance (ANOVA), followed by a Tukey-test for post-hoc comparison ($P<0.05$). The correlation between the measured PHB content in the *Artemia* nauplii used as live food and the survival of *M. rosenbergii* larvae fed with these *Artemia* was analyzed by bivariate correlation analysis (Pearson coefficient) at the 0.01 significance level. All the percentage data were arcsin transformed for statistical analysis, but only non-transformed means are presented.

5.3 Results

5.3.1 Experiment 1

Bacillus LT12 was cultured in LB medium (12 g/L salinity) and axenic hatching medium of *Artemia* separated from *Artemia* after 16, 20 and 24 h incubation (AHMA16, AHAM20 and AHMA24, respectively) for 24 h, and consequently enriched into axenic *Artemia* nauplii (Instar II) for 2 h and fed to *M. rosenbergii* larvae in the challenge tests with *Vibrio harveyi* BB120.

Cell density and PHB content of Bacillus *LT12 cultured in LB medium and AHMA16, 20 and 24*

Cell density and PHB content of *Bacillus* sp. LT12 grown for 24 h in axenic hatching medium collected after 16, 20 and 24 hours incubation (AHMA16, 20 and 24) was compared with bacteria grown in LB (Fig. 5.1). The percentage of PHB produced in bacterial cells was significantly higher when *Bacillus* sp. LT12 was cultured in AHMA16, 20 and 24 as compared to LB medium. The latter however resulted in a higher cell density compared to the other treatments. The three AHMA treatments were not significantly different from one another in both PHB content and cell density of *Bacillus* LT12.

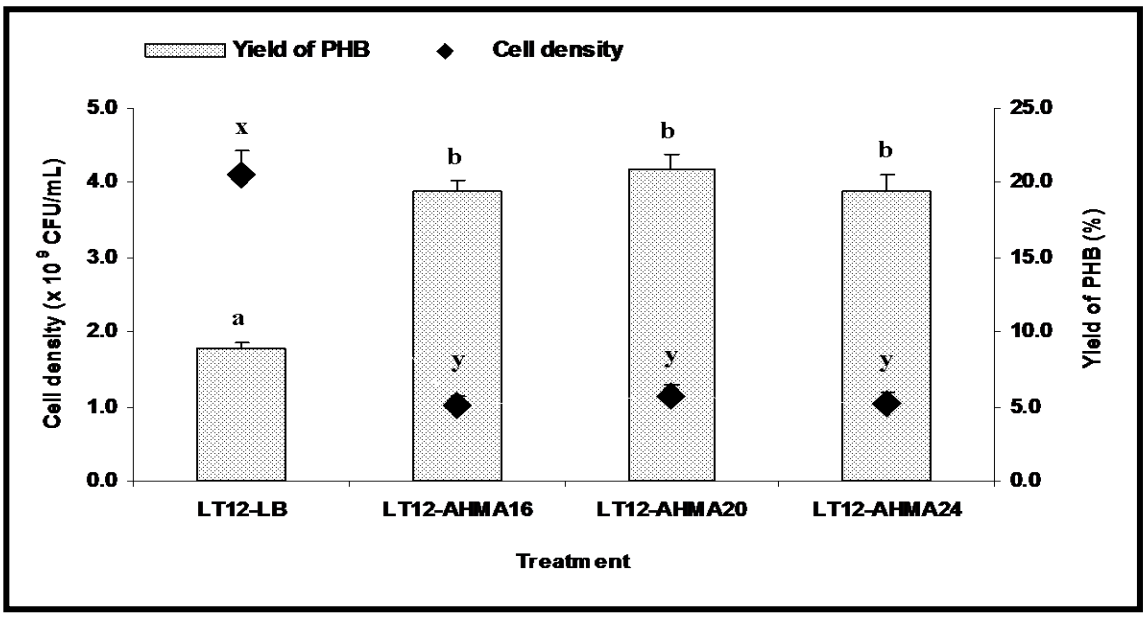

Figure 5.1 Cell density and PHB content of *Bacillus* LT12 (mean ± SD of three replicates) cultured for 24 h in LB medium, AHMA16, AHMA20 and AHMA24. Treatments not sharing the same superscript letter are significantly different ($P<0.05$).

PHB content in Artemia *nauplii enriched with different concentrations of* Bacillus *LT12 cultured in LB, AHMA16, 20 and 24 for 2 h*

The PHB content in *Artemia* nauplii enriched with different concentrations (10^8 and 10^9 CFU/ mL) of *Bacillus* LT12 grown in the different culture media (LB, AHMA16, 20 and 24) for 2 h is shown in Table 5.1. The PHB content in enriched *Artemia* nauplii increased from 9.8 ± 0.2 to 11.2 ± 0.1 (LT12-LB), 10.4 ± 0.2 to 12.8 ± 0.3 (LT12-AHMA16), 10.5 ± 0.3 to 12.9 ± 0.2 (LT12-AHMA20) and 10.6 ± 0.4 to 12.4 ± 0.2 (LT12-AHMA24) with the

corresponding increase in concentration of *Bacillus* LT12 from 10^8 to 10^9 CFU/mL. AHMA treatments mostly resulted in higher PHB levels in *Artemia* as compared to the corresponding LB treatments, although this was not always statistically significant. The highest PHB content in *Artemia* nauplii was recorded with the AHMA treatments at an enrichment dose of 10^9 CFU/mL *Bacillus* LT12. The PHB content in the non-enriched control *Artemia* nauplii (8.7 ± 0.5%) was the lowest.

Effect of feeding M. rosenbergii *larvae with* Artemia *enriched with different concentrations of* Bacillus *LT12 cultured in LB, AHMA16, 20 and 24 for 2 h on survival after challenge with* V. harveyi *BB120*

The survival in the non-challenged control group was significantly higher as compared to all challenged groups. Among the challenged groups, *M. rosenbergii* larvae from the LT12-AHMA16, LT12-AHMA20 and LT12-AHMA24 treatments at 10^9 CFU/mL showed a significantly higher survival compared to these from the treatments with a lower enrichment dose (10^8 CFU/mL) and the larvae fed non-enriched *Artemia* nauplii (control + BB120) (Table 5.1). There was no significant difference between the treatments LT12-AHMA at 10^8 CFU/mL and control treatment when challenged.

Table 5.1 PHB content in *Artemia* nauplii (means ± SD of three replicates) enriched with different concentrations of *Bacillus* sp. LT12 cultured in LB, AHMA16, AHMA20 or AHMA24 for 2 h and survival of *M. rosenbergii* larvae (mean ± SD of five replicates) fed with these *Artemia* nauplii and challenged with *Vibrio harveyi* BB120 during 8 days. Values within the same column not sharing the same superscript letter are significantly different ($P < 0.05$).

Treatment*	Enrichment dose of *Bacillus* sp. LT12 to nauplii (CFU/mL)	PHB content in enriched *Artemia* (% on CDW)	Estimated enrichment dose of PHB (mg/L)**	Challenge	Survival of *M. rosenbergii* larvae (%)
Control	0	8.7 ± 0.5 ^{a}	0	/	83 ± 10 ^{a}
Challenge	0	8.7 ± 0.5 ^{a}	0	+ *V. harveyi*	27 ± 3 ^{c}
LT12-LB	10^8	9.8 ± 0.2 ^{b}	8.7	+ *V. harveyi*	28 ± 3 ^{c}
LT12-LB	10^9	11.2 ± 0.1 ^{c}	87.0	+ *V. harveyi*	33 ± 5 ^{c}
LT12-AHMA16	10^8	10.4 ± 0.2 ^{b}	19.5	+ *V. harveyi*	29 ± 7 ^{c}
LT12-AHMA16	10^9	12.8 ± 0.3 ^{d}	195.0	+ *V. harveyi*	45 ± 5 ^{b}
LT12-AHMA20	10^8	10.5 ± 0.3 ^{bc}	21.0	+ *V. harveyi*	28 ± 4 ^{c}
LT12-AHMA20	10^9	12.9 ± 0.2 ^{d}	210.0	+ *V. harveyi*	56 ± 6 ^{b}
LT12-AHMA24	10^8	10.6 ± 0.4 ^{bc}	19.4	+ *V. harveyi*	30 ± 5 ^{c}
LT12-AHMA24	10^9	12.4 ± 0.2 ^{d}	194.0	+ *V. harveyi*	46 ± 5 ^{b}

** LB/AHMA16/AHMA20/AHMA24 denote the culture medium of* Bacillus *sp. LT12.*

*** Estimated concentration of PHB based on the PHB content in* Bacillus *sp. LT12 (in LB: 8.7% on CDW; AHAM16: 19.5% on CDW; AHAM20: 21.0% on CDW and AHAM24: 19.4% on CDW) and the assumption of 10^{12} CFU = 1 g CDW.*

Effect of feeding Artemia *enriched with different concentrations of* Bacillus *LT12 cultured in LB, AHMA16, AHMA20 and AHMA24 for 2 h on the TCBS counts in the gut of* M. rosenbergii *larvae challenged with* V. harveyi *BB120*

At day 8, the challenged larvae fed *Artemia* nauplii enriched with 10^9 CFU/mL of *Bacillus* sp. LT12 cultured in AHMA showed significantly lower numbers of TCBS colonies in the larval gut a factor 40 – 50 and 13 – 20 as compared to the larvae fed non-enriched *Artemia* and the AHMA treatment at an enrichment dose of 10^8 CFU/mL respectively (Table 5.2). The larvae fed *Artemia* enriched with 10^9 CFU/mL of *Bacillus* sp. LT12 grown in LB medium demonstrated significantly less TCBS colonies in the larval gut as compared with the challenged control treatment and both of the LB treatment and AHMA treatments at an enrichment dose of 10^8 CFU/mL. No significant differences in TCBS counts were observed among of the AHMA treatment at 10^9 CFU/mL and these were all significantly lower than the unchallenged control treatment.

Table 5.2 TCBS counts in the gut of *M. rosenbergii* larvae challenged with *V. harveyi* BB120 (mean ± SD of three replicates) and fed *Artemia* nauplii enriched with different concentrations *Bacillus* LT12 cultured in LB, AHMA16, AHMA20 and AHMA24 for 8 days. Treatments not sharing the same superscript letter are significantly different ($P<0.05$).

Treatment	Enrichment dose of *Bacillus* LT12 to nauplii (CFU/mL)	Challenge	TCBS count (log CFU/larva)
Control	0	/	2.7 ± 0.1^{b}
Challenge	0	+ *V. harveyi*	3.7 ± 0.1^{d}
LT12-LB	10^8	+ *V. harveyi*	3.4 ± 0.1^{c}
LT12-LB	10^9	+ *V. harveyi*	2.9 ± 0.1^{b}
LT12-AHMA16	10^8	+ *V. harveyi*	3.3 ± 0.1^{c}
LT12-AHMA16	10^9	+ *V. harveyi*	2.1 ± 0.1^{a}
LT12-AHMA20	10^8	+ *V. harveyi*	3.2 ± 0.1^{c}
LT12-AHMA20	10^9	+ *V. harveyi*	2.0 ± 0.1^{a}
LT12-AHMA24	10^8	+ *V. harveyi*	3.3 ± 0.1^{c}
LT12-AHMA24	10^9	+ *V. harveyi*	2.1 ± 0.1^{a}

5.3.2 Experiment 2

In this experiment, *Bacillus* sp. LT12 was directly co-cultured during gnotobiotic hatching of *Artemia* (LT12-co-culture) with or without addition of supplemental glycerol. After 26 h culture, these *Artemia* nauplii (Instar II) *in situ* enriched with *Bacillus* sp. LT12 in the gut were fed to *M. rosenbergii* larvae challenged with *Vibrio harveyi* BB120.

Cell dry weight and PHB content of Bacillus *sp. LT12 co-cultured for 26 h with hatching* Artemia *with or without addition of supplemental glycerol (0.17 or 0.51 g/L)*

The PHB content in bacterial cells of the treatments with supplemental glycerol (LT12-co-culture + 0.17 g/L glycerol and LT12-co-culture + 0.51 g/L glycerol) was significantly higher than without supplemental glycerol treatment (LT12-co-culture), but no significant differences were observed in the cell dry weight between these treatments (Fig. 5.2).

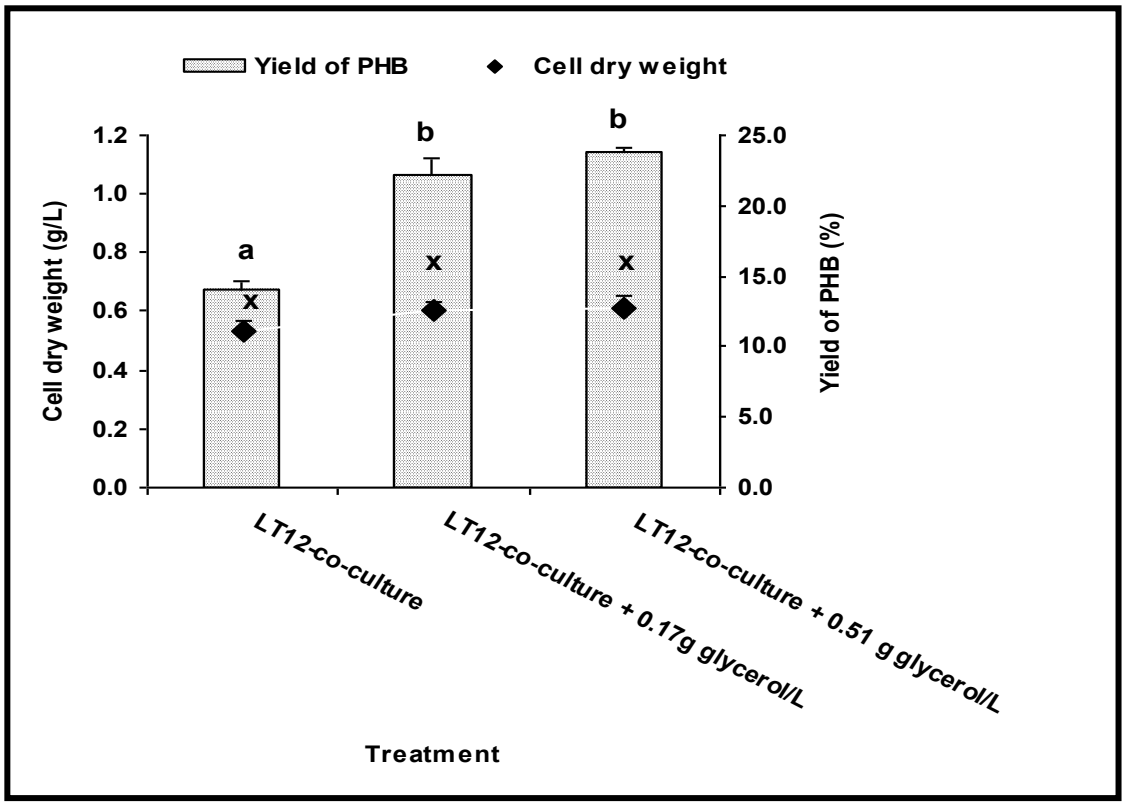

Figure 5.2 Cell dry weight and PHB content of *Bacillus* sp. LT12 (mean ± SD of three replicates) co-cultured for 26 h during axenic *Artemia* hatching with or without addition of supplemental glycerol. Treatments not sharing the same superscript letter are significantly different ($P<0.05$).

