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THE IMPACT OF PATHOGEN-PATHOGEN AND HOST-PATHOGEN SIGNALING ON LARVICULTURE OF GIANT FRESHWATER PRAWN

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ABBREVIATIONS

µl	Microliter
ABC	ATP binding cassette
AHL	Acyl Homoserine Lactone
AHLs	Acyl Homoserine Lactones
AI	Autoinducer
AIP	Autoinducing Polypeptide
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CAI	Cholera Autoinducer
CFU	Colony Forming Unit
CPS	Count per Second
cqsA	Cholerae quorum sensing Autoinducer
cqsS	Cholerae quorum sensing Sensor
GW	Green Water
GWC	Green Water Culture
h	Hour
HAI	Harveyi Autoinducer
HHL	N-hexanoyl-L-homoserine lactone
HPLC	High-performance liquid chromatography
HSL	N-(3-hydroxybutanoyl)-homoserine lactone
LB	Luria Bertani's
LSI	Larval Stage Index
$mg.l^{-1}$	Milligram per liter
min	Minute
mm	Millimeter
MOPS	3-(N-morpholino)propanesulfonic acid
nm	Nanometer
OD	Optical Density
ppm	Parts per million
ppt	Parts per thousand
QQ	Quorum Quenching
QS	Quorum Sensing
RifR	Rifampicin Resistance
rpm	Rotation per minute
sec.	Second
sp.	Species (singular)
spp.	Species (plural)
UV	Ultra-violet
v/v	Volume per volume

ABSTRACT

Prawn hatcheries still suffer major losses due to bacterial diseases caused by *Vibrio* spp. The pathogenicity of these *Vibrios* is determined by their production of putative virulence factors. Some of these factors are regulated by quorum sensing, a bacteria cell to cell communication. In this thesis work, we aimed at disrupting the quorum sensing (QS) signal HAI-1 of *Vibrio campbellii* BB120 using AHL degraders: *Pseudomonas sp*. NFMI-T and *Bacillus* sp. NFMI-C isolated from *Tetraselmis suecica* and *Chaetoceros muelleri* respectively. The impact of the AHL degraders and their cell-free extracts on the growth factor, quorum sensing regulated bioluminescence activity and production of virulence factors (caseinase, hemolysin and swimming motility) were investigated *in vitro*. Results showed that NFMI-C was efficient in degrading QS signal molecules of *V. campbellii* BB120 and the effective degradation took place through the production of intracellular enzyme. However, cell-free extracts of AHL degraders did not show any effect on the growth and virulence production of *V. campbellii* BB120. *In vivo* experiments were conducted with and without microalgae and the impact of live and dead (autoclaved) AHL degraders on larval survival and growth of freshwater prawn *Macrobrachium rosenbergii* larvae were evaluated. It was observed that in both experiments (with and without microalgae), the addition of autoclaved bacteria showed higher survival of prawn larvae. Similar result was obtained when the larvae were treated with coculture of microalgae and AHL degraders. However, the addition of AHL degraders without microalgae showed lower survival. On the other hand, the AHL degraders (live and autoclaved) had no impact on the larval growth in both experiments. Thus, the use of these AHL degraders could be suggested as a new anti-infective strategy for prawn larvae against infection caused by *V. campbellii* BB120.

1. INTRODUCTION

Aquaculture is expanding rapidly and making an important contribution to global food production. According to FAO projections, total aquaculture production reached 63.6 million tons in 2011, contributing about 41% of the total world fish production. It is expected to grow further by 33 percent to about 79 million metric tons until 2021 (FAO, 2012). This increasing production may contribute in filling the gap between the demand and supply of animal protein as a future food for the growing population.

Although the fish production is rising continuously, disease prevalence often due to bacterial infection has become a major constraint in producing high-quality seed of many cultured species of fish and shrimp (Phatarpeker *et al*., 2002). The frequent use of antibiotics to control bacterial infection in hatcheries has led to the development and spread of antibiotic-resistant bacteria (Karunasagar *et al*, 1994; Moriarty, 1998), and alternative methods are needed to control these problems.

Some studies showed that *Vibrio* spp. are a major cause of diseases in aquaculture (Austin and Zhang, 2006; Ruwandeepika *et al*., 2012; Tonguthai, 1997). One of the important aquaculture commodities that suffered from opportunistic luminous bacterium *Vibrio harveyi* is giant freshwater prawn *Macrobrachium rosenbergii* (Tonguthai, 1997; Pande *et al.,* 2013a). In general, vibriosis is prevalent in the early life stages (eggs, larvae, and postlarvae) of *M. rosenbergii* (Bhat and Singh, 1999), which need brackish water to survive (New *et al*., 2010).

Recent researches have clearly proved that the virulence of *V. harveyi* is under control of its multichannel quorum sensing (QS) system, a regulatory mechanism based on secreting and sensing small signal molecules called autoinducers (Cao and Meighen, 1989; Chen *et al*., 2002; Henke and Basler, 2004; Defoirdt *et al*. 2008; Natrah *et al*., 2011). According to Defoirdt *et al*. (2008), three autoinducers have been described so far: HAI-1, AI-2 and CAI-1, of which HAI-1 and the AI-2 mediated channels of the *V. harveyi* quorum sensing system are essential for full virulence in giant river prawn larvae (Pande *et al*., 2013)

Hence, interfering with quorum sensing and quorum sensing signal molecule, in particular, is found to be an attractive target to develop an antimicrobial method and can be a better strategy to fight against vibriosis (Du *et al*., 2014; Defoirdt *et al*., 2012). Few enzymes have been identified such as lactonases and acylases, which are secreted by certain bacteria that are able to inactivate QS molecules (Hong *et al*., 2012). Some examples are; lactonases produced by *Bacillus* spp. (Defoirdt *et al*., 2011; Dong *et al*., 2007) and acylases by *Pseudomonas* spp. (Chen *et al*., 2013). These enzymes hydrolyze or modify the signal molecules in the QS circuit

that inhibits the expression of bacterial virulence factors and provide a promising means to control bacterial infections (Du *et al*., 2014).

Moreover, microalgae such as *Nannochloropsis*, *Chlorella*, *Isochrysis* and *Tetraselmis* had shown a potential decrease in QS regulation of *V.harveyi* (Natrah *et al.,* 2011a). These microalgae species are widely used in green water culture system. The luminous bacterial count was reported lower in green water, suggests that microalgae are efficient in controlling growth of pathogenic bacteria (Tendencia *et al*., 2013). Several other beneficial effects of green water were identified that helps in improving larval survival, growth, water quality, assimilation efficiency, nutritional value of live prey, immune response, etc. Hence, this beneficial effect could be due to QS down-regulation or QS signals molecule's modification by microalgae.

In the present study, we aimed at investigating the impact of N-acyl homoserine lactone (quorum sensing signal molecule HAI-1) degrading bacteria isolated from microalgae (*Chaetoceros muelleri* and *Tetraselmis suecica*) on some of the virulence factors of *V. campbellii in vitro* and their impact on survival and growth performance of *M. rosenbergii* larvae challenged with pathogenic *V. campbellii in vivo.*

2. LITERATURE REVIEW

2.1. Giant freshwater prawn *Macrobrachium rosenbergii*

2.1.1. Taxonomy and distribution

Previous names of *M. rosenbergii* were; *Palaemon carcinus*, *P. dacqueti*, and *P. rosenbergii*. In 1959, the name *Macrobrachium rosenbergii* (De Man, 1879) was universally accepted. Its taxonomical classification is as follows (Fransen, 2013; De Grave *et al*., 2013):

Phylum:	Arthropoda
Subphylum:	Crustacea
Class:	Malacostraca
Order:	Decapoda
Family:	Palaemonidae
Genus:	*Macrobrachium*
Species:	*rosenbergii* (De Man, 1879)

About 200 species of *Macrobrachium* are distributed in the tropical and subtropical regions of the world (New, 2002), out of which only 25 species are significantly considered to have aquaculture potential (New and Nair, 2012). This species is found in the coastal belt (extending up to 200 km) of south-east Asian region (India, Thailand, Vietnam, and Philippines etc.), Northern Australia, New Guinea and western pacific islands (Holthuis, 1980). They are found in inland freshwater bodies of tropical areas like lake, river, streams, creeks, swamps, irrigation ditches, canals and ponds (Nandlal and Pickering, 2005).

2.1.2. Biology

The Adult *M. rosenbergii* is easily distinguished by its long chelipeds and rostrum. The rostrum is long and curved upward, carries 11-13 dorsal teeth and 8-10 ventral teeth. The body consists of cephalothorax (head) and abdomen (tail) and is covered by chitinous exoskeleton. The body is divided into 20 segments or somites (**Figure 2.1**). Of these segments, 14 are in the cephalothorax (6 segment in front portion and 8 segments in rear portion) and are covered by a shield known as carapace. The abdomen consists of 6 segments, each bearing a pair of swimming appendages known as pleopods or swimmerets. The sixth abdominal segment consists of uropods (stiff and hard) and telson, together forms the tail fan which is used to move or jerks backward. The body segments and function of appendages are as follows:

Front portion of head (cephalon): 6 somites

- Stalk eyes (1) - vision
- First antennae (2) } Tactile and sensory
- Second antennae (3) } perception
- Mandible (4) – grinding food
- First maxillae (5) } transfer food
- Second maxillae (6) } into mouth

Rear portion of head (thorax): 8 somites

- 3 pairs of maxillipedes (7-9) – function as mouth parts
- 5 pairs of pereiopods (10-14) – walking legs; female gonophores at the base of 3rd pereiopods and male gonophores at the base of 5rd pereiopods.

Abdomen: 6 somites

- 5 pairs of pleopods (15-19) – swimming legs
- A pair of uropod (20) - Propulsion together with telson

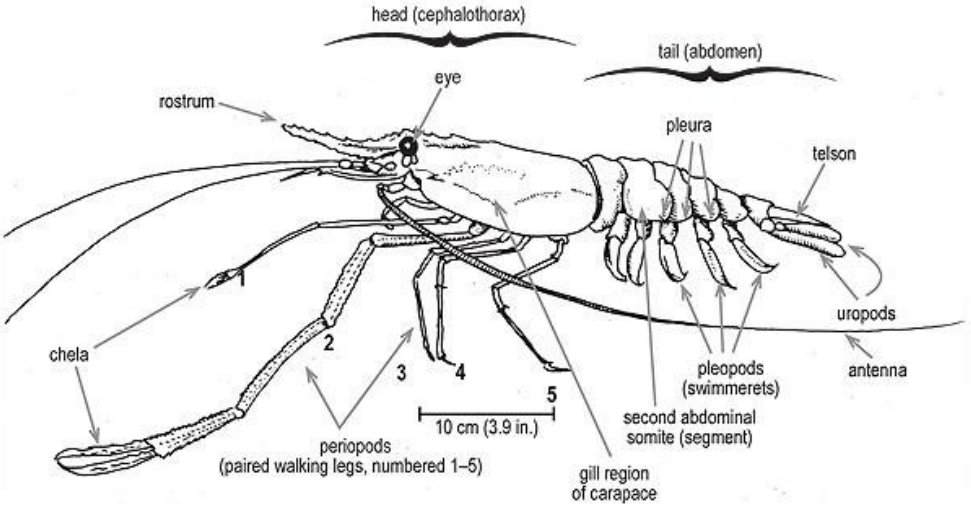


Figure 2.1. External morphology of *M. rosenbergii* (Hicks and Pierce, 2011).

Male and female can be classified based on their external characters. Males are generally larger than the females (**Figure 2.2**). Cephalothorax is proportionally larger than the abdomen. The chelipeds are long and massive which is the second pleopod. Genital pore is present at the base of 5th pereiopods. The pleura of the abdomen are shorter. The ventral side of the first abdominal segment has a lump (hard point) at the center. The appendix masculina, known as secondary sexual organ is involved in sperm transfer process (Christopher, 1979), which is situated in the second abdominal appendages (**Figure 2.3**).

Figure 2.2. Male and female *Macrobrachium rosenbergii.*

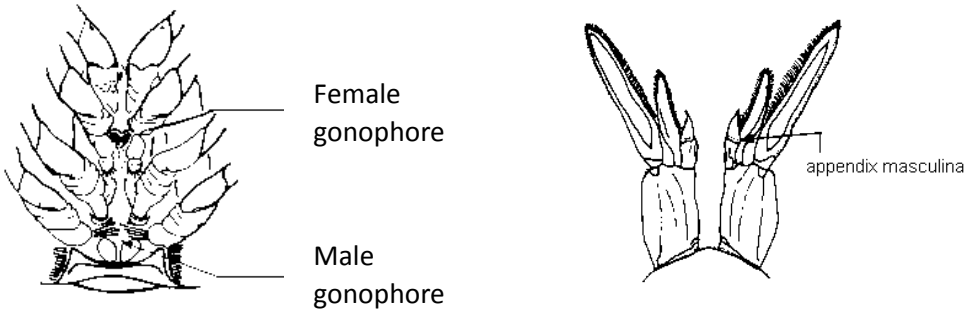


Figure 2.3. Position of male and female gonophore (at left), appendix masculina in male at the second pair of pleopods (at right).

Males grow faster than female if there is plenty of food and optimum environmental condition (especially dissolve oxygen and water temperature). It is considered as an ideal species for monoculture and polyculture. They are highly cannibalistic during larval stage when different age groups are put together. The dominant blue claw male shows territory behavior. They are nocturnal in habit and during day time, usually find shades and shelters for hiding themselves. Generally, they are omnivorous bottom feeder and love to stay in turbid water. In nature, they mostly eat aquatic insects and their larvae, small mollusks, crustaceans and carcasses of fishes and animals.

2.1.3. Life cycle and embryonic development

There are four distinct phases in the lifecycle of *M. rosenbergii*; egg, larva (zoea), postlarvae (PL) and adult (**Figure 2.4a**). Like other crustaceans, freshwater prawns also undergo molting throughout their life cycle for growth. These animals require brackishwater at the initial stages of their life and then post-larva moves to the freshwater where they grow into adult. The female gets matured when it attains a weight of about 15-20g within 4-6 months.

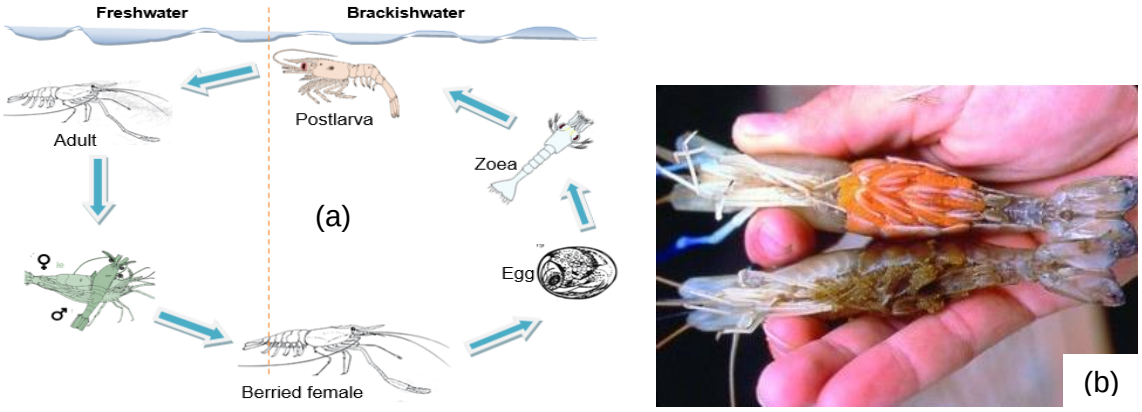


Figure 2.4. (a) Life cycle of *M. rosenbergii* (drawn based on concept given by Nandlal and Pickering, 2005; Bauer, 2013), (b) Difference between immature egg (bright orange) and matured egg (grayish black) (FAO, 2014).

Egg development starts with successful mating between ripe female and mature male. An incubation time for the fertilized eggs is temperature dependent. At 28-30^{o}C, it takes about 18-21 days for hatching. If the temperature is lower than the optimum, the incubation period is extended. Generally, eggs are yellow to bright orange in colour and gradually turn to grayish black in colour within 2-3 days before hatching (**Figure 2.4b**). This colour changes occurs as eyes get larger and embryos utilize their food reserves. During incubation, berried female aerate the eggs by vigorous movement of their pleopods. Details about embryonic development are provided in **Figure 2.5.**

2.1.4. Larval development

Generally, mating and incubation takes place in freshwater and for spawning berried female migrate to the coastal zones or brackishwater environment. Under hatchery conditions, they require 8-14 ppt (parts per thousand) within 1-2 days otherwise, they are prone to death. When larva hatches out, it starts swimming upside down with its eyes looking towards the surface. In nature, they feed on zooplanktons including rotifers, cyclopes, copepods, other minute crustaceans, small worms and larvae of other aquatic invertebrates.

There are 11 distinct larval stages of *M. rosenbergii.* These larvae undergo several morphological changes before they develop into postlarvae, and this takes about 22-35 days. The transition stage from larva to postlarva is called metamorphosis. As larvae grow or move to the next stage, they usually cast off their exoskeleton and attain a new body feature at each stage (**Table 2.1**).

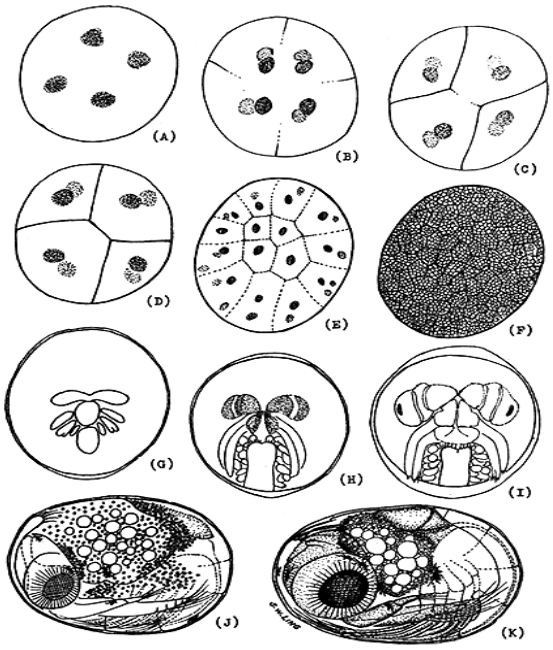


Figure 2.5. *Macrobrachium rosenbergii*, segmentation and embryonic development. Times refer to period since fertilization. (A) 7 h - completion of second nuclear division. (B) 8 h 45 min - third nuclear division nearly completed appearance of 4 cleavage furrows. (C) 8 h 55 min - third nuclear division completed tips of the 4 cleavage furrows have met at 2 points from which the median furrow is developing. (D) 9 h - complete formation of 4 quadrants (blastomeres). (E) 14 h - 32 nuclei. (F) 24 h - completion of segmentation. (G) 6 days - formation of caudal papilla. (H) 7 days - formation of optic vesicle. (I) 9 days - eye pigment developed. (J) 14 days - larva fully formed. (K) 19 days - larva ready to hatch. (Ling, 1968)

2.1.5. Current farming status of *Macrobrachium rosenbergii*

A rapid global expansion of freshwater prawn farming was noticed since 1995, mainly because of huge production from china and rapid take off of farming practices in India and Bangladesh. (New, 2005) Although, there are several species of *Macrobrachium* but at present *M. rosenbergii* (giant freshwater prawn) has a greater commercial importance. Particularly, in South-East Asia it is considerably gaining global attention for culture and export (New, 1990; FAO, 2012). It is mostly favored in at least 43 countries for both research and farming purpose (New, 2000). Freshwater prawn contributes about 5% of the total aquaculture production of shrimp and prawn (New, 1990). The global aquaculture production of *M. rosenbergii* has exceeded 200,000 tones/year in past few decades and recently in 2012, it reached to 220, 250 tones (FAO, 2014a; **Figure 2.6**). The farming of other *Macrobrachium* species has captured attention in some countries, *Macrobrachium nipponense* in China (Kutty and Weimin, 2010), *Macrobrachium malcolmsonii* in India (Kutty and Valenti, 2010) and *Macrobrachium amazonicum* in Amazonia (Moraes-Valenti and Valenti, 2010).

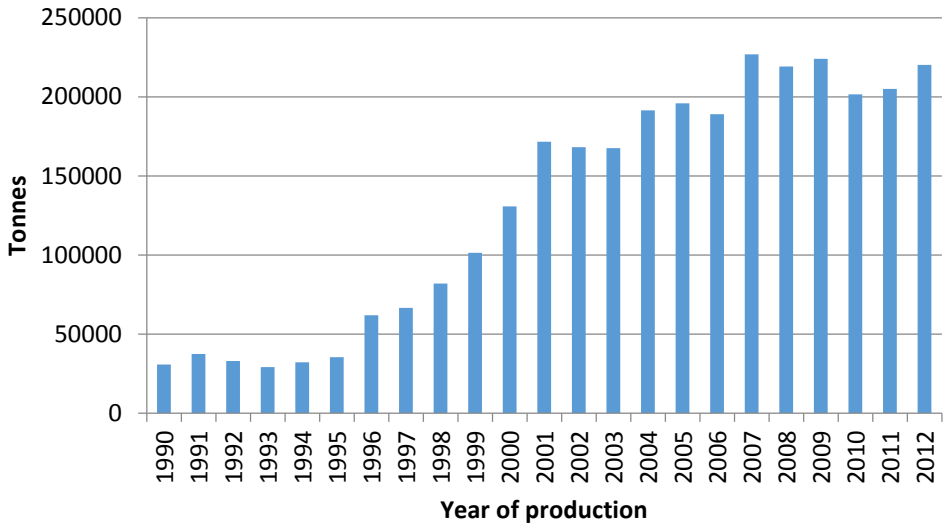


Figure 2.6. *M. rosenbergii* production during 1990-2012 (FAO, 2014a).