PHB content in Artemia *nauplii co-cultured with* Bacillus *sp. LT12 for 26 h with or without addition of supplemental glycerol*

The PHB content in *Artemia* co-cultured with *Bacillus* sp. LT12 ranged from 11.2 ± 0.4% without glycerol addition to 13.8 ± 0.5% when extra glycerol was added, while 8.7 ± 0.5% was measured in the control *Artemia* nauplii treatment (Table 5.3). The treatments with supplemental glycerol (LT12-co-culture + 0.17 g/L glycerol and LT12-co-culture + 0.51 g/L glycerol) showed a significantly higher PHB content in *Artemia* as compared to the treatments where no glycerol was added (LT12-co-culture and control). The control treatment resulted in a significantly lower percentage of PHB in *Artemia* as compared the LT12-co-culture treatments.

Effect of feeding Artemia *nauplii co-cultured with* Bacillus *sp. LT12 for 26 h with or without addition of supplemental glycerol on the survival of* M. rosenbergii *larvae challenged with* V. harveyi *BB120*

The challenged *M. rosenbergii* larvae supplied with the "LT12-co-culture + 0.17 g/L glycerol" and "LT12-co-culture + 0.51 g/L glycerol" treatments showed a significantly higher survival than the challenged larvae fed control *Artemia* nauplii (control + BB120), but a significantly lower survival as compared to the non-challenged larvae fed control *Artemia* nauplii (control) (Table 5.3). The treatment with *Bacillus* sp. LT12 co-culture without glycerol addition (LT12-co-culture) had a significantly lower larval survival compared to the non-challenged control treatment, but was not significantly different from the challenged control treatment.

Table 5.3 PHB content in *Artemia* nauplii (means ± SD of three replicates) co-cultured with *Bacillus* sp. LT12 for 26 h with or without addition of supplemental glycerol and survival of *M. rosenbergii* larvae (mean ± SD of five replicates) fed with these *Artemia* nauplii and challenged with *Vibrio harveyi* BB120 during 8 days. Values within the same column not sharing the same superscript letter are significantly different ($P<0.05$).

Treatment	PHB content in co-cultured *Artemia* (% on CDW)	Challenge	Survival of *M. rosenbergii* larvae (%)
Control	8.7 ± 0.5 [a]	/	87 ± 7 [a]
Challenge	8.7 ± 0.5 [a]	+ *V. harveyi*	36 ± 8 [c]
LT12-co-culture	11.2 ± 0.4 [b]	+ *V. harveyi*	54 ± 10 [bc]
LT12-co-culture + 0.17 g/L glycerol	13.7 ± 0.3 [c]	+ *V. harveyi*	63 ± 12 [b]
LT12-co-culture + 0.51 g/L glycerol	13.8 ± 0.5 [c]	+ *V. harveyi*	60 ± 14 [b]

Effect of feeding Artemia *co-cultured with* Bacillus *sp. LT12 with or without addition of supplemental glycerol for 26 h on TCBS counts in the gut of* M. rosenbergii *larvae challenged with* V. harveyi *BB120*

The larvae fed *Artemia* nauplii co-cultured with *Bacillus* sp. LT12 showed significantly lower numbers of TCBS colonies in the larval gut (a factor 60 – 230) as compared to the challenged larvae fed control *Artemia* (Table 5.4). No significant differences in TCBS counts were observed between the *Bacillus* sp. LT12 treatment without adding glycerol and the non-challenged control treatment, but the counts in these treatments were significantly higher as compared to the *Bacillus* sp. LT12 treatments with addition of supplementary glycerol.

Table 5.4 TCBS counts in the gut of *M. rosenbergii* larvae (mean ± SD of three replicates) challenged with *V. harveyi* BB120 fed control *Artemia* nauplii or nauplii co-cultured with *Bacillus* sp. LT12 for 26 h without or with addition of supplemental glycerol for 8 days. Treatments not sharing the same superscript letter are significantly different ($P<0.05$).

Treatment	Challenge	TCBS counts (log CFU/larva)
Control	/	2.1 ± 0.0^{bc}
Challenge	+ *V. harveyi*	3.9 ± 0.1^{d}
LT12-co-culture	+ *V. harveyi*	2.2 ± 0.1^{c}
LT12-co-culture + 0.17 g/L glycerol	+ *V. harveyi*	1.6 ± 0.1^{a}
LT12-co-culture + 0.51 g/L glycerol	+ *V. harveyi*	1.7 ± 0.1^{ab}

5.4 Discussion

Bacillus species are well known as PHB-accumulating bacteria (Thirumala et al. 2010). Several *Bacillus* strains isolated from soil have widely been used for PHB production (Aslim et al. 2002; Yilmaz et al. 2005). Recently, some *Bacillus* spp. that can produce PHB have been isolated from the gastrointestinal tract of aquatic animals such as various fresh water fish (Kaynar and Beyatli 2009) or aquaculture animals such as whiteleg shrimp (*Litopenaeus vannamei*) and European sea bass (*Dicentrarchus labrax*) (Tinh et al. 2007; Cam et al. 2009; Defoirdt et al. 2011). Since PHB has been suggested as a biocontrol agent against *Vibrio* disease in animal culture, the production of amorphous PHB from these *Bacillus* strains for inhibiting bacterial infection in aquaculture animals is interesting to investigate. In this study, we investigated the effect of the amorphous PHB content in *Bacillus* sp. LT12, isolated from the gut of *P. vannamei* shrimp, cultured in axenic hatching medium of *Artemia* either separate or in co-culture with *Artemia* and subsequently loaded into the live food *Artemia* nauplii, on the survival and putative *Vibrio* load in the gut of *M. rosenbergii* larvae challenged with *V. harveyi* BB120.

The results showed that *Bacillus* sp. LT12 can grow well in the hatching medium of *Artemia* (Fig. 5.3). The *Bacillus* strain apparently uses the carbon and nitrogen sources which are released into the hatching medium from the hatching *Artemia* cysts. Glycerol and glycogen

are the main carbonaceous substrates which have been determined in the hatching of decapsulated cysts (12 g/L salinity) with the highest concentration of 25.3 and 12.3 mg/g cysts, respectively (Chapter 3). Glycerol and glycogen are produced from trehalose (a respiratory substrate) during embryonic development of *Artemia* (Chapter 1) while the inorganic nitrogen that it is used by the bacterium can only originate from the metabolism-based excretion process of *Artemia*. In the first experiment, the cell density of *Bacillus* sp. LT12 cultured in AHMA16, 20 and 24 was significantly lower than when grown in LB medium (12 g/L salinity). The level of some unidentified nutrients in AHMA may have been restrictive for maximum growth of the *Bacillus* while LB medium is a specially formulated nutrient-rich microbial culture medium with optimal levels of nitrogen, carbon, sulfur, phosphorus, minerals and vitamins. Similar results were obtained in the second experiment, although a different culture technique was applied and the *Bacillus* was grown in the presence of the hatching *Artemia* cysts.

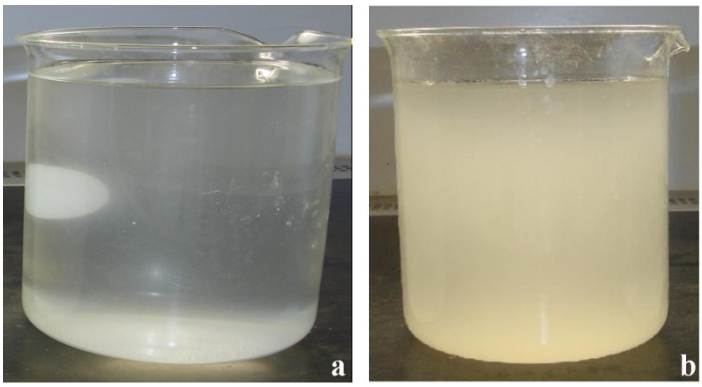

Figure 5.3 Turbidity of axenic hatching medium of *Artemia* separated from *Artemia* nauplii after 20 h of incubation (AHMA20) without *Bacillus* sp. LT12 (a) and with *Bacillus* sp. LT12 (b) grown into it for 24 h.

Despite a lower density of cell growth as compared to LB medium, using axenic hatching medium of *Artemia* as a culture medium for *Bacillus* sp. LT12 was shown to have a positive influence on the PHB content in the bacterial cells. During a 24 h culture period in axenic hatching medium of *Artemia* separated from the hatching *Artemia* after 16, 20 or 24 h incubation (AHMA16, 20 or 24) (Exp. 1), the PHB content in this bacteria increased over two times (19.5 ± 0.8%, 21.0 ± 0.9% and 19.4 ± 1.1% PHB, respectively) as compared with its culture in LB medium (8.7 ± 0.5% PHB). Most probably, *Bacillus* sp. LT12 has used the glycerol in the hatching medium for PHB production. The process of conversion of glycerol

to PHB was described by Jincy et al. (2013) and Deepthi et al. (2011), in which glycerol is converted into dihydroxyacetone by bacteria in a first step and through various biochemical reactions it is finally converted into acetyl-CoA, which serves as precursor to synthesize PHB through a number of reactions. In the co-culture of *Bacillus* sp. LT12 with hatching *Artemia* (Exp. 2), a significantly higher PHB content was obtained in this *Bacillus* when at least 0.17 g/L glycerol was added as supplemental carbon source. The higher addition of glycerol (0.51 g/L) did not substantially increase the PHB accumulation further. The cell growth can be considered similar with and without addition of glycerol to the medium. From this it is clear that the addition of glycerol has an effect on the PHB production but not on cell growth of the *Bacillus* strain. It can thus be stated that the addition of glycerol achieving a C/N ratio of about 20 is a promising strategy to boost PHB accumulation at similar cell yields. Lee (1996) reported that microorganisms accumulate PHB as an intracellular energy and carbon storage compound when external carbon is in excess and essential nutrients such as nitrogen become limiting. In the other case the bacterium grows biomass (Kadouri et al. 2005; Halet et al. 2007). In this case, LB medium (Exp. 1) is a less suitable source for PHB production in the *Bacillus* and the uptake of carbon is mainly channeled in the direction of cell growth. These results are in accordance with earlier studies that found that different carbon or nitrogen sources and especially the carbon to nitrogen ratio (C/N) in the bacterial culture medium affect PHB-accumulating bacteria (Yüksekdağ et al. 2004; Kaynar and Beyatli 2009; Supono et al. 2013).

Similarly as in previous studies (Defoirdt et al. 2007b; Nhan et al. 2010b), in both experiments of the current study, *Artemia* nauplii (Instar II) were used to deliver the amorphous PHB-containing *Bacillus* sp. LT12 to *M. rosenbergii* larvae, because *Artemia* nauplii are commonly used as live food for the larviculture of fish and shellfish (Sorgeloos and Léger 1992). In the first experiment, the PHB content of enriched *Artemia* showed a significant difference between the two treatments tested, 10^8 and 10^9 CFU/mL for 2 h, irrespective of the culture medium used. The concentration of *Bacillus* sp. LT12 used to enrich the *Artemia* thus affected the PHB content of the *Artemia* nauplii, resulting in a higher PHB content when an enrichment dose of 10^9 CFU/mL was used. At this enrichment dose, the PHB content in *Artemia* nauplii was 2.3 ± 0.6%, 4.1 ± 0.2%, 4.2 ± 0.3% and 3.7 ± 0.7% for LB, AHMA16, AHMA20 and AHMA24 respectively (Exp. 1). In experiment 2, co-culture of the *Bacillus* strain during *Artemia* hatching resulted in a PHB level in the *Artemia*

of 2.5 ± 0.9%, 5.0 ± 0.8% and 5.2 ± 0.9%, without additional glycerol addition, with 0.17 g/L and with 0.51 g/L additional glycerol respectively. These levels were significantly higher as compared to the control *Artemia* nauplii without co-culture of the *Bacillus*. The survival of *M. rosenbergii* larvae challenged with *V. harveyi* BB120 showed a significant positive correlation with the PHB content of the *Artemia* nauplii in the different treatments. A Pearson product moment correlation coefficient *(r)* of 0.9035 and 0.9624 ($P<0.01$) (Fig. 5.4) was found in experiment 1 (*Bacillus* sp. LT12 grown in AHMA) and in experiment 2 (*Bacillus* sp. LT12 co-cultured during *Artemia* incubation) respectively. These results are in agreement with the findings in Chapter 2.1 & 2.2 showing that the effect of amorphous PHB application through *Artemia* as a carrier on the survival of the predator depends on the combination of the enrichment dose and PHB content of the PHB-accumulating bacteria. In this respect the current study showed that increasing the enrichment dose of *Artemia* with *Bacillus* sp. LT12 to 10^9 CFU/mL as was tested in experiment 1 or adding supplemental glycerol to increase the PHB-content of the *Bacillus* delivered to the prawn larvae through *Artemia* as was tested in experiment 2, both had a positive effect on the survival of the prawn larvae. The antimicrobial properties of PHB, a chemical polymer, are activated when it is converted into the monomer (β-hydroxybutyrate). This short chain fatty acid (SCFA) was found by Defoirdt et al. (2007b) to inhibit the growth of *Vibrio campbellii* when it was challenged with *Artemia* nauplii in gnotobiotic condition. The degradation of PHB occurs in the gut as was previously documented by Defoirdt et al. (2009). For illustrating the bacteriostatic action of the PHB-accumulating *Bacillus* sp. LT12, TCBS counts were used for tentatively estimating the number of vibrios in the gut of the prawn larvae (López-Torres and Lizárraga-Partida 2001). In this study, in both experiments significantly lower TCBS counts were observed in all treatments where *Bacillus* sp. LT12 was applied as compared to the control challenge treatment without the application of this bacterium. It seems that the PHB-accumulating *Bacillus* strain can inhibit the growth of vibrios in larval gut of *M. rosenbergii* and that these decreases in general correspond to increased survival of the larvae. In addition, recently it was also reported that immunostimulation of the treated host is involved in the antimicrobial effect of PHB (Suguna et al. 2014, Baruah et al. 2015). Although the role of PHB contained in *Bacillus* sp. LT12 on the disease resistance of *Artemia* is undeniable in this study, *Bacillus* sp. LT12 is also known to have quorum sensing interfering activity by which it can protect *Artemia* from infection with bacterial pathogens

(Defoirdt et al. 2011). It is a beneficial feature that may contribute additionally to the value of *Bacillus* sp. LT12 as a disease protecting agent.