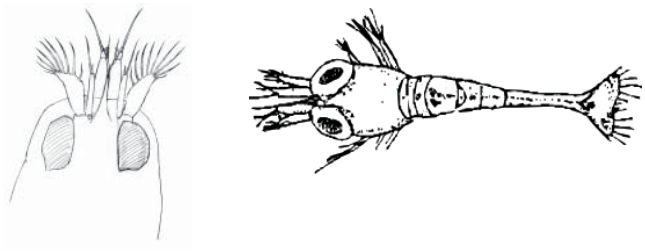
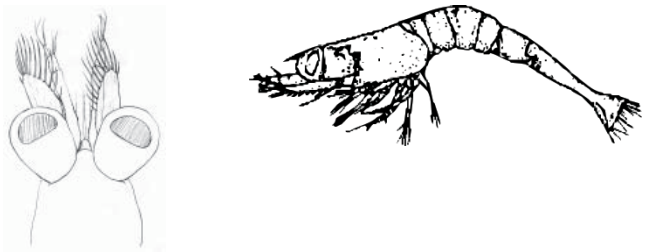
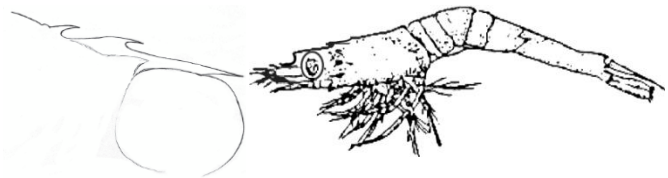
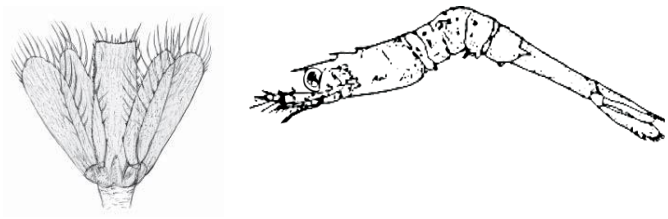
2.1.6. Diseases in freshwater prawn aquaculture

The giant freshwater prawn *M. rosenbergii*, is one of the most important inland culture species from a commercial point of view (Cheng and Chen 2000). In the recent years, traditional aquaculture has turned into science-based commercial aquaculture with heavy inputs and high intensity of production (Kongkeo, 2004). Therefore, diseases are more of a concern in hatchery based culture system (McVey, 1993).

Disease outbreak, rarely result from simple contact between a host and pathogen but unstable environmental changes often act as a trigger for diseases development (Plumb and Hanson, 2011). An undesirable changes in the environment such as stocking density; poor water quality; undesirable temperature and pH changes; turbidity; ammonia and nitrite levels; inadequate nutrition and poor sanitation cause physiological stress and physical injury, which make the host weak and susceptible to diseases (Rottmann *et al.,* 1992).

The common diseases occurring in prawn larvae and post larvae include infestation of ciliates, bacterial, fungal and viral diseases (Soundarapandian and Varadharajan, 2013). The most predominating bacterial disease of the prawn culture environment is vibriosis, causing a significant mortality in larviculture and grow-out phases of crustacean aquaculture (Gomez-Gil *et al.,* 2000). There are several bacterial diseases reported to have a significant effect on growth and survival of *M. rosenbergii*, are summarized in the given **Table 2.2.**

Table 2.1. Different larval stages and characteristics of *M. rosenbergii* (Nandlal and Pickering, 2005).

Larval stage	Age (days)	Chararastristics	Picture
I	1	Sessile eyes	
II	2	Stalked eyes	
III	3-4	Uropods present	
IV	4-6	2 dorsal teeth	
V	5-8	Telson narrows and elongated	

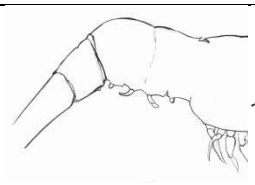
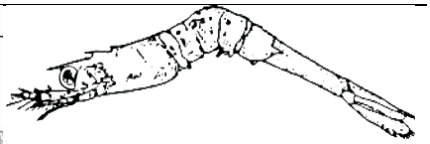
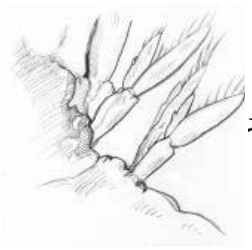
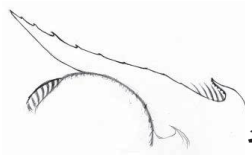
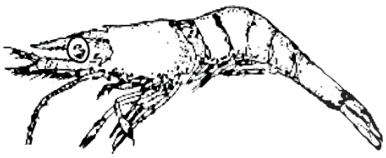
VI	7-10	Pleopod buds present		
VII	11-17	Pleopods biramous		
VIII	13-20	Pleopods with setae		
IX	15-22	Endopods of pleopods with appendices internae		
X	17-23	3-4 dorsal teeth on rostrum		
XI		Teeth on half of upper dorsal margin		
PL		Adult behaviour, benthic, swims forwards with dorsal side uppermost. Teeth on upper and lower margin of rostrum (also behavioural changes mainly in swimming)		

Table 2.2. List of the bacterial diseases, their causative agents, symptoms and the infected larval stages of *M. rosenbergii*.

Diseases	Causative agent	Symptoms	Affected stages	References
Tail rot diseases	*Vibrio parahaemolyticus, V. anglinotenus, Pseudomonas aeroguinosa, Aeromonas sps, Staphylococcus aureus*	Similar to shell diseases; colour patches on tail that later spread over the body; perforated exoskeleton and tail	Spent females	Dinakaran *et al.,* 2013
Muscles Necrosis	*Enterococcus*-like bacterium closely related to *Enterococcus seriolicida*	Opaque and whitish musculature; melanized hemocytic granulomas; hepatopancrease necrosis	Adults	Cheng and Chen, 2002; 1998
Shell disease, brown/black spot, black rot/erosion, blisters, necrosis of appendage	*Vibrio*, *Pseudomonas* or *Aeromonas sp*. groups (Chitinoclastic bacteria)	Melanised lesions on the carapace, abdominal segments, rostrum, tail, gills, and appendages; Necrosis	All life stages; frequent in juveniles and adults	Dinakaran *et al.,* 2013; Pillai and Bonami, 2012; El-Gamal *et al.,* 1986; Hipólito *et al.,* 1996; Dugan and Frakes, 1973
Larval Mid Cycle Diseases (MCD)	*Enterobacter aerogenes*	Similar to bacterial necrosis	IV to XI stages	Johnson, 1978; Brock, 1988; Tonguthai, 1997

Diseases	Causative agent	Symptoms	Affected stages	References
Bacterial necrosis	Secondary infection; Mixed bacterial infections: *Leucothrix* spp., non-filamentous bacilli and cocci (*Pseudomonas* sp.)	Similar to black spot diseases in terms of gross sign; bluish colour or discoloration, brown spots on antennae and newly formed appendages	IV and V stages	Pillai and Bonami, 2012; McVery, 1993; New *et al.,* 2010; Tonguthai, 1997; FAO, 2014
White post larval diseases	*Rickettsia*	White larvae, atrophy of the hepatopancrease	IV and V stages	FAO, 2014, McVery, 1993; De Silva *et al.,* 1989; Lacroix *et al.,* 1994
Vibriosis	Non-luminescent bacteria *Vibrio cholera*, *V. alginolyticus*, *V. carcharine* and *V. mimicus*	Weak and moribund larvae, intermittent and weak swimming movement	Post-larvae and Adults	New *et al.,* 2010; Oanh *et al.,* 2001;
Tuberculosis	*Mycobacterium* sp.	Focal granuloma	Adults	Lewis and chinabut, 2011; Diggles *et al.,* 2002; Brock *et al.,* 2006

Diseases	Causative agent	Symptoms	Affected stages	References
Enterococcosis	*Enterococcus faecium* associated with the yeast (*Metschnikowia bicuspidate*) infection diseases	yellowish-brown body color; milky hemolymph; opaque or whitish muscles; necrotic lesions in the muscles, hepatopancreas and other internal organs	Juvenile and Adults (7 months old)	Chen *et al.,* 2003; Pillai and Bonami, 2012
Unknown	*Lactococcus garvieae*	Opaque and whitish muscles; necrotic lesions in the muscles, hepatopancreas and other internal organs	Adults (2 months old)	Chen *et al.,* 2001
white muscle disease	*Lactococcus lactis lactis*	opaque and whitish muscles	Adult (3-5 months old)	Wang *et al.,* 2008a
Filamentous bacterial diseases	*Leucothrix* sp.	Periopods, gills, uropods infection	Larvae	Shailender *et al.,* 2012; Sandifer *et al.,* 1975
Luminous bacterial diseases or Luminescent larval syndrome	*Vibrio harveyi*	Greenish luminescence of moribund and dead larvae	Egg, larvae and post-larvae	New *et al.,* 2010; Tonguthai, 1992; 1997

2.2. *Vibrio* species

2.2.1. Biology of Vibrio spp.

The genus *Vibrio* belongs to *Vibrio*naceae, the largest family of gram-negative gammaproteobacteria. More than 20 species of *Vibrio* were recognized, out of them some are pathogenic to human (*V. cholerae, V. parahaemolyticus and V. vulnificus);* some are pathogenic to aquatic animals, including shrimps and prawn (*V. harveyi, V. splendidus, V. anguillarium, V. parahaemolyticus, V. penaecida, V. vulnificus*) (Dinakaran *et al.,* 2013) and some non-pathogenic (*Vibrio natriegens and Vibrio mytili*) (Ruwandeepika *et al*., 2012). According to Bergey's manual of Determinative Bacteriology, *Vibrios* are usually motile, rod shape, non-spore formers, mesophilic, chemo-organotrophic and have biochemical characters like facultative fermentative, oxidase positive (Breed *et al.,* 1957).