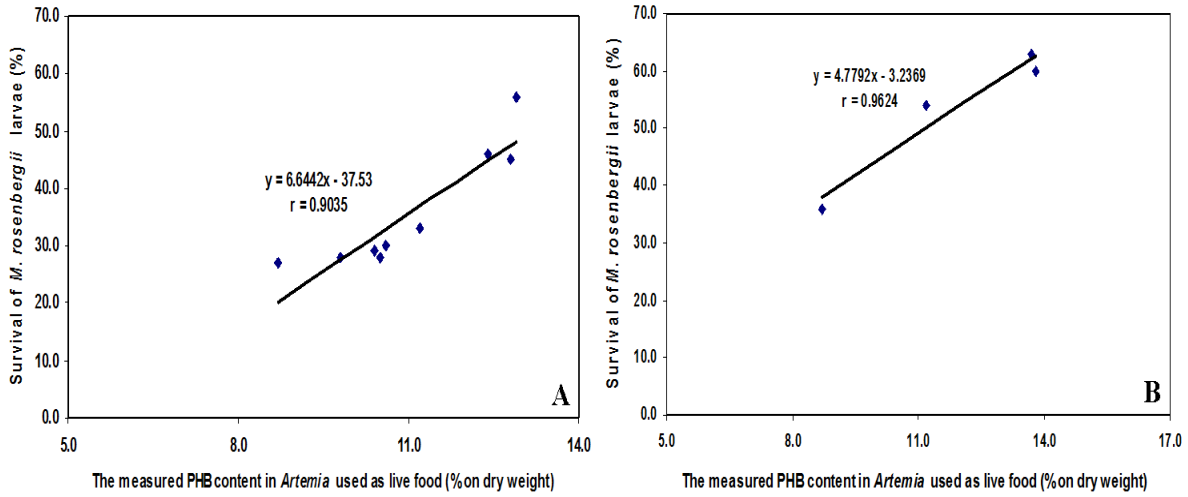

Figure 5.4 Correlation between the PHB content in *Artemia* nauplii: enriched with different concentrations of *Bacillus* sp. LT12 cultured in LB medium, AHMA16, AHMA20 and AHMA24 for 2 h (A); or co-cultured for 26 h with *Bacillus* sp. LT12 during incubation with or without addition of supplemental glycerol (B) and the survival of *M. rosenbergii* larvae fed these *Artemia* in a challenge test with *V. harveyi* BB120 during 8 days of culture.

Economic and ecological benefits are the most important considerations for application of biological disease control agent candidates. The high cost of commercial PHB products is a main obstacle for its application in aquaculture. In this respect, the use of waste water such as the hatching medium of *Artemia* to produce PHB is a new strategy to reduce its cost. Phuong et al. (2006) have shown that the cost for producing one million postlarvae in a *M. rosenbergii* hatchery in the Mekong Delta consists for 42% of operational costs, in which the *Artemia* cost is approximately 25% corresponding to 3 kg *Artemia* cysts. It can be calculated that the incubation of 1 kg of *Artemia* cysts at the standard density of 2 g cysts/L seawater requires 500 L seawater. Therefore, the amount of PHB that can be produced from one kg *Artemia* cysts is about 35 g (based on around 0.5 g/L CDW and 14% PHB in bacteria after 26 h co-culture with *Artemia*). Consequently, the use of the hatching medium of *Artemia* to culture *Bacillus* sp. LT12 not only increases the PHB content of this bacteria but also enhanes the survival and disease resistance of *M. rosenbergii* larvae through feeding enriched *Artemia* carrier LT12. Additionally, co-culture of *Bacillus* strains with hatching

Artemia may reduce the load of pathogenic bacteria in this live prey through exclusion and prevent their transfer to larval prawn.

In conclusion, our study has provided a new practical strategy for applying *Bacillus* sp. LT12 containing PHB through its culture in axenic hatching medium of *Artemia* and feeding the latter to *M.* larviculture through enrichment of *Artemia* nauplii. The application of PHB-accumulating bacteria as probiotics in aquaculture cultured in hatching water of *Artemia* may improve the economic efficiency and ecological sustainability of fish and shellfish hatcheries. Further tests are however still needed to validate this technique at the hatchery level.

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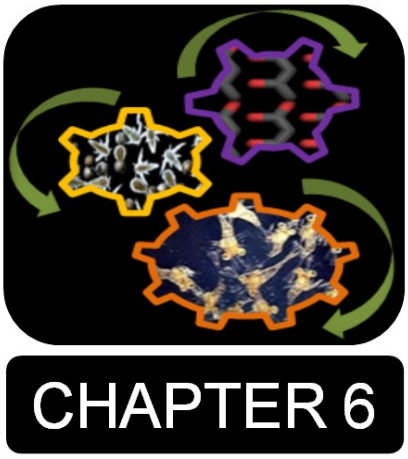

GENERAL DISCUSSION

CONCLUSIONS AND FURTHER PERSPECTIVES

6.1 General discussion

Poly-β-hydroxybutyrate (PHB) is known from recent studies to inhibit the growth of pathogenic bacteria, and to enhance the growth performance and also to stimulate the immune system of both larval and juvenile stages of some aquatic animals (Defoirdt et al. 2007b; Nhan et al. 2010a; De Schryver et al. 2010b; Suguna et al. 2014; Baruah et al. 2015). In almost all these studies, crystalline PHB particles (extracted from bacterial cells) have been used. Besides the undoubtedly beneficial effects of crystalline PHB particles, there are some weak points associated with this PHB form that impede its practical application in larviculture, the most important ones being the size of the particles, their bioavailability and the production cost. Therefore, in this PhD research it was first assessed if the use of amorphous PHB contained in whole cells of the bacterium *Alcaligenes eutrophus* either in lyophilized form or as freshly grown forms can induce similar or even better beneficial effects than crystalline PHB particles on *Artemia* nauplii and *M. rosenbergii* larvae. Secondly, the reuse of the hatching medium of *Artemia* was investigated as an alternative cost-efficient strategy to culture PHB accumulating bacteria.

In this chapter, the importance of the obtained results of these studies is highlighted and discussed by integrating them with literature information. Through the text, parts of Fig. 1.11 are highlighted to be able to relate the discussion part to specific research chapters and to illustrate how this section fits in the overall research scheme. Further research strategies for the practical application of the strategy in fish and shellfish hatcheries are also suggested.

6.1.1 The importance of the poly-β-hydroxybutyrate form for application at the larval stage

In this work, whole bacterial cells of *Alcaligenes eutrophus* (both lyophilized and living cells) were supplied to an actual aquaculture animal, namely *M. rosenbergii* larvae (Fig. 6.1). In Chapter 2 – section 1 it was found that the supplementation of amorphous PHB in lyophilized bacterial cells (100 mg/L & 1000 mg/L – 80% PHB on CDW) through *Artemia* nauplii as carrier, improved the performance and disease resistance of the prawn larvae in comparison to the use of crystalline PHB. The use of amorphous PHB in the form of freshly grown bacteria cultured in LB medium also significantly protected *Artemia* nauplii from *V. campbellii* infection, while *M. rosenbergii* larvae could be protected against *V. harveyi*

infection (Chapter 2 – section 2). Overall, it could thus be shown that the use of the amorphous form of PHB resulted in a higher treatment efficiency than the use of the crystalline form of PHB.

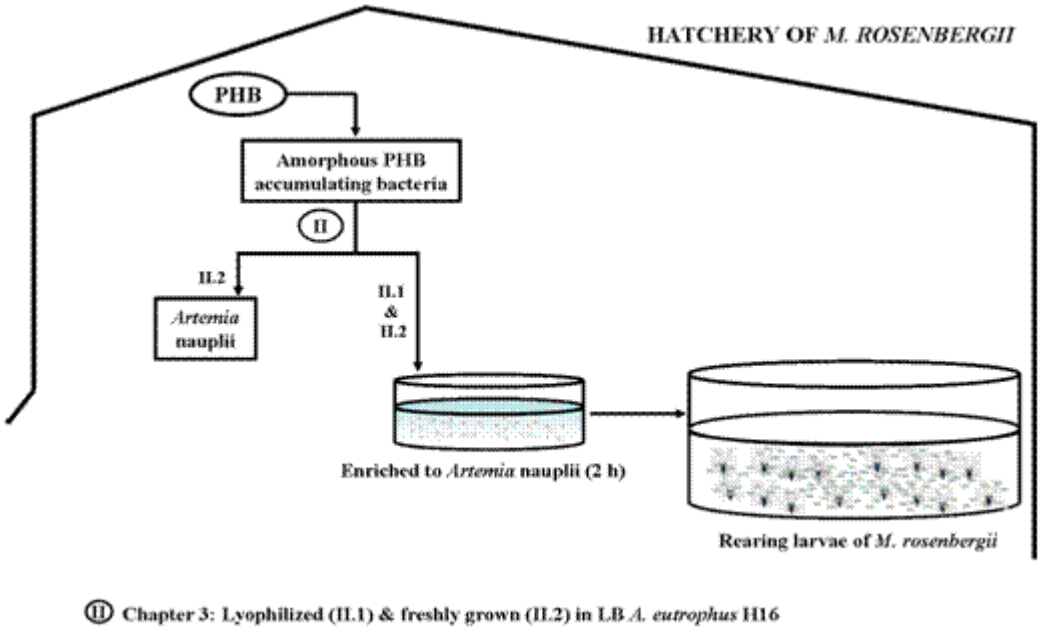

Figure 6.1 The scheme of the experiments performed for assessing the effect of lyophilized and freshly grown *A. eutrophus* on the performance of *Artemia* nauplii and *M. rosenbergii* (Chapter 2).

Increased efficiency

In previous studies, crystalline PHB particles have been supplied to aquatic animals such as *Artemia* nauplii (Defoirdt et al. 2007b), *M. rosenbergii* larvae (Nhan et al. 2010a), European sea bass juveniles (De Schryver et al. 2010b), Chinese mitten crab larvae (Sui et al. 2012) and Siberian sturgeon juveniles (Najdegerami et al. 2012) and have been shown to induce beneficial effects in terms of growth performance, survival and disease resistance. In our work, similar health and performance effects could be observed in Chapters 2, 4 & 5 when PHB in amorphous form was used, either as lyophilized or freshly grown *A. eutrophus* or as *Bacillus* sp. LT12 grown in *Artemia* hatching medium. The results showed that the concentration of amorphous PHB needed was lower than for crystalline PHB particles. The difference in the size between crystalline PHB and amorphous PHB could be the first reason to explain this result. In fact, the size of amorphous PHB inclusions in bacterial cells is typically 0.2 to 0.5 µm in diameter (Anderson and Dawes 1990), while the crystalline PHB

particles used in our study were much larger (approximately 30 μm on average according to the manufacturer). Assuming that the shape of amorphous PHB inclusions and crystalline PHB particles is a spherical, it means that the surface to volume ratio of amorphous PHB can be a factor 15 – 140 larger than of crystalline PHB. As a result the susceptibility of amorphous PHB to enzymatic and microbial degradation can be considerably increased as compared to crystalline PHB particles (Anderson and Dawes 1990). A second reason to mention is the different biophysical state of crystalline PHB as compared to amorphous forms. When in the inclusions (then called intracellular PHB), the PHB exists in amorphous state and is covered by a surface layer consisting of proteins and phospholipids. Barton (2004) stated that these proteins are assumed to be involved with the PHB metabolism, while more specifically according to Horowitz and Sanders (1994) they function as active PHB polymerase. The second component of the surface layer, the phospholipids, has a main role as chemically competent coating for the maintenance of the physical state of the amorphous PHB inclusions (Horowitz and Sanders 1994). Upon damage of the surface layer of the vacuoles resulting from physical or chemical strees, the polymer adopts an ordered helical structure leading to an (at least) partially crystalline polymer (called extracellular PHB – typical degree of crystallinity is 50 – 60%) (Jendrossek and Handrick 2002). The metabolisation efficiency of PHB by aquatic animals and/or the gut microbiota has been suggested to depend on its crystallinity (Defoirdt et al. 2009). These assumptions are supported by the finding of Yu et al. (2005) that the amorphous polymer decomposed 30 times faster than the crystalline PHB because of hydrolytic enzymes diffusing faster in the amorphous phase than in the crystalline phase. In support of this, Tokiwa et al. (2009) stated that the degree of crystallinity is a crucial factor affecting biodegradability as enzymes mainly attack the amorphous domains of PHB which are less resistant than crystalline regions. The amorphous regions of the molecules are loosely packed, and thus more susceptible to degradation. Biodegradation is considerably affected by pH, because under more alkaline conditions hydroxyl anions lower the energy barriers of ester bonds leakage and as such the required activation energy for biodegradation (Yu et al. 2005). Based on literature, it can be assumed that ingested PHB is substantially degraded in the animal gut via bacterial enzymatic hydrolysis and more specifically by two types of bacterial enzymes: intracellular PHB depolymerases or extracellular PHB depolymerases (Defoirdt et al. 2009). As intracellular PHB depolymerases are normally active within bacterial cells for metabolisation of accumulated PHB, their activity is likely to be less important than the

action of extracellular PHB depolymerases that bacteria excrete to degrade and utilize PHB present in the environment as a carbon source (Jendrossek and Handrick 2002). In the gut of the host, the enzymatic degradation process is hypothesized to result in the release of 3-HB from the PHB polymers which then plays a central role in the beneficial effect of PHB on the interaction between the microbiota and the host's gut and the action of PHB as a biocontrol agent (Defoirdt et al. 2006; 2007b; Chapter 2, 4 & 5). Beside microbial enzymes, enzymes originating from the host itself may also contribute to the metabolisation of PHB in the gut. Therefore, the effects of PHB on the digestive enzyme activity in aquatic animals have been a topic of study. A recent study on Siberian sturgeon (*Acipenser baerii*) larvae showed that PHB increased the lipid content in the body and decreased total saturated, monoenoic, n-3, n-6 and decoxahexanoeic acid (DHA). In addition, the activity of pepsin was significantly increased in the digestive extracts of fish being fed with PHB (Najdegerami et al. 2012). A study on the effect of PHB on Nile Tilapia (*Oreochromis niloticus*) found that the activity of lipase significantly increased in fingerlings fed with 25 g PHB/kg and 50 g PHB/kg diets (Situmorang et al. unpublished).

Nutritional contribution

Another factor contributing to the higher efficiency of whole cells containing amorphous PHB as compared to extracted crystalline PHB may not even be PHB related. Upon feeding of whole cells, other nutrients contained within the bacterial cells may also benefit the host animal. Brown et al. (1996) have reported that the nutrients contained in bacteria consist of 25 – 49% protein (a major constituent), 2.5 – 11.0% carbohydrate, 4.7 – 14.0% ash and 2.5 – 9.0% lipid (a minor component) on cell dry weight. A calculation of the extra nutrients that PHB containing bacterial biomass may bring to the *Artemia* nauplii used as live food is presented in Box 1. It illustrates that each gram of *Artemia* dry biomass which is co-cultured with *Bacillus* sp. LT12 also carries an increased amount of nutrients originating from bacteria corresponding to 14.8% protein; 6.9% lipid; 10.1% carbohydrate; and 19.6% ash from the bacteria. The calculation has been performed for these main nutritional components but it should be taken into consideration that other fractions such as essential amino acids may also be increased in the enriched *Artemia* nauplii. The levels of essential amino acids in *Artemia* are generally not a major problem in view of its nutritional value, but sulphur amino acids, like methionine, are the first to be limiting (Merchie 1996). Bacteria such *Bacillus thuringiensis* are known to be able to overproduce methionine (Willke 2014) and as such

could be of added value to the diet of aquatic animals Apart from the PHB, the extra nutrients may thus also contribute to the metabolic activity of the host. Evidently, this contribution will mainly depend on the bacterial strain used.

Box 1: **Calculation of the increase in main nutritional components of *Artemia* biomass by enrichment with PHB accumulating bacteria (*Bacillus* sp. LT12)**

1) PHB increase in *Artemia* biomass when co-cultured with *Bacillus* sp. LT12 (Chapter 5):
5% on dry biomass ≈ 50 mg PHB/g dry *Artemia* biomass
2) PHB content *of Bacillus* sp. LT12 co-cultured with *Artemia* (Chapter 5):
24% on dry biomass
From (1) and (2) the amount of bacterial biomass enriched in *Artemia* can be estimated:
208 mg dry biomass of bacteria / g dry *Artemia* biomass

Average composition of bacteria (Brown et al. 1996)*:	Protein: 37%	Lipid: 6%	Carbohydrate: 7%	Ash: 9%
Nutrients in dry bacteria per g *Artemia* nauplii biomass (3):	Protein: 77 (mg/g)	Lipid: 13 (mg/g)	Carbohydrate: 15 (mg/g)	Ash: 19 (mg/g)
Average composition of *Artemia* nauplii biomass (Léger et al. 1987) (4):	Protein: 52.2% (522 mg/g)	Lipid: 18.9% (189 mg/g)	Carbohydrate: 14.8% (148 mg/g)	Ash: 9.7% (97 mg/g)
Nutritional increase (%) in *Artemia* biomass enriched with PHB accumulating bacteria ((3)/(4) x100)	Protein: 14.8%	Lipid: 6.9%	Carbohydrate: 10.1%	Ash: 19.6%

* Other bacterial components such as (essential) amino acids, fatty acids, nucleic acids, etc. (making up about 40% of the cell dry weight) are not mentioned in this box, but may also contribute to a nutritional increase in enriched *Artemia* biomass

Probiotic effect

In case the PHB containing bacterial cells are freshly grown and thus alive, depending on the strain, they may bring additional benefits for the animals in their fight against pathogens as probiotics (Laranja et al. 2014). The beneficial effects of probiotics for the host result from interactions between the probiotic and the pathogen such as the competition for nutrient substrate and adhesion sites or by production of inhibitory substances acting bactericidal/bacteriostatic towards the pathogen. Also the stimulation of immunity (by increasing macrophage activity, producing of systematic antibodies and increasing local

antibodies) is an example of how probiotics may help the host (Verschuere et al. 2000b; Ali Farzanfar 2006). In case of *Bacillus* sp. LT12, this bacterium was originally isolated for its potential quorum sensing interfering effect which is another example of how probiotics may protect host health (Defoirdt et al. 2011).

Production technical aspects

A major difference in the production of PHB containing bacteria as compared to crystalline PHB is the avoidance of a PHB extraction step. According to Suriyamongkol et al. (2007) there are two common methods to extract PHB from bacteria. The conventional one uses large amounts of hazardous solvents (e.g. chloroform, methanol, etc.), which is repeated several times within the same PHB extraction process (Kessler et al. 2001). These compounds do not only pose a potential risk for the aquatic animals (residues in the PHB) but can also be problematic when they would end up in the environment. The second method is designed to avoid the use of organic solvents. A cocktail of enzymes, including proteases, nucleases and lysozymes is used to treat the bacterial cells and in a later phase proteins, nucleic acids, and cell walls are removed by the use of detergents to ensure the PHB is left intact. The cost for this extraction method is, however, still an impediment. As mentioned, the problems of the extraction step can be avoided when amorphous PHB is used.

No matter if PHB would be applied in bacterial form or in extracted form, the production cost of PHB remains an issue. The cost of raw materials is highly significant in the total production cost of PHB. For example, the cost of C-source for producing 1 kg of PHA in 2010 was € 0.87 (sucrose), € 1.07 (glucose), € 0.63 (ethanol), € 0.58 (methanol), € 0.94 (cassava starch), € 0.24 (cane molasses), € 1.22 (palm oil) and € 1.31 (soya oil) while the commercial price of PHA was in range of € 1.5 – 5.0/kg (Chanprateep 2010). For the production of PHB out of the two bacterial species *Bacillus megaterium* and *Cupriavidus necator* using the raw material crude glycerol (after purification at the price of € 0.112/kg), the production cost was about € 2.9 – 3.1/kg (*B. megaterium*) and € 1.4 – 1.8/kg (*C. necator*) (Posada et al. 2011). Several solutions have been suggested for solving the cost problem such as the improvement of the culture process, extraction and purification technology as well as the development of superior bacterial strains (Chaitanya et al. 2014). However, the reduction of the PHB production cost by the use of cheaper carbon sources (Kim 2000), is the most straightforward and feasible strategy. Consequently, the re-use of the hatching

medium of *Artemia* which is currently considered a waste carbon source in the operation of crustacean hatcheries was suggested in this study as an alternative carbon source for the culture of amorphous PHB accumulating bacteria (see further).

In view of all the above, it seems logic to pursue the use of PHB containing bacteria rather than the use of crystalline PHB. There are, however, some issues that need to be considered for the application of the PHB-bacteria approach in aquaculture. The most important issue includes *the selection of proper bacterial strains*. It is an important step because not all bacterial strains can be used in aquaculture even though they can produce high concentrations of PHB in the cells. Based on the properties for selecting probiotics in aquaculture as described by Kesarcodi-Watson et al. (2008) they need to be (i) harmless to the host; (ii) accepted by the host, e.g. through ingestion; (iii) reach the location where the effect is required to take place; and (iv) preferably not contain antibiotic bacterial resistance genes. In addition, a main problem for introducing PHB accumulating bacteria in the aquaculture market are safety regulations such as the European Union (EU) safety regulations on probiotic use for animal nutrition (no. 1831/2003) (Anadón et al. 2006) or the United States Code of Federal Regulations (Balcázar et al. 2006). The introduction of new microorganisms for commercial applications in animal culture in Europe requires an assessment as microbial feed additives. The European Food Safety Authority (EFSA) has worked out specific criteria that should be met and specific guidelines on how the approval for the use of microorganisms as feed additives for animals should be achieved (Anadón et al. 2006) (see EFSA documentation in appendix A). When approved, such microorganisms then receive the Qualified Presumption of Safety (QPS) status that was introduced by EFSA (EFSA 2007). As illustrated by Balcázar et al. (2006) a list of microorganisms authorized as probiotics in feeding stuffs under Council Directive 70/524/EEC exists. However, some probiotics are commercialized on the market that has been notified, but that do not appear in the last authorized list of feed additives published by the Commission. As the acceptance of a bacterial strain as marketable probiotic is not straightforward, the traditional strategy is to isolate this bacterium from within an aquaculture system and even more preferred from within an intestine of a healthy aquaculture animal (Rollo et al. 2006; Ige 2013). As this strategy is assumed to increase the chances of receiving EFSA approval, PHB accumulating strains from such environments will also have a higher change for acceptance as a feed additive. The selection of strains belonging to the *B. cereus* taxonomic group for direct use

in animal production is considered inadvisable. If, however, they are proposed for use then the full genome (including chromosome and plasmids) should be sequenced and bioinformatic analysis should be performed made to search for genes coding for enterotoxins and cerelide synthase (Appendix A). If there is evidence of homology, the non-functionality of the genes (e.g., mutation, deletion) should be demonstrated (EFSA 2014).

6.1.2 Reuse of Artemia *hatching medium to culture PHB-accumulating bacteria*

The change in carbonaceous compounds (trehalose, glycerol, glycogen and total organic carbon) and nitrogenous compounds (total nitrogen) in the axenic hatching medium of decapsulated *Artemia franciscana* cysts was determined throughout the hatching process. It was found that the average C/N ratio at a salinity of 12 g/L was around 10 throughout the hatching period (Chapter 3 – Fig. 6.2). The axenic hatching medium of *Artemia* was then harvested and used to culture *Bacillus* sp. LT12, which was selected from a variety of *Bacillus* sp. for its ability to serve as a sole food for *Artemia* nauplii and to accumulate PHB in the cell (up to 22% on cell dry weight). Feeding freshly grown *Bacillus* sp. LT12 resulted in an increase in growth and survival of the *Artemia* nauplii (Chapter 4 – Fig. 6.3). The supplementation of *Bacillus* sp. LT12 cultured in the hatching medium of the *Artemia* also significantly protected the nauplii from challenge with *Vibrio campbellii*.

To further explore the strategy of culturing PHB accumulating *Bacillus* sp. LT12 in the hatching medium of *Artemia*, it was attempted to perform a co-culture of the bacterium during hatching of the *Artemia* cysts. Extra glycerol was added to optimize the C/N ratio for bacterial growth and PHB accumulation. This strategy resulted in the efficient production of biomass with a PHB content up to about 24% on cell dry weight. Combining the production of *Artemia* with the culture of *Bacillus* sp. LT12 in the same medium implies that when the nauplii enter the instar II stage they become immediately enriched with the freshly grown bacterium. Feeding of these enriched nauplii to *M. rosenbergii* larvae resulted in a significant protection of the larvae against pathogenic infection by *V. harveyi* (Chapter 5 – Fig. 6.4).

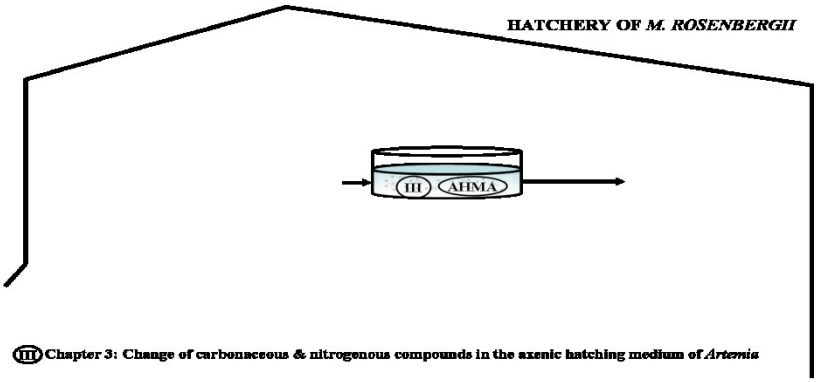

Figure 6.2. Schematic representation of the experiments performed to investigated the change of carbonaceous and nitrogenous compounds in the hatching medium of decapsulated *Artemia* cysts.