The family *Vibrio*naceae (including *Vibrio* and photobacteria) commonly possess two different chromosomes (Okada *et al.,* 2005). The larger chromosome (chromosome I) is conserved and carry most of the essential gene. The smaller chromosome (chromosome II) carries more species-specific gene and is essential for growth and viability. The presence of these two replicons may be a strategy to replicate rapidly and colonize, which give the bacteria fitness to overwhelm other bacterial populations (Reen *et al.,* 2006)

2.2.2. Virulence factors of *Vibrios*

Vibrios are the most important bacterial pathogen commonly observed in prawn hatcheries, grow-out ponds and culture water sediments (Vaseeharan and Ramasamy, 2003; Haldar *et al.,* 2011) and cases of mortality were already reported from Asian countries (Sunaryanto and Mariam, 1986; Karunasagar *et al.,* 1994). Diseases caused by *Vibrio spp.* have been observed in several other aquatic animals such as shrimp, crayfish, fish, oysters and lobster (Chrisolite *et al.,* 2008; Raissy *et al.,* 2011). Vibrosis normally occurs during the warm summer months when the salinity and organic load are high (Caipang and Aguana, 2011).

Bacteria use an array of virulence factors to infect host and cause diseases. However, pathogenicity in *Vibrios* are not clearly defined. There are several factors mentioned which are involved in virulence or pathogenesis in *Vibrios* include; adhesion, lytic enzyme production (hemolysins, protease, chitinase and lipase) (Mok *et al.,* 2003; Ruwandeepika *et al.,* 2011; Defoirdt *et al.,* 2010; Natrah *et al.,* 2011; Defoirdt, 2014), production of siderophore (Lilley and Bassler, 2000, Defoirdt, 2014), type III secretion (Henke and Bassler, 2004b), Extracellular polysaccharide and biofilm formation (Anetzeberger *et al.,* 2009; Defoirdt, 2014), bacteriophage association (Salcedo and Owens, 2013) and flagellar motility (Yang and Defoirdt, 2014). Some of the virulence factors described in brief are as follows:

a. Hemolysin

Hemolysin is one of the major exotoxins responsible for initiation of a virulence factor in *Vibrios*. Hemolysin activity is generally referred as the capacity to lyse erythrocytes and hence two different lytic activities were observed, either by forming pores or by breaking down cell membrane (Sun *et al.,* 2007). It was reported that the phospholipase activity of *V. harveyi* haemolysin (VHH) plays a major role in haemolysis (Sun *et al*. 2007). Furthermore, thermostable direct haemolysin (TDH) secreted by *V. parahaemolyticus* showed hemolytic activity by forming pores in the plasma membrane of red blood cells and enterocytes. This allows an influx of multiple ionic species which result in osmotic imbalance and cause cell death (Raimondi *et al.,* 2000). However, hemolysin has also been shown to have cytotoxic, enterotoxic and cardiotoxic activity (Baffone *et al.,* 2005; Hiyoshi *et al.,* 2010).

In vitro, hemolytic activity can be screened using agar plate containing blood cells. Three different types of hemolytic activity can be observed on agar plates, which are; *α*-hemolysis (agar under the colony is dark and greenish), *β*-hemolysis (lysis of blood cell under and around the colonies, and area appear transparent or light yellow) and *γ*-hemolysis (agar under and around the colony is unchanged) (Ray and Rayan, 2004).

b. Protease

Protease is considered as another important group of lytic enzyme, often show virulence activity towards both fish and shrimp (Lee *et al*., 1997; Kahla-Nakbi *et al.* 2009; Rui *et al.* 2008). Protease has been reported in *Vibrio* spp. such as *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus* (Ruwandeepika *et al.,* 2012). It has been found to digest a range of host proteins including gelatin, fibronectin and collagen, suggesting a potential role in pathogenesis of *vibrios* (Toe *et al.,* 2003). In order to cause diseases different forms of proteolytic enzymes are secreted. These include; metalloprotease, serine protease, cysteine protease, collagenase, caseinases, gelatinases (Ruwandeepika *et al.,* 2012; Defoirdt, 2014). Several authors have reported the presence of proteases in *Vibrios*. Lee *et al.* (1997a) isolated alkaline serine protease from diseased *Penaeus japonicus* (Kuruma prawn). Haopeng *et al.* (2009), studied alkaline serine protease from a strain isolated from moribund *Epinephalus coioides* showing virulence. Lee *et al.* (2002), purified serine protease extracted from the extracellular proteins of a clinical isolate of V parahaemolyticus. A study showed that cysteine protease and the metalloprotease used by *V. harveyi* as a virulence factor (Lee *et al.* 1999; Teo *et al.,* 2003), on the other hand metalloprotease and alkaline serine protease shown to be a virulence factor of *Vibrio alginolyticus* (Haopeng *et al.,* 2009; Aguirre-Guzman *et al.,* 2004; Deane *et al.,* 1989; Hare *et al.,* 1983).

In vitro, proteolytic activity can be screened using agar containing skimmed milk (caseinase activity) or gelatin (gelatinase activity).

c. Motility

Bacterial motility is considered as an important virulence factor in many pathogens (Yang and Defoirdt, 2014). They use flagella to move and adhere to the host cell where they start colonizing to express its pathogenesis (Josenhans and Suerbaum, 2002). The ability to cause disease or pathogenesis is directly proportional to the swimming motility; faster the swimming greater the adhesion factor or pathogenesis (Koger, 1998). *Vibrio* species such as *V. harveyi*, *V. parahaemolyticus* possess dual flagellar system. Polar flagella involved in motility in liquid environment (swimming), while lateral flagella enable to move on solid or viscous environment (swarming) (Yang and Defoirdt, 2014; Belas *et al*., 1986). *V. cholera* is highly motile by means of single polar flagella (Butler and Camilli, 2004), whereas *V. parahaemolyticus* is superior swarmer and use lateral flagella (McCarter, 1999). A study showed that swarming organisms require production of extracellular molecules (capsular polysaccharide), capable of altering surface tension (McCarter, 1999).

2.3. Quorum Sensing and Quorum Quenching

2.3.1. Quorum sensing

Quorum sensing is a specific type bacterial cell-cell communication depending on the density of a bacterial population (Bassler, 2002). This regulatory mechanism was first discovered in luminous marine bacteria *Vibrio fischeri* with symbiotic association with squid *Euprymna scolopes* (Nealson *et al.* 1970; Nealson and Hastings, 1979). *Euprymna scolopes* acts as a host to *Vibrio fischeri*, which provide light to avoid predator and to assist in feeding (Visick and McFall-Ngai, 2000), whereas *V. fischeri* benefits through high nutrient supplies and enabling them to grow to very high density upto 10^{11} cells/ml (Nyholm and McFall-Ngai, 1998). Several phenotypes were shown to be regulated by quorum sensing activity are depicted in **Figure 2.7**.

Quorum sensing mechanism has been identified in many different groups of bacteria that produces and releases specific chemical signal molecules called “autoinducers”. The production of signal molecules takes place during specific stage of growth or in response to particular environmental changes. The signal molecule diffuses freely in and out of the cell and increases in concentration with increasing cell density (Waters and Bassler, 2005; **Figure 2.8**). These signals get accumulated in the extracellular environment and at certain time period it reaches a threshold concentration, which is sensed by specific bacterial receptor present on

the cell surface. As a consequence, specific regulator gets triggered and their quorum sensing control of gene expression starts (Winzer *et al.* 2002).

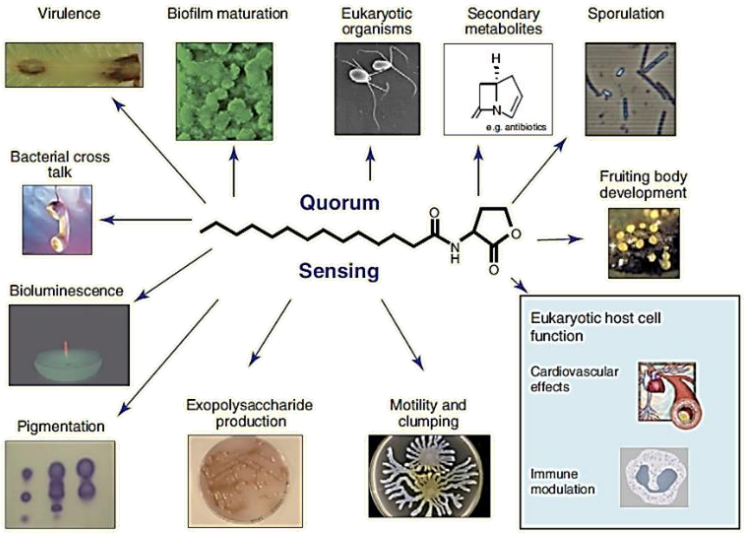


Figure 2.7. Phenotypes shown to be regulated by quorum sensing in Gram-negative and Gram-positive bacteria (Diggle *et al.,* 2007).

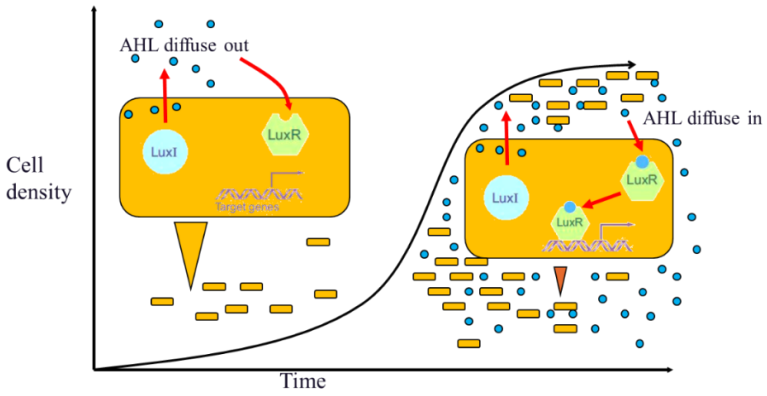


Figure 2.8. Correlation between bacterial cell density and the concentration of AHL signal molecules.

2.3.2. Different quorum sensing signaling mechanisms

Quorum sensing (QS) mechanism has been reported in both Gram-negative and Gram-positive bacteria (Diggle *et al.* 2007; Williams *et al.* 2007). Apparently, they use different type of signal molecules to sense population and initiation of virulence gene expression. Gram-negative bacteria use acyl homoserine lactones (AHLs, acyl-HSLs, or HSLs) as signal molecules, also called autoinducer 1 (AI-1), whereas Gram-positive bacteria use autoinducing oligopeptide (AIP). Another signal molecules called autoinducer 2 or AI-2 used by Gram-

positive as well as Gram-negative bacteria. A poorly characterized autoinducer 3 or AI-3 has been also reported in several enteric bacterial species such as Enterohaemorrhage *Escherichia coli* (EHEC) (Boyen *et al.,* 2009; Asad and Opal 2008; Kendall and Sperandio, 2007).

a. AHL mediated signaling

AHLs (N-acylhomoserine lactones) of different species differ with the acyl side chain and it varies between C_4 and C_{14} which have an oxo or a hydroxyl substitution at the third position (Defoirdt *et al.,* 2004; **Figure 2.9**). This mechanism is also reported in many Gram-negative bacteria, including aquaculture pathogens such as *V. anguillarum*, *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Edwardsiella tarda* and *Yersinia ruckeri* (Defoirdt *et al.,* 2011a, Natrah *et al.,* 2011b).