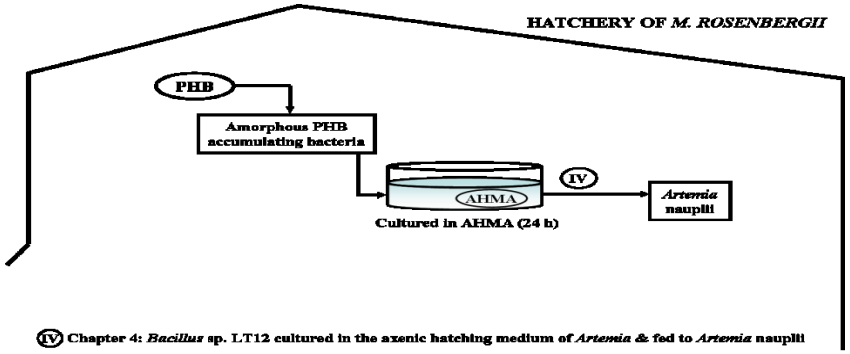

Figure 6.3. Schematic representation of the experiments performed to evaluate the effect of *Bacillus* sp. LT12 cultured in the axenic hatching medium of *Artemia* as feed for *Artemia* nauplii.

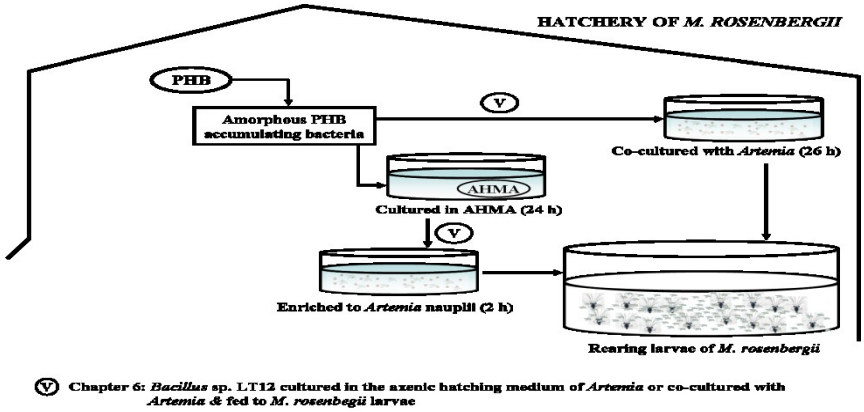

Figure 6.4. Schematic representation of the experiments performed to evaluate the effect of *Bacillus* sp. LT12 cultured in the axenic hatching medium of *Artemia* or co-cultured with *Artemia* on *M. rosenbergii* larvae.

The findings of Clegg (1964) suggested that trehalose as the respiratory substrate being present in dormant cysts is converted into glycogen and glycerol during embryonic development, and that the extent of these conversions is controlled by the external osmotic pressure. This author also described that when the embryo emerges from the cyst, glycerol is released into the hatching medium. Similarly, the results from our work (Chapter 3) showed that there is a substantial amount of organic carbon released from the cysts into the hatching medium during incubation. The main carbonaceous compound in the hatching medium was glycerol, followed by glycogen and a very small amount of trehalose. These compounds have so far been considered as waste resulting from the hatching of *Artemia* cysts for the production of nauplii as a live food for the larval stages of fish and crustaceans (Sorgeloos et al. 1993). Considering that during normal hatchery operation, hatching of *Artemia* is performed on a daily basis at large scale, this represents a considerable amount of organic substrate that is being wasted and which imposes a substantial burden on the environment. Taking shrimp hatcheries in Vietnam as an example, the amount of *Artemia* cysts required for producing 1 million postlarvae is approximately 5 – 10 kg for *P. monodon* shrimp, 1 – 5 kg for *L. vannamei* shrimp and about 3 kg for *M. rosenbergii* prawn (Gervais and Zeigler 2013; Phuong et al. 2006). Therefore, the estimated amount of total organic carbon that is released during the hatching of *Artemia* cysts for the rearing of larvae in shrimp hatcheries is about 275 – 550 g (*P. monodon*), 55 – 275 g (*L. vannamei*) and 165 g (*M. rosenbergii*) per one million postlarvae (assuming that the total organic carbon release is around 55 mg/g *Artemia* cysts – Chapter 3).

In this PhD research, it was investigated if the hatching medium of *Artemia* cysts containing this organic substrate can be reused as a nutrient medium for growing PHB accumulating bacteria. In our work, this approach was attempted using *Bacillus* sp. LT12 which was isolated from the gut of healthy whiteleg shrimp *(L. vannamei)* (Tinh et al. 2007; Defoirdt et al. 2011). This *Bacillus* strain is very unlikely to be suitable for use in aquaculture because of its close phylogenetic relationship with bacilli from the *Bacillus cereus* complex based on the 16S rRNA gene (and thus unlikely to be accepted by EFSA regulations). Further genetic and metabolic characterization is therefore needed for this bacterium. The safety of *Bacillus* will be assessed through the guidance of EFSA regulations (Appendix A) based on the toxigenic potential of *Bacillus* species for use in animal nutrition (EFSA 2014). Nevertheless, this bacterium was shown to be a good model to illustrate the strategy of using

Artemia hatching medium to culture PHB accumulating bacteria which can then later be applied for other newly isolated bacteria that do fulfill EFSA requirements. The results showed that *Bacillus* sp. LT12 was able to grow well in the *Artemia* hatching medium and moreover showed a higher production of PHB as compared to its culture in LB medium (more than a factor 2 increase) (Chapter 4).

The synthesis of PHB in bacteria depends on several factors, of which one is the source of carbon and nitrogen. In Chapter 3, glycerol was determined as the main carbon component present in the hatching medium of *Artemia*. The relation between glycerol and PHB synthesis has been reported by Deepthi et al. (2011) and Jincy et al. (2013) stated that glycerol is the most energetically favorable substrate for the formation of acetyl-CoA. The latter chemical compound serves as the main precursor for the synthesis of PHB within the bacterial cell. Furthermore, the C/N ratio of the bacterial culture medium is another factor determining PHB synthesis by bacteria. Patnaik (2005) suggested that the optimum C/N ratio for PHB accumulation is about 20. At C/N ratios considerably below this value, good growth of the bacteria may still be supported. The PHB accumulation will, however, decrease due to a shortage in the excess of carbon source. As a result, the overall effeciency of PHB production (depending on growth and PHB concentration in the biomass) will decrease as well. The C/N ratio in the hatching medium of *Artemia* (at 12 ppt) during the incubation process was around 10 (Chapter 3) which is an aspect that allows to explain the difference in PHB accumulation as compared to when *Bacillus* sp. LT12 is cultured in LB medium. Although no information about the C/N ratio of LB is available, LB medium is renowned as a specially formulated nutrient-rich microbial culture medium with no limiting factors (Berezina 2013) composed of tryptone, an enzymatic digest of protein that provides amino acids for the bacteria, yeast extract and sodium chloride (MacWilliams and Liao 2006). It thus seems rather optimized in composition for bacterial growth with a balanced availability of carbon and nitrogen. In this respect it seems logic that a lower PHB accumulation is achieved than in media with unbalanced nutritional composition. Specifically interesting for the case of *Bacillus* sp. LT12 is that it can grow in a wide range of salinities of the *Artemia* hatching medium, making the strategy advantageous to apply for aquatic animals in both brackish and sea water. Overall, the strategy of reusing the hatching medium of *Artemia* as a microbial growth source creates perspectives from an economic and an environmental point of view.

6.1.3 The economics and sustainability of reusing **Artemia** *hatching medium for the production of PHB accumulating bacteria*

The selection of bacteria with a high capacity for PHB accumulation is an important step in amorphous PHB production. Examples of wild type bacteria strains that have been found to accumulate high proportions of PHB in their biomass include *Alcaligenes eutrophus* (> 80% PHB on cell dry weight), *Alcaligenes latus* (> 75% PHB on cell dry weight), *Aeromonas hydrophila* (< 50% PHB on cell dry weight) and *Pseudomonas putida* (> 60% PHB on cell dry weight) (Chen 2009). However, the main goal of producing PHB from these bacterial species is to provide a material for the packaging and medical industry (Chen 2009; Brigham and Sinskey 2012), and not for the aquaculture industry. *Bacillus* spp. have also long been found to produce PHB as an intracellular reserve for carbon and energy (Williamson and Wilkinson 1958; Macrae and Wilkinson 1958). According to Singh et al. (2009), the level of PHB accumulated in bacilli can be increased from 11% up to as high as 70% on cell dry weight if cultured under optimized condition. In the genus *Bacillus*, the synthesized PHB mainly serves as an energy source to be able to enter into the sporulation process (Valappil et al. 2007). During the sporulation process the PHB that is maximally accumulated during or shortly after the cessation of logarithmic growth, provides the fuel that the cells need to produce spores (Kominek and Halvorson 1965; Singh et al. 2009).

Besides finding a suitable strain with high PHB accumulating capacity, another main obstacle to the application of PHB in aquaculture is the production cost. There are several options to decrease the high production cost of PHB such as improving culture process, separation processes and using a cheap carbon source. Especially the latter can aid to decrease the high production cost of the PHB (Kim 2000). Attempts in this sense include the use of molasses media that were used to cultivate *Bacillus* sp. JMa5 under fed-batch conditions to 70.0 g/L CDW after 30 h of culture and 25 – 35% PHB (on cell dry weight) (Wu et al. 2001). Crude glycerol generated as a by product from biodiesel production was used to culture *Bacillus firmus* NII 0830 to a maximum of 3.0 g/L CDW and 53% PHB (on cell dry weight) (Deepthi et al. 2011) while *Bacillus subtilis* NG05 was cultured in sugar industry waste water to 10 g/L CDW and 50% PHB (on cell dry weight) (Singh et al. 2013a). In aquaculture, *Artemia* cysts are one of the most commonly used materials for diet preparation of larval fish and shrimp. These first larval stages of larvae benefit highly in

terms of health and growth upon the use of *Artemia* nauplii in the diet (Litvinenko et al. 2014). In a standard *M. rosenbergii* hatchery in the Mekong Delta, the expenditure for *Artemia* cysts is quite high as compared to total operating costs and was estimated to more than 25% of total cost (Phuong et al. 2006). Normally, at the end of the *Artemia* hatching process, *Artemia* nauplii are separated from the hatching medium that includes the empty cyst shells, bacteria and hatching metabolites (Sorgeloos et al. 1993). Then this waste medium is directly released into the environment without treatment in most current crustacean hatcheries. In the concept of this PhD research this waste stream would be considered as an alternative source of carbon for the production of PHB-accumulating bacteria. The economic value of reusing the hatching medium of *Artemia* simulated for a standard *M. rosenbergii* hatchery in the Mekong Delta as described by Phuong et al. (2006) is presented in Box 2. In monetary value, the benefit is relatively small and estimated at only 0.3 – 0.6 % of the cost of *Artemia* cysts. However, the added values of the strategy (increase in growth and disease resistance of the larvae, lowered environmental impact, lowered *Vibrio* load of the *Artemia* nauplii, avoiding treatment cost of the *Artemia* hatching medium) need to be taken into consideration as well.

Box 2: Calculation of the economic value of reusing the hatching medium of *Artemia* nauplii for the production of PHB accumulating *Bacillus* sp. LT12 in a standard *M. rosenbergii* hatchery

According to Phuong et al. (2006): 3 kg *Artemia* cysts are needed to produce 1 million postlarvae (PL). At standard conditions, 2 g *Artemia* cysts are incubated per liter seawater, so the total volume for incubation of 3 kg *Artemia* cysts is 1.5 m^3 ≈ 1500 L. Assuming a hatching efficiency of 75% and a weight of 280.000 dry cysts/g, this represents 630.000.000 nauplii that are fed to the postlarvae.

Calculation 1: the value of PHB produced

1. In the hatching medium of *Artemia*, enough nutrients are released for the culture of 0.53 g/L bacterial CDW with a PHB content of 14% (see results Chapter 5):

   in 1500 L this makes (0.53 g/L * 1500 L) = 795 g CDW (at 14% PHB) ≈ 111.3 g PHB

   At a market price of € 5/kg PHB (Biotechnoly Co., Germany in 2010), this represents a value of € 0.6 for the PHB produced out of 3 kg cysts. At a cost of € 75/kg *Artemia* cysts, this is 0.3% on the feed cost for 1 million PL.

2. Upon addition of 0.17 g/L glycerol to the hatching medium, 0.6 g CDW/L can produced with a PHB content of 22% (see results Chapter 5):

   - Following a similar calculation as above, this represents a PHB production of 198 g PHB out of 3 kg cysts, or a value of € 1.

   - The cost for glycerol by-product is € 0.3/kg (Yang et al. 2012), so the total glycerol cost per 3 kg cysts is (0.17 g/L * 1500 L * 0.3/1000) = € 0.08.

   Overall, the added value of PHB produced out of 3 kg cysts using the glycerol strategy is thus (€ 1 - € 0.08) = € 0.92, or 0.4% on the feed cost for 1 million PL.

Calculation 2: the value of avoidable purchase of PHB

The effects on the *M. rosenbergii* larvae achieved with *Bacillus* sp. LT12 cultured in the hatching medium of *Artemia* cysts (see results Chapter 5) are comparable to those for which 100 mg/L of externally produced PHB accumulating bacteria are needed to enrich *Artemia* nauplii (see results Chapter 2.1). To enrich the 630 million nauplii that are fed to the postlarvae at an enrichment density of 100 – 300 nauplii/mL, the volume of enrichment water is about 3150 L.

With a need for 100 mg/L PHB bacteria with 80% PHB (or 80 mg PHB/L), and for a total volume of 3150 L enrichment medium:

total need for 252 g PHB to enrich the nauplii.

At a market price of € 5/kg PHB (for PHB in crystalline form), this represents € 1.26 of PHB accumulating bacteria that does not need to be purchased. At a cost of € 75/kg *Artemia* cysts, this is 0.6% on the feed cost for 1 million PL.

Summarizing, rather than seeing it only as waste, the released carbon from *Artemia* cysts can bring an economic benefit through decreasing the cost of amorphous PHB procurement. Furthermore, ecological benefits are achieved by avoiding pollution caused by the hatching medium of *Artemia* which contains organic carbon and nitrogen.