Gram-negative bacteria use LuxI/LuxR type quorum sensing (**Figure 2.10**). An AHL synthase LuxI involved in biosynthesis of AHL molecules. These molecules accumulated in the extracellular environment either via diffusion or active transport (Defoirdt *et al*, 2011a). When a certain threshold is reached, the signals are sensed by LuxR, and AHL binds to LuxR receptor present in bacterial cell membrane.

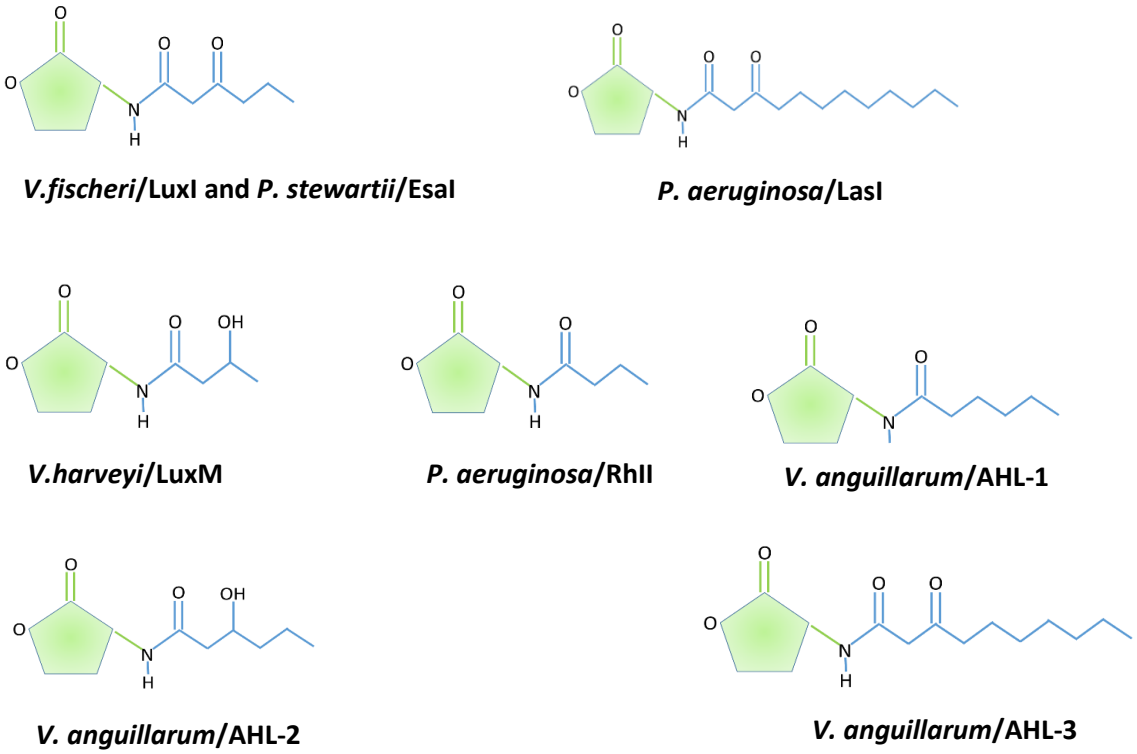


Figure 2.9. Structure of different AHL molecules produced by different Gram-negative bacteria (redrawn from Bassler, 2002).

The LuxR-AHL complex binds to LuxICDABE promoter of quorum sensing-regulated target genes and transcription process of this operon is activated through intracellular biochemical pathways. The LuxR signal receptor is highly selective for its cognate AHL signal molecule and only binds to signal molecule produced by LuxI (Bassler, 2002; Natrah *et al.,* 2011b). Due to its specificity this system is mainly used for intra-species communication.

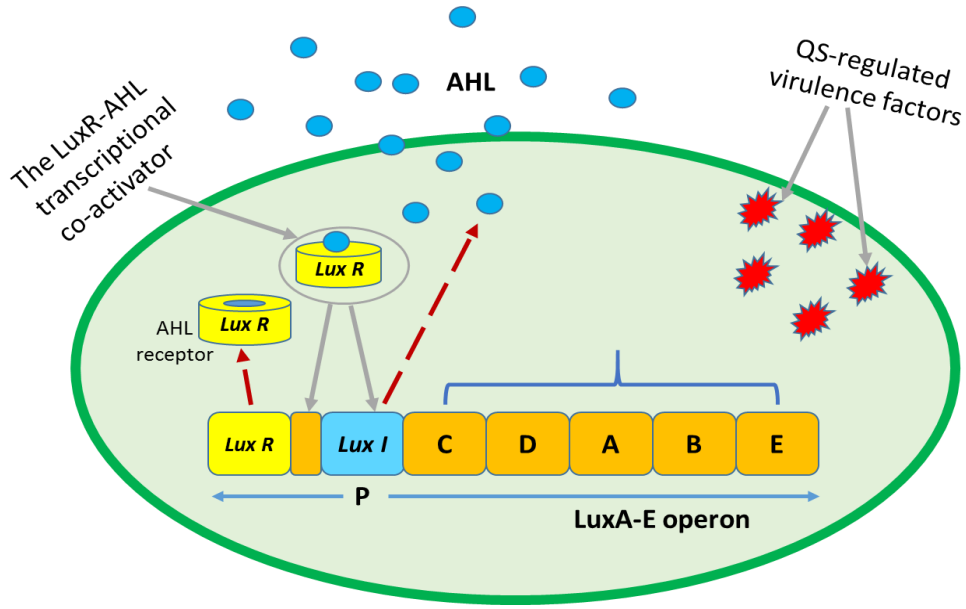


Figure 2.10. AHL mediated signaling in Gram-negative bacteria (redrawn from Asad and Opal, 2008).

b. AIP (autoinducing polypeptides) mediated signaling:

Autoinducing polypeptides are short amino acid or peptides, exclusively used by Gram-positive bacterial species (**Figure 2.11**). AIP signal is produced by the peptide signal precursor locus which is then processed, modified and matured oligopeptide autoinducers exported to extracellular environment by the ATP-binding cassette (ABC transporter) (Sturme *et al.,* 2002; **Figure 2.12**). When AIP molecules reach a threshold concentration, the autoinducer is detected by 2-component receptor complex (sensor kinase) (Simon *et al.,* 2007), and subsequently autophosphorylates at a conserved histidine residue (H). The phosphoryl group is then transferred to a cognate response regulator protein. The response regulator is phosphorylated on a conserved aspartate residue (D). The phosphorylated response regulator activates the transcription process of targeted genes (Henk and Bassler, 2004; Sturme *et al.,* 2002; Miller and Bassler, 2001). Few Gram-positive bacteria which are pathogenic to human, found to produce virulence factors that are controlled by QS activity such as *Staphylococcus aureus*, *Listeria monocytogens*, *Enterococcus faecalis, Streptococcus pneumonia* and *Clostridium perfringens* (Rutherford and Bassler, 2012). *Bacillus subtilis,* a non-pathogenic

Gram-positive bacterium is widely used in aquaculture as a probiotics (Vaseeharan and Ramasamy, 2003).

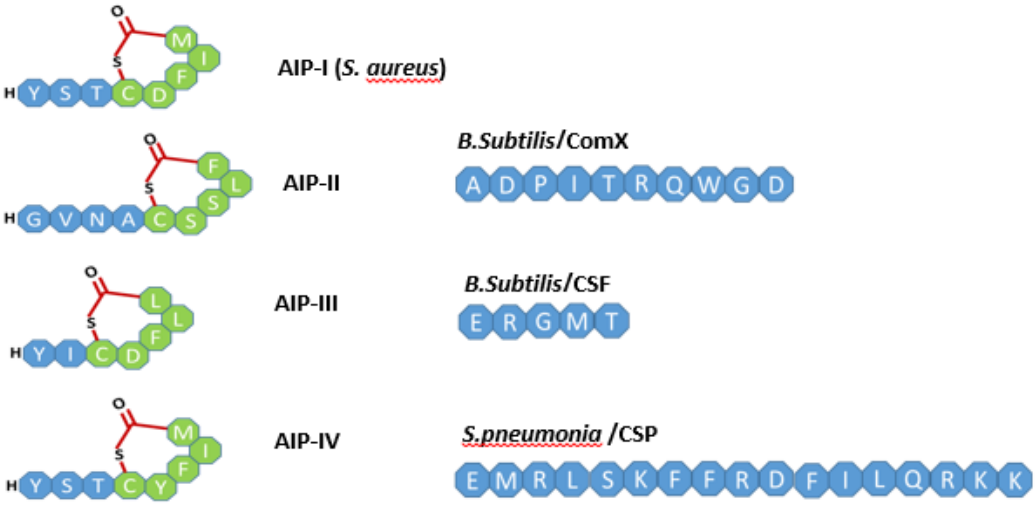


Figure 2.11. Different oligopeptide base autoinducers used by Gram-positive bacteria for quorum sensing (redrawn from Ryan and Dow, 2008).