6.1.4 The proposed model for application of amorphous poly-β-hydroxybutyrate on crustacean larviculture in the future

In crustacean hatcheries, the preparation of live food (*Artemia* nauplii) for larvae is a daily practice. The proposed use of PHB has so far almost exclusively focused on the use of crystalline PHB. In this PhD research, it was shown that PHB containing bacteria are a good alternative. Based on current knowledge and practices, it seems evident to culture such PHB accumulated bacteria in culture units from the shrimp production unit. The farmers would then need to purchase this PHB at an as yet relatively high cost. The results of our research have demonstrated, however, that it is possible to integrate the production of live food with the production of PHB biomass within the crustacean hatchery, including even an automatic enrichment of the live food with this biomass (Fig. 6.5). The integration of amorphous PHB accumulating bacteria culture with *Artemia* nauplii production brings some advantages such as saving on labour, equipment, time and also cost. In addition to this, the applied strategy not only benefits the cultured animals in terms of survival, growth and disease resistance but also controls the number of potential pathogenic bacteria loaded on the *Artemia* nauplii and thus reduces their introduction in the larval rearing water. This application has focused, within the frame of this PhD research, on the production of PHB accumulating bacteria. It could also be applied, however, for other types of beneficial bacteria such as probiotic bacteria or N-acyl homoserine lactone (AHL) degrading bacteria.

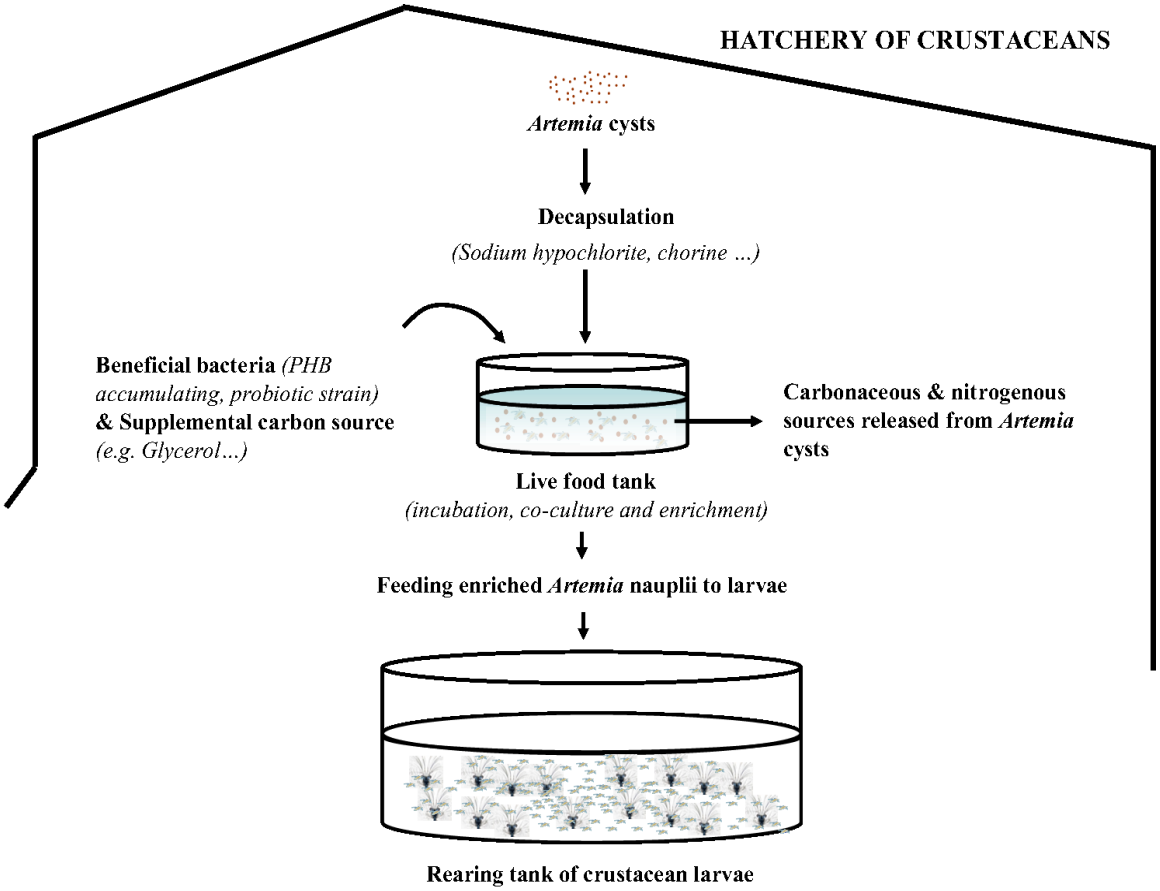

Figure 6.5 A schematic representation of the application of amorphous PHB accumulating bacteria in the hatchery of crustaceans

6.2 General conclusions

Figure 6.6 provides a schematic overview of the research performed within the frame of this PhD research and its most important conclusions. In summary, these are:

- The amorphous PHB strategy resulted in a significantly enhanced survival, development and/or resistance to pathogenic bacteria of *Artemia* nauplii and *M. rosenbergii* larvae. Additionally, this strategy also inhibited the growth of vibrios in the host gut.
- Lower amounts of amorphous PHB accumulating bacteria were needed as compared to crystalline PHB to enrich *Artemia* nauplii to obtain similar beneficial effects on *M. rosenbergii* larvae.
- During *Artemia* cyst incubation, a variety of carbon and nitrogen compounds is released from the *Artemia* cysts. The carbon compounds include glycerol, glycogen and trehalose, and the resulting C/N ratio in the hatching medium is on average 10 at a salinity of 12.
- The hatching medium of *Artemia* can be reused to culture *Bacillus* sp. LT12 with the following beneficial effects:
    - To increase the amorphous PHB content of the bacteria.
    - To use the cultured *Bacillus* sp. LT12 to protect *Artemia* nauplii and *M. rosenbergii* larvae against pathogenic *V. campbellii* LMG21363 or *V. harveyi* BB120, respectively.
    - To lead to a higher economic efficiency in hatcheries by increasing the quantity and quality of seed, decreasing the amorphous PHB production costs and also the price of seed. Furthermore, the hatching medium of *Artemia* could be converted from a waste to a useful substrate and as such increase the ecological sustainability of the aquaculture activity.

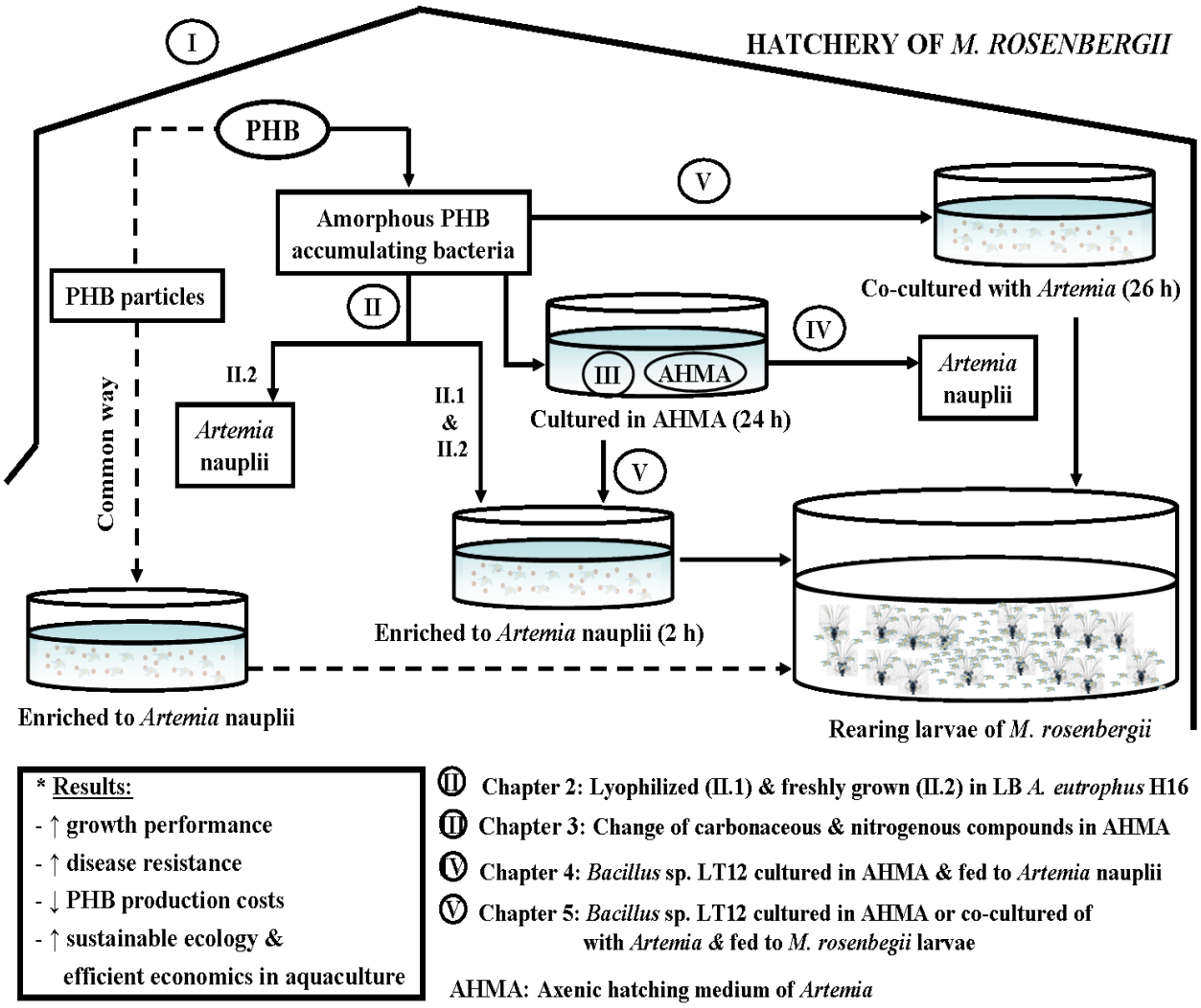

Figure 6.6 Schematic overview of the research performed within this PhD study and the most important conclusions.

6.3 Further perspectives

1. To isolate new beneficial bacteria from healthy aquatic animals for further application in fish and shrimp hatchery practices. These target bacteria need to fulfill the requirements of safety and have no negative impact on the environment and the health of animals and humans.

2. To look for new bacteria with more than one beneficial feature (e.g. PHB accumulation, AHL degradation, etc.) or a combination of more than one bacterium with a beneficial effect to obtain a synergistic effect on production efficiency of the fish and shrimp hatcheries in the future.

3. The model application of amorphous PHB accumulating bacteria production in hatching medium of *Artemia* for *M. rosenbergii* hatcheries needs to be validated under full-scale hatchery conditions. The parameters that need to be assessed under normal hatching conditions include growth, initial bacterial density, PHB yield, etc., of the target bacteria as well as culture performance of the cultured animals.

4. The potential and effect of adding the *Artemia* hatching medium (i.e. the water phase) following co-culture *of Artemia* with PHB accumulating bacteria to the culture water of the animals should be investigated as well. Indeed, this hatching medium still contains a high concentration of the PHB accumulated bacteria and upon addition in the culture water they may still benefit (directly or indirectly) the cultured animals. The effects in terms of hygiene, culture performance, etc. are aspects that need to be considered.

APPENDIX A

SCIENTIFIC OPINION

Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition[1†]

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)[2,3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

Bacillus species are used in animal production directly as microbial feed additives or as the source of other feed additives, notably enzymes. The principal safety concern for consumers and, to a lesser extent livestock, associated with *Bacillus* is a capacity for toxin production. However, the capacity for toxin production and the nature of the toxins produced is unevenly distributed over the genus, occurring frequently in some species and more rarely in others. In principle, the selection of strains belonging to the *B. cereus* taxonomic group for direct use in animal production is considered inadvisable. If, however, they are proposed then the full genome should be sequenced and a bioinformatic analysis made to search for genes coding for enterotoxins and cereulide synthase. If there is evidence of homology, the non-functionality of the genes (e.g. mutation, deletion) must be demonstrated. For other species, concerns appear to be associated to the production of surfactin like-lipopeptides, although the relation between the presence of these compounds and/or other toxic factors and the risk of illness in human has not yet been established. In the absence of animal models shown to be able to distinguish hazardous from non hazardous strains, the FEEDAP Panel relies on the use of *in vitro* cell-based methods to detect evidence of a cytotoxic effect. Such tests should be made with culture supernatants since the concentration of cells obtained in a broth culture would always exceed that found in animal food products. If the strain proves to be cytotoxic it is not recommended for use.

KEY WORDS

Bacillus species, enterotoxin production, emetic toxin, cereulide, surfactin-like lipopeptides

[1] On request from EFSA, Question No EFSA-Q-2013-00303, adopted on 8 April 2014.

[†] This guidance document replaces the previous EFSA Technical Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition, adopted in November 2011 (EFSA-Q-2009-00973). The requirements for the assessment of species belonging to the *Bacillus cereus* group are unchanged.

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3 Guidance on Bacillus safety, including Andrew Chesson, Pier Sandro Cocconcelli, Per Einar Granum and Christophe Nguyen-Thé, for the preparatory work on this scientific opinion.

SUMMARY

The European Food Safety Authority (EFSA) asked the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) to update the FEEDAP Panel Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition published in 2011.

Bacillus species are used in animal production directly as microbial feed additives or as the source of other feed additives, notably enzymes. The principal safety concern for consumers and, to a lesser extent livestock, associated with *Bacillus* is a capacity for toxin production. However, the capacity for toxin production and the nature of the toxins produced is unevenly distributed over the genus, occurring frequently in some species and more rarely in others.

In principle, the selection of strains belonging to the *B. cereus* taxonomic group for direct use in animal production is considered inadvisable. If, however, they are proposed then the full genome should be sequenced and a bioinformatic analysis made to search for genes coding for enterotoxins and cereulide synthase. If there is evidence of homology, the non-functionality of the genes (e.g. mutation, deletion) must be demonstrated.

For other species, concerns appear to be associated to the production of surfactin like-lipopeptides, although the relation between the presence of these compounds and/or other toxic factors and the risk of illness in human has not yet been established. In the absence of

animal models shown to be able to distinguish hazardous from non hazardous strains, the FEEDAP Panel relies on the use of *in vitro* cell-based methods to detect evidence of a cytotoxic effect. Such tests should be made with culture supernatants since the concentration of cells obtained in a broth culture would always exceed that found in animal food products. If the strain proves to be cytotoxic it is not recommended for use.

BACKGROUND AS PROVIDED BY EFSA

Regulation (EC) No 1831/20031 establishes the rules governing the Community authorisation of additives for use in animal nutrition. Moreover, Article 7(6) of this Regulation provides for the European Food Safety Authority (EFSA) to publish detailed guidance to assist applicants in the preparation and presentations of applications.

EFSA has the responsibility to assess the safety of feed additives before an authorisation is granted. A considerable amount of feed additives are composed by microorganisms. As a tool to simplify and harmonise within EFSA the assessment of microorganisms used in food and feed, the Scientific Committee published in 2007 one opinion on the introduction of a Qualified Presumption of Safety (QPS) approach for the assessment of selected microorganisms.

The list of microorganisms included in such opinion and considered to qualify for the QPS approach to safety assessment is updated regularly by the Biological Hazards (BIOHAZ) Panel. The last update is from 2012. The QPS approach is regularly used by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) in the assessment of microbial products subject to a pre-authorisation assessment.

Bacillus species are widely used as feed additives, and several of them are considered to qualify for the QPS approach to safety assessment, provided that the qualification of the absence of food poisoning toxins, surfactant activity or enterotoxic activity is met. In 2000, the Scientific Committee for Animal Nutrition (SCAN) adopted an opinion on the safety of use of *Bacillus* species in animal nutrition. This opinion was revised in 2011 by the FEEDAP Panel in the form of the Technical Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition (EFSA FEEDAP Panel, 2011), and updated according to the most recent scientific and technical developments. The aim of this

document, which complements the QPS opinion, is to provide applicants with proportionate and up-to-date guidance on how to conduct the safety assessment of *Bacillus*-based products.

This Guidance makes a clear difference between the *Bacillus cereus* group (including known human enteropathogens) and other *Bacillus* species.

Science evolves fast and since the Guidance document was issued, new information on the toxicity and prevalence of these toxins has become available. Therefore, the FEEDAP Panel in view of this and of the experience gained so far from the assessment of the toxigenic potential of products based on *Bacillus* species (other than *B. cereus*) is intended to produce an update of the Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition. This output is aimed at highlighting the uncertainties and making proposals to address them in the context of the assessment of the dossiers of non-*Bacillus cereus* based products.

TERMS OF REFERENCE AS PROVIDED BY EFSA

The FEEDAP Panel is requested to update the Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition.

Introduction

A number of strains of *Bacillus* species are used in animal production either directly as microbial feed additives or as the source of other feed additives, notably enzymes. Regulation (EC) No 1831/2003 requires that all feed additives, including microorganisms, are assessed for safety before being placed on the market. The principal safety concern for consumers and, to a lesser extent livestock, associated with *Bacillus* (and related genera) is a capacity for toxin production. However, the capacity for toxin production is unevenly distributed over the genus, occurring frequently in some species and more rarely, if at all, in others. For this reason, the Scientific Committee on Animal Nutrition (SCAN), when first developing guidance in this area, recommended that the use of strains of the *Bacillus cereus* taxonomic group, a group containing many known pathogenic strains, be strongly discouraged. However, the Committee recognised that strains from other *Bacillus* species may be considered safe (EC, 2000). The FEEDAP Panel concurs with this general position.

The Qualified Presumption of Safety (QPS) approach to the safety assessment of microorganisms adopted by EFSA is considered applicable to most of the commercially relevant *Bacillus* species (EFSA, 2007; EFSA BIOHAZ Panel, 2013). This approach requires the unambiguous identification of the strain being assessed, a demonstration of susceptibility to clinically relevant antibiotics and, in particular, evidence that the strain lacks a capacity for toxin production. Any other strain of *Bacillus* or related genera not falling within the scope of the QPS approach would also require an assessment of toxigenic potential. This document is intended to provide technical guidance for the assessment of any toxigenic potential for strains of *Bacillus* intended to be used directly as a feed additive or indirectly as a source of such additives.

The scope of the guidance

Although a number of species earlier considered to belong to the genus *Bacillus* have been transferred to other genera, to date none has been the subject of a feed additive assessment. Since relatively little is known about the toxigenic capacity of the genera related to *Bacillus* (i.e., *Geobacillus, Aneurinibacillus* and *Paenibacillus*) and, consequently, whether the approach to safety assessment described would fully apply, it is considered prudent to restrict this guidance to bacterial strains belonging to *Bacillus sensu strictu.*

Safety concerns caused by Bacillus species

* Identification

Characterisation of *Bacillus* strains according to Claus and Berkeley (1986) and Bergey's manual of

Systematic Bacteriology (2009) must be completed by molecular methods to identify strains to the species level. This is essential as it determines whether the current guideline applies and, if so, the nature of the testing recommended. Partial sequences (approximately 500 bp) of the 16S rRNA gene can be amplified using methods described in Guinebretière et al. (2001) and From *et al*. (2005) and compared to sequences from databases. If the partial sequence does not provide a definitive identification, then the 16S rRNA gene should be fully sequenced (Guinebretière *et al*., 2001). To differentiate species within the *B. subtilis* group, partial sequences of the *gyrA* gene or *gyrB* genes may be needed in addition to the

16S rRNA gene sequences. These can be obtained using methods described in Chun and Bae (2000) and From et al. (2005) for *gyrA* and Wang et al. (2007) for *gyrB*.

* Assessment of *Bacillus* species other than the *Bacillus cereus* group

Bacillus species other than members of the *B. cereus* group are a rare cause of foodborne diseases. In such events, the food contained high numbers (between 10^5 and 10^9 CFU/g) of the suspected *Bacillus* spp. (Kramer and Gilbert, 1989, From et al., 2007a). The production of the *B. cereus*-like diarrhoeal enterotoxins by some strains of other *Bacillus* species was described in the SCAN opinion (EC, 2000), although such strains have so far not been associated with foodborne diseases. The current view is that the very few reports of *B. cereus*–like enterotoxins occurring in other species of *Bacillus* are likely to have resulted from a misidentification of the strain involved (From et al., 2005). The few incidents of food poisoning investigated where non-*B. cereus* group strains were determined to be the causative organism suggest an association with surfactin-like lipopeptides (From et al., 2007b). However, the capacity for cyclic lipopeptides production appears widely distributed, if not universal amongst strains of *B. subtilis* (Apetroaie-Constantin et al., 2009, From *et al*., 2007a, Hwang et al., 2009, Mikkola et al., 2007), *B. licheniformis* (Nieminen et al., 2007, Dybwad et al., 2013, Madslien et al., 2013), *B. pumilus* (Taylor et al., 2005, From et al., 2007b) and *B. mojavensis* (From et al., 2005). The relation between the presence of surfactin-like lipopeptides and/or other toxic factors and the risk of illness in human has not yet been established.

In the absence of animal models shown to be able to distinguish hazardous from non-hazardous strains, the FEEDAP Panel relies on the use of *in vitro* cell-based methods to detect evidence of a cytotoxic effect. Such tests should be made with culture supernatants since the concentration of cells obtained in a broth culture would always exceed that found in animal food products.

Accordingly, the following is recommended for the assessment of non-*B. cereus* group species:

A cytotoxicity test made preferably with Vero cells or other epithelial cell lines using culture supernatant following the protocol described in the Appendix. Detection based on ^{14}C-leucine uptake is described but the use of other methods such as those based on lactate

dehydrogenase release or propidium iodide uptake could be used as an alternative (Fagerlund et al., 2008).

If the strain proves to be cytotoxic it is not recommended for use.

The FEEDAP Panel recognises that the underlying cause of the rare outbreaks of food poisoning attributed to non-*B. cereus* species is currently poorly understood. Accordingly, the Panel would be open to alternative approaches to establish the safety of these *Bacillus* strains.

* Assessment of species belonging to the *Bacillus cereus* group

A review of the virulence factors involved in the gastro-intestinal infections caused by *B. cereus* can be found in Stenfors-Arnesen et al. (2008):

- The role of hemolysin BL (Hbl) and of the non-hemolytic enterotoxin (Nhe) in diarrhoeal outbreaks has been confirmed (Stenfors-Arnesen et al., 2008). In particular the mode of action of Nhe on the cell membranes has been described (Lindbäck et al., 2010). Genes coding for Nhe, unlike those coding for Hbl, are present in most, if not all, strains of *B. cereus* (Guinebretière et al., 2010, Fagerlund et al., 2007) and the amount of Nhe produced at 32 °C by *B. cereus* strains was correlated with their cytotoxic activities (Moravek et al., 2006).
- The toxin previously named 'Enterotoxin K' (now cytotoxin K) has been characterised as a beta-barrel cytotoxin now called CytK (Lund et al., 2000). Two forms are distinguished (Fagerlund et al., 2004), CytK1 being more cytotoxic than CytK2.
- Enterotoxin T has now been identified as the result of a cloning artefact (Hansen et al., 2003) and should no longer be considered as a virulence factor
- Enterotoxin FM has been identified as an endopeptidase (Tran et al., 2010) which does not show direct toxic activity on epithelial cells.
- Emetic toxin (cereulide) is still the only toxin identified in *B. cereus* causing the emetic disease. Its potent toxic effect on liver cells and various mammalian cell lines has been shown (Andersson et al., 2007). Fatal or very severe *B. cereus* emetic outbreaks have been reported since 2000 (Shiota et al., 2010; Posfay-Barbe et al., 2008; Dierick et al., 2005). The non-ribosomal peptide synthase producing

cereulide has been identified (Ehling-Schulz et al., 2005) and characterised (Magarvey et al., 2006).

Other factors produced by *B. cereus* with various toxic activities have been characterised (Hemolysin II and several metalloproteases) but there is no evidence so far of their implication in gastro-intestinal diseases (Cadot et al., 2010). The toxic effect some of them show on macrophages may rather indicate a role in clinical infections.

In summary, diarrhoeal disorders produced by *B. cereus* result from the production of toxins Nhe, Hbl and CytK, alone or in combination in the intestine (Table 1). The emetic disease results from the production of cereulide by *B. cereus* cells in the food.

Table 1: *Bacillus cereus* toxins which can be considered as the causative agents of gastro-intestinal diseases (Stenfors-Arnesen et al., 2008)

Toxin	**Genes/operons**	**Nature**	**Foodborne infection/intoxication**
Nhe (non hemolytic enterotoxin)	*nhe*	Protein (three components)	diarrhoeal
Hbl (hemolysin BL)	*hbl*	Protein (three components*)	diarrhoeal
CytK (cytotoxin K)	*cytK*	Protein	diarrhoeal
Cereulide	*ces*	Cyclic peptide	emetic

* *The production of a fourth component, whose role has not been elucidated, was shown by* Clair et al., 2010.

In principle, the selection of strains belonging to the *B. cereus* taxonomic group for direct use in animal production is considered inadvisable.

If, however, they are proposed for use then the full genome (including chromosome and plasmids) should be sequenced and bioinformatic analysis made to search for genes coding for enterotoxins and cereulide synthase (Table 1). If there is evidence of homology, the non-functionality of the genes (e.g., mutation, deletion) should be demonstrated.

Strains harbouring a toxigenic potential should not be used as feed additives.

Annex

Recommended procedure for the detection of cytotoxicity using epithelial cell lines

- *Preparation of test substance*

Bacterial cells should be grown in brain heart infusion broth (BHI) at 30 °C and harvested after 6 h when it is anticipated that cells will have reached a density of at least 10^8 CFU/mL. Cells should be removed by centrifugation at room temperature. Toxicity is determined using 100 µL of supernatant in the Vero cells assay.

- *Cell assay*

Vero cells are grown in MEM medium supplemented with 5 % foetal calf serum. Cells are seeded into 24-well plates two-three days before testing. Before use, check that the growth of the Vero cells is confluent. If so, remove the medium and wash the cells once with 1 mL preheated (37 ºC) MEM medium.

- Add 1 mL preheated (37 ºC) low-leucine medium to each well and then add the test substance (1-100 µL of *Bacillus* supernatant), incubate the cells for 2 hours at 37 ºC.
- Remove the low-leucine medium with the toxin, wash each well once with 1 mL preheated (37 ºC) low-leucine medium. Mix 8 mL preheated low-leucine medium with 16 µL ^{14}C-leucine and add 300 µL of this mixture to each well, incubate the cells for 1 hour at 37 ºC.
- After removing TCA, add 300 µL 0.1 M KOH and incubate at room temperature for 10 minutes. Transfer the content of each well to liquid scintillation tubes with 2 mL of liquid scintillation cocktail. Vortex the tubes, and count the radioactivity in a scintillation counter for 1 minute.
- Percentage inhibition of protein synthesis is calculated using the following formula: ((Neg. ctrl – sample)/Neg. ctrl) × 100; the negative control is Vero cells from wells without addition of sample. Above 20 % inhibition is considered to indicate cytotoxicity.

For the alternative method with propidium iodide uptake or lactate dehydrogenase, values above 20 % of the fluorescence/absorbance obtained from the positive control (usually detergent treated cells) are considered to indicate cytotoxicity.