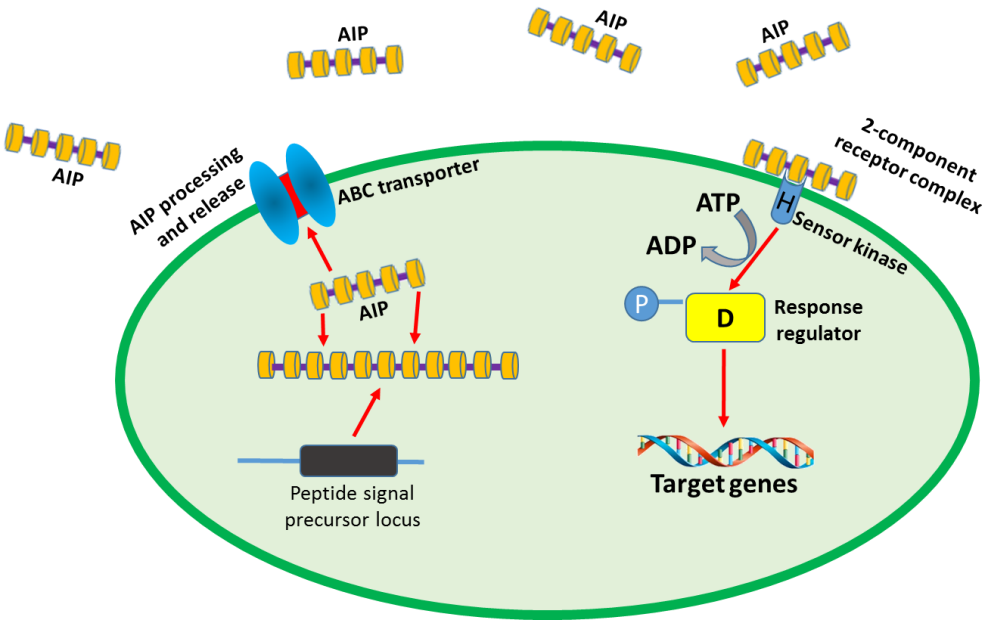


Figure 2.12. Autoinducing polypeptide (AIP) mediated signaling in Gram-positive bacteria (modified from Miller and Bassler, 2001).

c. Quorum sensing in *Vibrio harveyi*

Multichannel QS system have been reported in *Vibrios* such as *V. anguillarum*, *V.harveyi* and *V. vulnificus*, *V. parahaemolyticus* which are considered as a potent aquaculture pathogen (Defoirdt *et al.,* 2011a; Defoirdt *et al.,* 2008; Ruwandeepika *et al.,* 2012). The QS system of a particular *V.harveyi* strain BB120 (ATCC BAA-1116); recently classified as *V. campbellii* (Lin *et al.,* 2010) has been extensively studied (Yang and Defoirdt, 2014). This species use three different signal molecules namely harveyi autoinducer 1 (HAI-1), autoinducer 2 (AI-2) and cholera autoinducer 1 (CAI-1) (Cao and Meighen, 1989; Chen *et al.,* 2002; Higgins *et al.,* 2007; **Figure 2.14**). These autoinducers are detected by distinct membrane bound sensor kinase present on the cell surface, feeding a common phosphorylation/de-phosphorylation signal transduction cascade (Natrah *et al.,* 2011b; **Figure 2.13**). Due to specificity, AI-1 (as AHL) signal is used for intraspecies communication. On the other hand, AI-2 signal is use for interspecies communication (Defoirdt *et al.,* 2004; Sun *et al.,* 2004).

Harveyi autoinducer 1 (an AHL; 3-hydroxybutanoyl-homoserine lactone) is produced by LuxLM (Freeman and Bassler, 1999a; Miller and Bassler, 2001), share no homology to LuxI type enzyme but catalyze the identical biochemical reaction to generate a specific AHL (Waters and Bassler, 2005) and AI-2 (Furanosyl borate diester; 3a-methyl-5,6-dihydrofuro-[2,3-d][1,3,2]dioxaborole-2,2,6,6a-tetraol) by LuxS (Chen *et al.,* 2002; Waters and Bassler, 2005; Miller *et al.,* 2002) . Both HAI-1 and AI-2 is detected by their cognate sensors LuxN and LuxQ (Natrah *et al.,* 2011b; **Figure 2.13**). The LuxN and LuxQ are two component proteins, each contain a sensor kinase domain and response regulator domain. Another protein is required in conjugation with LuxQ to transduce the AI-2 signal (Miller and Bassler, 2001) called LuxP. Phenotypical analysis of LuxN and LuxQ mutant suggests that the two signaling systems function in parallel to regulate the density-dependent gene expression of luminescence, as the elimination of either signaling system alone does not abolish density sensing (Freeman and Bassler, 1999a). An Interaction of LuxN and LuxPQ sensor with their cognate autoinducing legands AI-1 and AI-2 stimulates a phosphatase activity in the sensors and transduce a signal to shared integrator protein LuxU (Freeman and Bassler, 1999). LuxU is a phosphotransferase protein, transfers the signal to the response regulator protein LuxO (Bassler *et al.,* 1994). LuxO action is indirect and it was confirmed that LuxO together with the alternative sigma factor σ54 controls the expression of the luciferase structural operon luxCDABE. A transcriptional activator protein LuxR is also required for the expression of luciferase structural operon (Miller *et al.,* 2002). HAI-1 activity is restricted to *V.harveyi* and closely related bacterium *V. parahaemolyticus*, indicating that this signal is species specific (Henke and Bassler, 2004a). AI-2 has been reported to regulate specific target gene in many pathogenic bacteria such as *V. harveyi*, *Vibrio cholerae*, *Escherichia coli*, *Salmonella*

typhimurium, *Porphyromonas gingivalis*, *Shigella flexneri*, *Streptococcus pyogenes*, *Neisseria meningitidis*, *Actinobacillus actinomycetemcomitans, Borrelia burgdorferi*, and *Clostridium perfringens* (Bassler, 2002).

A third kind of signaling molecule reported in *V.harveyi* is cholera autoinducer 1 (CAI-1; 3-hydroxytridecan-4-one) which is synthesized by CqsA (not similar to LuxM) and is detected by sensor protein CqsS (Liu *et al.,* 2013; **Figure 2.13**). Cqs system function in parallel with HAI-1 and AI-2 which controls the QS target genes and all three autoinducers together display synergistic activity. However, the *V. harveyi* CAI-1-CqsS system appear to be different from LuxN and LuxPQ system by responding to autoinducer at much lower cell densities (Henke and Bassler, 2004). By the time, CAI-1 is only known to be present in *Vibrio* species and considered to be a part of *Vibrio*-specific interspecies language (Liu *et al.,* 2013). As like *V.harveyi*, three parallel signal transduction pathways have been also characterized in *V. alginolyticus* (Liu *et al.,* 2011).

At low signal molecule, LuxN, LuxPQ and CqsS receptors function as kinases. LuxO is phosphorylated via LuxU, sRNAs are transcribed and LuxR protein is not produced. In presence of high signal molecules LuxN, LuxPQ and CqsS receptors function as phosphatase, LuxO is dephosphorylated, sRNAs are not transcribed and LuxR is produced which activates the expression of target genes by binding to their promoter regions (Yang and Defoirdt *et al.,* 2014; **Figure 2.13**).

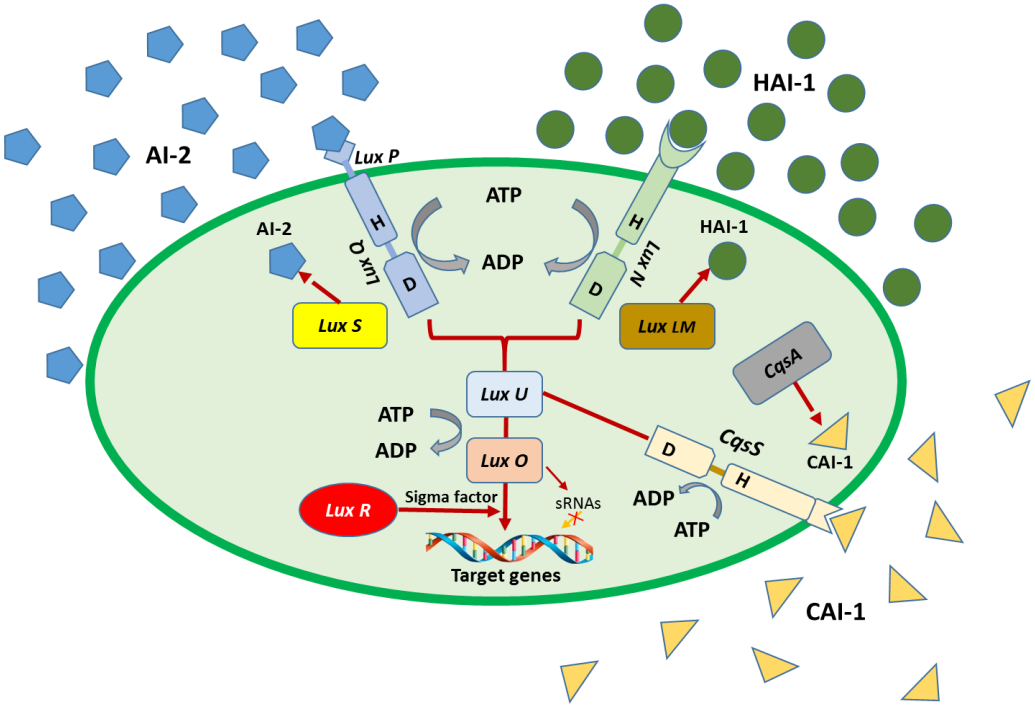


Figure 2.13. Multi-channel signaling pathways in *Vibrio harveyi* (modified from Defoirdt *et al*., 2008).

AI-2 **CAI-1** **HAI-1**

Figure 2.14. Quorum sensing signal molecules produced by *Vibrio harveyi* (redrawn from Natrah *et al*., 2011b).

2.3.3. Quorum Quenching

The wide and frequent use of antibiotics in the past has brought various complications in resistance development among pathogens (Defoirdt *et al.,* 2010a; 2011a) and now it has become one of the greatest challenge (Rasko and Sperandio, 2010). However, the increasing knowledge of bacterial pathogenesis and cell-to-cell communication mechanism, suggest a new anti-infective strategy i.e. “Quorum Quenching” (Chu *et al.,* 2013). Quorum quenching referred as the process of disruption of quorum sensing signaling molecules to control pathogenic bacteria without interfering with their growth (Tinh *et al.,* 2007; Finch *et al.,* 1998; Hentzer *et al.,* 2003). In particular, this strategy might be useful to aquaculture (Defoirdt *et al.,* 2004). Another control strategy is aimed at killing bacteria or preventing their growth by anti-virulence strategies. This may be useful in developing novel drugs to treat bacterial-mediated infectious diseases (Rasko and Sperandio, 2010).

Quorum quenching can be achieved in several ways such as inhibition of signal molecule synthesis, application of quorum sensing antagonists, chemical inactivation of QS signals, biodegradation of signal molecule by bacterial lactonases and acylases, and by application of QS agonists (Defoirdt *et al.,* 2004).

a. Targeting signal molecule synthase (Lux I)

An inhibition of signal molecule synthesis can be achieved by inhibiting the enzyme involved in the biosynthesis of acyl chain (acyl-acyl carrier protein) (ACP) or S-adenosylmethionine (SAM) synthase or LuxI homologous protein (Parveen and Cornell, 2011; Defoirdt *et al.,* 2004).

b. Targeting the receptor (Lux R) using QS antagonists

Natural or synthetic halogenated furanones and undefined exudate of higher plants and algae can be used as a QS antagonistic molecule (Defoirdt *et al.,* 2004). A best example of anti-virulence compound i.e. brominated furanone (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2-

(5H)-furanone, which is secreted by the red marine algae *Delisea pulchra* to prevent biofilm formation (Maeda *et al.,* 2012). It was reported that the furanone block all three channels of *V. harveyi* at once by acting at the end of the QS signal transduction cascade (Defoirdt *et al.,* 2008). This natural compound was reported to inhibit acyl-homoserine lactone based and autoinducer-2 based QS in other pathogenic bacteria (Ren *et al.,* 2001; Manefield *et al.,* 1999; 2002; Defoirdt *et al.,* 2006). Synthetic analogues of AHLs, such as N-acyl-3-amino-5H-furanone effectively block LuxR protein preventing cognate signal molecules binding (Czajkowski and Jafra, 2008). Other compound such as cinnamaldehyde, (Z-)-4-bromo-5-(bromomethylene)-2(5H)-furanone & (Z)-4-((5-(bromomethylene)-2-oxo-2,5-dihydrothiophen-3-yl)metoxy)-4-oxobutanoic able to disrupt QS in *V. harveyi* when challenged to *M. rosenbergii* larvae (Pande *et al.,* 2013a). A variety of higher plants such as pea, soyabean, rice, tomato, crown vetch and *Medicago truncatula* were found to secrete specific QS mimics to affect specific bacterial pathogens (Bauer and Robinson, 2002; Teplitski *et al.,* 2000). A Gram-positive marine *Actinomycetes* bacteria releases antagonistic molecule that inhibit biofilm formation of *V. harveyi*, *V. vulnificus* and *V. anguillarum*, without affecting the growth (You *et al.,* 2007). Recently, a marine *Halobacillus salinus* has been reported that secrete secondary metabolite capable of QQ in several Gram-negative reporter strain by blocking QS regulated phenotype (Teasdale *et al.,* 2011, 2009). Group of bacterial spp. related to *Actinomycetes*, *Streptomyces*, *Micromonospora*, *Salinispora* have been reviewed with respect to their broad distribution and their potential for use as probiotics (Das *et al.,* 2008; Hirsch and Valdes, 2010)

c. Biodegradation of signal molecules

Based on AHL structure, Dong and Zhang (2005) suggested that four different ways of degradation may occur mediated by lactonase, decarboxylase, acylase and deaminase. Of these only two types of QQ enzyme have been found, namely lactonase and acylase (Hong *et al.,* 2012; **Figure 2.15**). These two enzyme used by bacteria as a defense mechanism against antibiotic-producing bacteria in the ecological niche (Gonzalez and Keshavan, 2006), source of energy in form of carbon and nitrogen (Stephane *et al.,* 2003) and as a bio-control agent (Rasmussen *et al.,* 2005; Dong and Zhang, 2005).

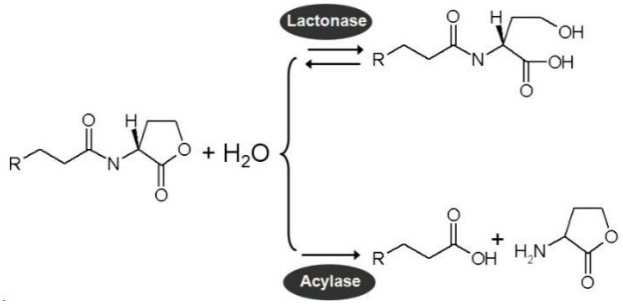


Figure 2.15. Degradation of AHL molecules by the action of lactonase and acylase enzyme.

AHL-lactonase

The enzyme lactonase hydrolyzes the ester bond of the lactone ring forming acyl homoserine, which renders the signaling molecules incapable of binding to their target transcriptional regulator (Dong *et al.,* 2001; 2000). This phenomenon was first reported in *Bacillus sp.* isolates 240B1 encoded by gene AiiA (Lee *et al.,* 2002; Defoirdt *et al.,* 2011; Dong *et al.,* 2007; 2002). AHL lactonases appears to be a potent enzyme and effectively hydrolyze four different type of AHL C4-HSL, 3-oxo-C6-HSL, 3-oxo-C8-HSL, and 3-oxo-C12-HSL (Wang *et al.,* 2004). Few other bacteria are also reported to possess an ability to disrupt QS by opening lactone ring such as *Agrobacterium*, *Arthrobacter*, *Bacillus thuringiensis*, *B. cereus*, *B. mycoides*, *B. anthracis*, and *Klebsiella* spp. (Chan *et al.,* 2010; Chu *et al.,* 2011; Kalia *et al.,* 2011; Dong and Zhang*,* 2005). Recently a bacillus strain MBG11 with polymorphism has been reported which may confer increased stability to AHL lactonase and can be a potential candidate for heterologous expression (Huma *et al.,* 2011). Different QQ enzymes with lactonase activity were characterized from various microorganisms are summarized in the given **Table 2.3**.

AHL acylase

An AHL acylase enzyme cleave the peptide bond of the lactone ring, releases a fatty acid and homoserine lactone, and thus cause significant reduction in effectiveness of the signaling molecule (Lin *et al.,* 2003). Many different type of acylase reported, differ in term of substrate specificity for side chain and nucleus. A well know example is β-lactam acylase which has been mainly targeted for the production of semisynthetic β-lactam antibiotics (Sio *et al.,* 2006; Bruggink *et al.,* 1998; Sio and Quax, 2004). Different QQ enzymes with acylase activity were characterized from various microorganisms are summarized in the given **Table 2.4**.

Other AHL degrading/inactivation strategies

The use of chemical agents, such as oxidized halogen, antimicrobials hypochlorous and hypobromous acids could be an alternate strategy to inactivate AHLs. These chemicals rapidly react and destroy 3-oxo-acyl HSLs activity (Borchardt *et al.,* 2001). The pH and temperature also play a role in AHL inactivation. At alkaline conditions (pH>7) and high temperature (>37^{o}C) lactone ring of AHL molecule become unstable, causing lactonolysis (Yates *et al.,* 2002). The pH mediated inactivation by hydrolysis is restricted only to the AHL molecule with shorter acyl-chain (C<10) (Voelkert and Grant, 1970; Michels *et al.,* 2000; Yates *et al.,* 2002). Therefore, long chain AHLs are more stable than short chains (Yates *et al.,* 2002; Natrah *et al.,* 2011b). It was reported that the dial pH fluctuation determines different acyl-chain in natural microbial mat (Decho *et al.,* 2009).

Table 2.3. Characterization of AHL- lactonase enzymes in various microorganisms.

Bacterial Strain	QQ enzymes	Role of QQ enzymes	AHL degradation	References
Agrobacterium tumefaciens c58	AttM	AiiB modulates the conjugation frequency of Ti plasmid and the emergence of tumour; AttM enhances the fitness of *A. tumefaciens* in the plant tumour	3-oxo-C8-HSL, C6-HSL	Zhang *et al*., 2002; Haudecoeur *et al*., 2009 ; Haudecoeur and Faure, 2010
Arthrobacter sp. IBN110	AhlD	Metabolism of AHLs as carbon and nitrogen sources	3-oxo-C6-HSL, C4-HSL, C6-HSL, C8-HSL, C10-HSL	Park *et al*., 2003
Bacillus sp. 240B1	AiiA	Microbial competition; Control of the toxicity effects of AHLs and tetramic acid derivatives, Competition for iron from the environment	3-oxo-C6-HSL, 3-oxo-C8-HSL, 3-oxo-C10-HSL	Dong *et al*., 2000; Park *et al*., 2008; Kaufmann *et al*., 2005
Geobacillus kaustophilus strain HTA426	GKL	unknown	C6-HSL, C8-HSL, C10-HSL, 3-oxo-C8-HSL and 3-oxo-C12-HSL	Chow *et al*., 2010
Microbacterium testaceum StLB037	AiiM	Providing protection to the plant from pathogens for the purpose of symbiotic interaction with the host	3-oxo-C6-HSL, C6-HSL, 3-oxo-C8-HSL, C8-HSL, 3-oxo-C10-HSL, C10-HSL	Wang *et al*., 2010

Bacterial Strain	QQ enzymes	Role of QQ enzymes	AHL degradation	References
Mycobacterium avium subsp. *Paratuberculosis* K-10	MCP	Unknown	C7-HSL, C8-HSL, 3-oxo-C8-HSL, C10-HSL, C12-HSL	Chow *et al*., 2009
Mycobacterium Tuberculosis	PPH		C4-HSL, C10 HSL, 3-oxo- C8-HSL	Afrait *et al*., 2006
Ochrobactrum sp. T63	AidH	Unknown	C4-HSL, C6-HSL, 3-oxo-C6-HSL, 3-oxo-C8-HSL, C10-HSL	Mei *et al*., 2010
Rhodococcus erythropolis W2	QsdA	Involvement in fatty acid metabolism	AHLs with or without substitution on carbon 3 and with an acyl chain ranging from 6 to 14 carbons	Afrait *et al*., 2006; Uroz *et al*., 2008
Solibacillus silvestris StLB046	AhlS		C6-HSL, 3-oxo-C6-HSL, C10-HSL, 3-oxo-C10-HSL	Morohoshi *et al*., 2012
Sulfolobus solfataricus strain P2	SsoPox		3-oxo-C8-HSL, C8-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL	Merone *et al*., 2005; Elias *et al*., 2008

Table 2.4. Characterization of AHL-acylase enzymes in various microorganisms.

Bacterial Strain	QQ enzymes	Role of QQ enzymes	AHL degradation	References
Anabaena sp. PCC7120	AiiC	Interference with the communication system within the complex microbial communities; control of the cytotoxicity effect of AHLs	AHLs with or without substitution on carbon 3 and with an acyl chain ranging from 4 to 14 carbons	Romero *et al.*, 2008
Comamonas sp. strain D1	unknown		AHLs with or without substitution on carbon 3 and with an acyl chain ranging from 4 to 16 carbons	Uroz *et al.,* 2007
Pseudomonas aeruginosa PAO1	PvdQ; High homology with AiiD	Regulation of pyoverdine biosynthesis; utilization of AHL; involvement in the maturation of pyoverdine siderophore; regulation of 3-oxo-C12-HSL, regulation of virulence phenotype; playing a role in iron sequestration; regulation of flagellum-dependent motions; development of antibiotic resistance; development of pyoverdine I	AHLs with or without substitution on carbon 3 and with an acyl chain ranging from 10 to 14 carbons	Huang *et al.,* 2003; Sio *et al*., 2006; Jimenez *et al*., 2010; Wang *et al*., 2011; Hannauer *et al*., 2012
Pseudomonas aeruginosa PAO1	QuiP		AHLs with or without substitution on carbon 3 and with an acyl chain ranging from 7 to 14 carbons	Huang *et al*., 2006
Pseudomonas syringae strain B728a	HacA		C8-HSL, C10-HSL and C12-HSL	Shepherd and Lindow, 2009