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SUMMARY

The giant freshwater prawn, *Macrobrachium rosenbergii*, is commercially one of the most important crustaceans. It is not only of high value to humanity as a food source, but also has a high economic value as an export product. The global production of *M. rosenbergii* reached over 220,000 tonnes in 2012. However, a major constraint in the large scale aquaculture of this species is not only the inadequate supply but also the poor quality of seed. This latter problem occurs particularly when the natural seed is over-exploited and the demanded seed is produced by *M. rosenbergii* hatcheries. In the hatchery, besides other factors such as broodstock, husbandry techniques and nutrition, the outbreak of diseases (caused by e.g. vibrios) is a main factor affecting the seed production and quality. Antibiotics have been a traditional method to control disease in aquaculture, but are being limited in their use these days. Several strategies, including water treatment, immunostimulation, vaccination, etc. have been investigated and developed with the purpose of replacing the use of antibiotics. Emerging nearly 10 years ago in the field of aquaculture, the use of poly-β-hydroxybutyrate has been assessed as a potential alternative strategy to control luminescent vibriosis. In almost all the PHB related studies crystalline PHB particles (extracted from bacterial cells) have been the used PHB form. Besides the undoubtedly beneficial effects of crystalline PHB particles, there are some weak points associated with this PHB form that impede its application in practical larviculture, the most important ones being bioavailability (size and cystallinity) and production cost.

Therefore, in this PhD research it was first illustrated that the use of amorphous PHB contained in whole cells of the bacterium *Alcaligenes eutrophus* in lyophilized and freshly grown cell forms induced similar and even better beneficial effects on the growth performance or disease resistance of *Artemia* nauplii or *M. rosenbergii* larvae than crystalline PHB particles (Chapter 2). In the second part, the change in carbonaceous compounds (trehalose, glycerol, glycogen and total organic carbon) and total nitrogen in the axenic hatching medium of decapsulated *Artemia fransicana* cysts was determined throughout the hatching process. Three different salinities (5, 12 and 35 g/L) of the incubation medium were applied. Trehalose appeared in the medium in small quantities (maximally 2.6 mg C/g incubated dry cysts) as compared to glycerol and glycogen (maximally 28.5 ± 1.2 and 13.8 ± 1.0 mg C/g incubated dry cysts, respectively) (Chapter 3).

Overall, the C/N ratio in *Artemia* hatching medium at a salinity of 12 g/L (which is most relevant for practice) was about 10 throughout incubation. In the final part of this work, the reuse of the hatching medium of *Artemia* was investigated as a cost-efficient strategy to culture the PHB accumulating bacterium *Bacillus* sp. LT12 and supply it to *Artemia* nauplii or *M. rosenbergii* laviculture as an antimicrobial agent. The PHB level in *Bacillus* sp. LT12 was increased about 2-fold when this bacterium was cultured in the axenic hatching medium of *Artemia* (AHMA) harvested at the different times points during the incubation process (16, 20 & 24 h). Adding the bacterium which was cultured in AHMA harvested at 16 or 20 h, into the culture water of *Artemia* nauplii challenged with *Vibrio campbellii* LMG21363 showed to completely protect the nauplii when they were added at a density of 5 x 10^7 CFU/mL (Chapter 4). Moreover, the disease resistance of *M. rosenbergii* larvae in the challenge test with *Vibrio harveyi* BB120 was significantly increased when feeding *Artemia* nauplii enriched with 10^9 CFU/mL of *Bacillus* sp. LT12 grown as well in the hatching medium of *Artemia* harvested at 16, 20 or 24 h. When *Bacillus* sp. LT12 was co-cultured with *Artemia* during cyst incubation and supplementary glycerol was added at 0.17 and 0.51 g/L, the results showed that the PHB content in the bacteria and the disease resistance of *M. rosenbergii* larvae were significantly increased as compared to control treatment (without adding extra glycerol) (Chapter 5).

In conclusion, the results in this PhD work illustrate that whole bacterial cells containing amorphous PHB are more promising to use as a disease control strategy in crustacean larviculture than crystalline PHB. The reuse of *Artemia* hatching medium in crustacean hatcheries as the nutrient medium to culture PHB accumulating bacteria to be fed to the cultured animals does not only result in an increased culture efficiency of the larvae but also reduces the load on the environment by reducing the volume of waste water originating from crustacean hatcheries.

SAMENVATTING

De reuzenriviergarnaal, *Macrobrachium rosenbergii*, is commercieel gezien één van de meest belangrijke schaaldieren. Ze heeft niet enkel belang voor de mens als voedselbron, maar heeft eveneens een hoge economische waarde als export product. De productie van *M. rosenbergii* op wereldvlak bereikte 220,000 ton in 2012. Er zijn echter belangrijke remmende factoren voor de grootschalige aquacultuur van dit species, namelijk de onvoldoende beschikbaarheid en de lage kwaliteit van jonge dieren. De lage kwaliteit vindt vooral zijn oorsprong wanneer de natuurlijke stock aan jonge dieren overgeëxploiteerd is en deze daarom in *M. rosenbergii* kwekerijen moeten worden geproduceerd. In de kwekerijen is, naast de invloed van broedvissen, huishoudtechniek en nutritie, de uitbraak van ziekten (veroorzaakt door bv. vibrios) een belangrijke factor die de productie van jonge dieren en hun kwaliteit bepaalt. In het verleden werd het gebruik van antibiotica standard aangewend om dit probleem te verhelpen, maar dit wordt huidig minder en minder toegepast. Verschillende strategieën waaronder waterbehandeling, immunostimulatie, vaccinatie, etc. werden daarom onderzocht en ontwikkeld als alternatief voor antibioticagebruik. Ongeveer 10 jaar geleden werd het gebruik van poly-β-hydroxybutyrate in de aquacultuur voorgesteld als een nieuwe methode om luminescente vibriosis te controleren. In bijna alle PHB gerelateerde studies werd echter het gebruik van PHB in kristallijne vorm (geëxtraheerd uit de bacteriële cel) toegepast. Hoewel kristallijn PHB ontegensprekelijk een positieve werking heeft zijn er een aantal negatieve punten verbonden met zijn gebruik voor larvicultuur in de praktijk. De belangrijkste hiervan zijn biobeschikbaarheid (grootte en kristalliniteit) en productie kost.

In het kader van dit doctoraatsonderzoek werd eerst aangetoond dat het gebruik van amorf PHB nog steeds vervat in volledige cellen van de bacterie *Alcaligenes eutrophus* in gevriesdroogde en vers gegroeide toestand leidde tot gelijkaardige en zelfs betere effecten dan kristallijn PHB op de groeiperformantie en ziekteresistentie van *Artemia* nauplii of *M. rosenbergii* larven (Hoofdstuk 2). In het tweede deel werd vervolgens de vrijstelling van koolstofbronnen (trehalose, glycerol, glycogeen en totaal organisch koolstof) en totaal stikstof in het axenische hatching medium van gedecapsuleerde *Artemia franciscana* cysten doorheen het hatching proces onderzocht. Er werden hierbij drie verschillende saliniteiten (5, 12 en 35 g/L) in het hatching medium toegepast. Trehalose werd vrijgesteld in kleine

hoeveelheden (maximaal 6 mg C/g geïncubeerde droge cysten) in vergelijking met glycerol en glycogeen (respectievelijk maximaal 28.5 ± 1.2 and 13.8 ± 1.0 mg C/g geïncubeerde droge cysten) (Hoofdstuk 3). Algemeen gezien was de C/N verhouding in het *Artemia* hatching medium bij een saliniteit van 12 g/L (de meest relevante voor de praktijk) ongeveer 10 doorheen het hatching proces. In het laatste deel van dit werk werd dan het gebruik van het hatching medium van *Artemia* onderzocht als een kost-effectieve strategie om de PHB accumulerende bacterie *Bacillus* sp. LT12 op te kweken en toe te passen op *Artemia* nauplii or *M. rosenbergii* larvicultuur als een antimicrobieel agentia. Het gehalte aan PHB in *Bacillus* sp. LT12 nam toe tot een factor 2 wanneer deze werd opgegroeid in het axenische hatching medium van *Artemia* (AHMA) geoogst op diverse tijdspunten doorheen het hatching process (16, 20 & 24h). Het toevoegen van de bacteriën opgegroeid in AHMA van 16 of 20 h aan 5 x 10^7 CFU/mL in het kweekwater van *Artemia* nauplii blootgesteld aan de pathogeen *Vibrio campbellii* LMG21363 resulteerde in een complete bescherming (Hoofdstuk 4). Daarbovenop kon het gebruik van deze bacteriën aangerijkt in *Artemia* nauplii aan 10^9 CFU/mL de resistentie van *M. rosenbergii* larven tegen de pathogeen *Vibrio harveyi* BB120 significant verhogen. Wanneer *Bacillus* sp. LT12 werd opgegroeid gelijktijdig met *Artemia* nauplii productie in éénzelfde *Artemia* hatching medium en additioneel glycerol werd toegevoegd aan 0.17 en 0.51 g/L kon de ziekteresistentie van *M. rosenbergii* larven significant worden verhoogd in vergelijking met wanneer geen extra glycerol werd toegevoegd (Hoofdstuk 5).

Als conclusie kan gesteld worden dat dit doctoraatsonderzoek het gebruik van volledige bacteriële cellen die amorf PHB bevatten naar voor schuift als geprefereerde strategie tov het gebruik van kristallijn PHB om ziekten in de larvicultuur van schaaldieren te bestrijden. Het hergebruik van *Artemia* hatching medium in schaaldierkwekerijen als een nutriëntenmedium voor de kweek van PHB bevattende bacteriën en het voeden daarvan aan de gekweekte dieren verhoogt niet alleen de kweekefficiëntie maar reduceert eveneens de druk op het milieu door de hoeveelheid aan afvalwater geproduceerd in deze schaaldierkwekerijen te reduceren.

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Education

1993 – 1998

Undergraduate study in University of Nha Trang, Nha Trang, Vietnam

Bachelor of Aquaculture

Thesis: *"The effect of fatty vitamins (A, E and K) on the growth performance of juvenile shrimp* Penaeus monodon*"* performed at Cua Be research station.

Promoter: Assoc. Prof. dr. Lai Van Hung.

2004 - 2006:

Master study in Ghent University, Faculty of Bioscience Engineering

Master of Science in aquaculture

Thesis: "*Immunoprotection of* Artemia *by an isogenic yeast mutant"* performed at the Laboratory of Aquaculture & *Artemia* Reference Center.

Promotor: Prof. dr. ir. Peter Bossier, supervisor: PhD student Siyavash Soltanian.

Professional activities

1998-2008: Researcher at Research Institute for Aquaculture No.3

- Participated in the governmental project: "To study the breeding of mud crab - *Scylla paramamosain* Estampador 1949" (1998 – 2000).

- Participated in the international project: "Development of leading centers for mud crab aquaculture in Indonesia, Viet Nam and Australia" (2000 -2001).
- Participated in the governmental project: "The experimental seed production of mud crab - *Scylla paramamosain* Estampador 1949" (2001 – 2003).
- Participated in the governmental project: "To study the grow-out culture of mud crab - *Scylla paramamosain* with high productivity in the experimental stage" (2003 – 2004).
- Participated in the governmental project: "To study the breeding biology of red slipper lobster *Cyllarides squammosus"* (2006 – 2008).

2008 – 2009: Researcher and Head of Biotechnology department at Mariculture Research & Development Center, RIA3.

- Leader of the governmental project: "To study the characteristics of breeding biology and test of seed reproduction of mantis shrimp *Harpiosquilla harpax* de Haan 1844*"*.

2010 – present: Doctoral research with Prof. dr. ir. Peter Bossier and Dr. ir. Peter De Schryver (Laboratory of Aquaculture and *Artemia* Reference Center, Ghent University) as scientific promoters. This PhD research was supported by Vietnamese government.

Publications

Publication in international peer-reviewed journals

Soltanian S, **Thai TQ**, Dhont J, Sorgeloos P, Bossier P (2007) The protective effect against *Vibrio campbellii* by pure beta-glucan and isogenic yeast cells differing in beta-glucan and chitin content operated with a source-dependent time lag. Fish Shellfish Immun 23(5):1003-14.

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Thai TQ, Nhon NT (2009). Results of trials of artificial breeding in headlines shrimp (*Harpiosquilla harpax* De Haan 1844). Research Institute for Aquaculture No. 3 Newsletter. Khanh Hoa publishing house, pp. 11 – 16.

Participation in national and international scientific congress (*Oral presentation* - *presentation given by* *author in bold and conference paper)*

Thach NC, **Thai TQ** (2003) Effect of salinity and food types on the development of fertilised eggs and zoea larvae of mud crab *(Scylla paramamosain)*. Conference: Mud crab aquaculture in Australia and Southeast Asia, At Proceedings of the ACIAR Crab Aquaculture Scoping Study and Workshop, 28–29 April 2003, Volume No 54. Joondooburri Conference Centre, Bribie Island, Australia.

Thach NC, **Thai TQ** (2007) The status of mud crab culture in Vietnam. The Asian-Pacific Aquaculture conference from 5 – 8, August in Hanoi, Vietnam.

Thai TQ (2008) Vaccine development for aquaculture. The vaccine conference of Ministry of Science & Technology from 2 – 3, December in Hanoi, Vietnam.

Thai TQ, Wille M, De Schryver P, Bossier P (2013) The effect of dried bacterial accumulating poly-β-hydroxybutyrate *Alcaligenes eutrophus* H16 on the larviculture of the giant freshwater prawn *Macrobrachium rosenbergii*. Asian-Pacific Aquaculture conference from 10 – 13, December in Ho Chi Minh City, Vietnam.

Training

Participation in the training of the international project: “Development of leading centers for mud crab aquaculture in Indonesia, Viet Nam and Australia” in Bribie Island, Brisbane, Australia (January – February, 2001).

Participation in the workshop "Population genetics in wild fisheries and aquaculture" at RIA3, Vietnam, 2008 of School of Natural Resource Sciences, Queensland University of Technology, Brisbane, Australia.

Award

Co-author of the best invention award of the World Intellectual Property Organization (Hanoi, April 2004).

Co-author of the best Scientific and Technological Innovation Vietnam - 2003 prize of the Ministry of Science & Technology and Vietnam Union of Science & Technology Associations.

Thesis supervision

Kha NK (2010) Influence of bacterial PHB content (as manipulated by growing them in media with different C/N ratio) on freshwater prawn (*Macrobrachium rosenbergii*) larval performance. Master of Sciences in Aquaculture at the Faculty of Agricultural and Applied Biological Sciences, Ghent University, Belgium.