Bacterial Strain	QQ enzymes	Role of QQ enzymes	AHL degradation	References
Pseudomonas syringae strain B728a	HacB		AHLs with or without substitution on carbon 3 and with an acyl chain ranging from C_6 to C_{12}	Shepherd and Lindow, 2009
Ralstonia sp. XJ12B	AiiD	Oligotrophic nutrient scavenging from the natural environment	3-oxo-C8-HSL, 3-oxo-C10-HSL and 3-oxo-C12-HSL (less activity against 3-oxo-C6-HSL)	Lin *et al*., 2003
Ralstonia solanacearum GMI1000	Aac	Metabolism of AHL as source of carbon and nitrogen; Modulation of the QS pathways or as a signal turnover mechanism	C7-HSL and C8-HSL, 3-oxo-C8-HSL and C10-HSL	Chen *et al*., 2009 Lin *et al*., 2003
Rhodococcus erythropolis W2	unknown	Involvement in fatty acid metabolism	3-oxo-C10-HSL	Uroz *et al*., 2005 Uroz *et al*., 2008
Shewanella sp. strain MIB015	Aac	Unknown	C8-HSL, C10-HSL and C12-HSL	Morohoshi *et al*., 2008
Streptomyces sp. strain M664	AhlM	Unknown	C8-HSL, C10-HSL, 3-oxo-C12-HSL	Park *et al*., 2005
Tenacibaculum maritimum strain NCIMB2154(T)	unknown		C10-HSL	Romero *et al*., 2010
Variovorax paradoxus VAI-C		Metabolism of AHLs as carbon and nitrogen sources	C4-HSL, C6-HSL, 3-oxo-C6-HSL, C8-HSL, C10-HSL, C12-HSL,C14-HSL	Leadbetter and Greenberg, 2000

2.4. Microalgae and Green water system in aquaculture

2.4.1. Commonly used microalgae for green water culture and its beneficial effects in aquaculture.

Green water culture technique is the way of adding microalgae to the system during larval rearing process (Conceicao *et al*, 2010). Microalgae species generally used in green water culture include; *Nannochloropsis spp., chlorella spp*., *Isochrysis spp*., *Tetraselmis spp*. and *Dunaliella* spp. with a cell density ranging from 2,000 cells/ml to 50,000 cells/ml. In general *N. oculata* tends to be the dominant algal species used for GWC (Fotedar and Phillips, 2011; Khan *et al.,* 2002). *Spirulina*, a blue-green algae is cultured in a mass scale and used as animal feed in aquaculture (Roychoudhury and Mukherjee, 2013).

Green water culture technique reported to have a beneficial effect on the larval growth and survival in several ways. It was found that bacteria isolated from green water system has a potential ability to nitrify ammonia and nitrite aerobically (Velusamy and Krishnani, 2013). Krishnani *et al.* (2010), isolated sulphur oxidizing bacteria from green water system which maintains safe level of sulfide concentration within system. Luminous bacterial count was reported lower in green water than non-green water (Tendencia *et al.,* 2013). Lio-po *et al*. (2002), suggested that extracellular and intracellular metabolites of microalgae are efficient in controlling growth of pathogenic bacteria. Tendencia *et al.* (2012), confirmed that the abundance of chlorophyceae in GW improves the shrimp immune competence to resistance diseases, resulting in higher survival. An *in vitro* study confirms that use of *Tetraselmis suecica* as GW inhibits the growth of *Vibrio spp.* (Regunathan and Wesley, 2004). Green water system were also reported to improve assimilation efficiency of animal, increase nutritional value of live prey and enhance visual contrast allowing better visibility of prey organism (Reitan *et al*., 1997; Papandroulakis *et al.* 2002).

2.4.2. Microalgae growth dynamics

There are five well defined growth phases of axenic culture of microalgae as mentioned by Coutteau, 1996 (**Figure 2.16**).

1. ***Lag phase:*** also known as adaptation phase. The algal cells adjust itself to the altered conditions and the specific growth rate is significantly lower. They use new nutrients to replenish their internal pools of nitrogen and phosphorus. The physiological age of the cell affects their capability of multiplication, such as cells taken from late lag phase, log phase or early stationary phase will produce a shorter lag time than cells from late stationary phase. Lag phase are much more sensitive to temperature or other environmental changes (Becker, 1994).

2. ***Exponential phase:*** At this phase cell divide and multiply rapidly and the cell density increases as a function of time according to logarithmic function.

$$C_t = C_0.e^{mt}$$

Where, C_t and C_0 being the cell concentration at time t and 0 respectively and m is the specific growth rate depend on algal species, light intensity and temperature (Coutteau, 1996). If cell numbers are plotted on log scale versus time, the increase of cell number is linear.

The generation time or doubling time of the microalgae can be calculated mathematically using this formula (Lee and Shen, 2004):

$$N_t = N_0.2^n$$

Where, N_t: no. of cells at time t, N_0: no. of cells at time 0 and n is the no. of generations.

3. ***Phase of declining growth rate:*** Cell division slows down when nutrients, light, pH, carbon dioxide or other physical and chemical factors begin to limit growth.
4. ***Stationary phase or linear growth phase:*** Culture enters stationary phase when net growth is zero with a relatively constant cell density. Oxidative breakdown of synthesized substances begin to reduce the constant increment of the cell density and equilibrium is reached between the maximum algal biomass concentration and loss due to degradation process. Light become limited and respiration plays and increasing role (Becker, 1994).
5. ***Death phase or crash phase:*** Nutrients are exhausted, cells starve, cell division stops, cell density decreases rapidly, water quality deteriorates and the culture eventually collapses (Coutteau, 1996).

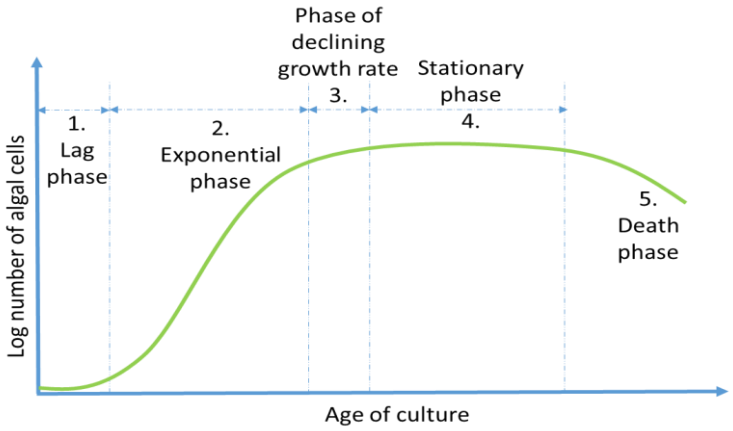


Figure 2.16. Algal growth curve.

2.4.3. Potential role of algae in quorum quenching

Microalgae and bacteria co-exist in aquatic environment and form a unique symbiosis association called phycosphere (Sapp *et al.,* 2007). In this association, algae releases large amounts of organic compound (extracellular product) that can be assimilated by the bacteria and in turn, bacteria provide inorganic nutrient and growth factors to algae (Bi *et al.,* 2012). Such interaction play and important role during biogeochemical cycle within the microbial loop (Azam, 1998; Sapp *et al.,* 2007).

In some cases, bacteria and micro-algae are able to raise the pH (particularly during late growth phase), which can affect the stability of quorum sensing molecules in their surroundings (Natrah *et al.,* 2011b). Micro-algae can mimic the AHL signal molecule which can block the receptor either through competition or displacement of original AHL that inhibit QS activity. One such example is a unicellular soil-freshwater alga *Chlamydomonas reinhardtii*, found to secrete substances that mimic the activity of the AHL (Teplitski *et al.,* 2004; Natrah *et al.,* 2011b). The ability to interfere with QS using different microalgae were tested. It was suggested that the extract of marine algae *Nanochloropsis*, *Isochrysis* sp., *T. suecica*, *T striata*, *T. tetrathele* and freshwater algae *C. reinhardtii* significantly decrease QS regulated green fluorescence protein production (Natrah *et al.,* 2011a), Whereas *Chlamydomonas reinhardtii* and *chlorella* spp. were reported to stimulated QS regulated luminescence in *V. harveyi*, indicating that algae may produce compounds that affect the AI-2 mediated QS of *Vibrio* spp. (Teplitski *et al.,* 2004).

Macroalgae have been shown to produce chemical compounds that interfere with QS. *Delisea pulchera*, a red alga known to synthesize halogenated furanones which inhibit QS activity (Natrah *et al*., 2011b). A marine macroalgae *Asparagopsis taxiformis* showed antibacterial, as well as anti-QS activity (Jha *et al*., 2013). *Laminaria digitata* produces oxidized halogen compounds that can deactivate AHL molecules (Natrah *et al*., 2011a)

3. MATERIALS AND METHODS

3.1. *In vitro* experiments

3.1.1. Bacterial strains and growth conditions

The bacterial strains used in this study are listed in **Table 3.1**. The bacterium stock was stored in 40% sterile glycerol at -80ºC. The stored stocks were aseptically streaked onto Luria-Bertani (LB) agar plate containing 12 $g.l^{-1}$ of NaCl (Prolabo®, Belgium), so called LB_{12}. After 24 hours of incubation at 28ºC, a single colony was picked and inoculated into 5 ml LB_{12} broth. The bacterial cultures were incubated overnight at 28ºC under constant agitation (100 min^{-1}) and cell density was measured with the spectrophotometer (Thermo Spectronic) at 600 nm (OD_{600}).

Table 3.1. The bacterial strains used in this study.

Strains	Relevant information	References
Vibrio campbellii BB120*	Quorum sensing bacterium	Bassler *et al*., 1997
NFMI-T	Acyl-Homoserine Lactone (AHL) degrader	This study
NFMI-C	Acyl-Homoserine Lactone (AHL) degrader	This study
Vibrio campbellii JAF548	Mutation in luxO (AHL and AI-2 signal transduction)	Defoirdt *et al*., 2005
Vibrio campbellii BB120* RifR	Spontaneous rifampicin resistant mutant of BB120	This study
NFMI-T RifR	Spontaneous rifampicin resistant mutant of NFMI-T	This study
NFMI-C RifR	Spontaneous rifampicin resistant mutant of NFMI-C	This study

*_Vibrio campbellii_ BB120 with genomic sequence ATCC BAA-1116 previously designated _Vibrio harveyi_. (Lin *et al*., 2010).

3.1.2. Selection of natural rifampicin resistant mutants

Initially, 5000 ppm rifampicin (Sigma Aldrich) stock was prepared in 10 ml of 99.9% methanol (HPLC grade) and wrapped with aluminum foil. Stock solution of rifampicin was then kept at 4ºC for further use. From the streaked LB_{12} plates of different bacterial strains, a single colony was picked and inoculated into 5 ml LB_{12} broth containing 2 ppm rifampicin. The concentration of rifampicin was gradually increased from 2 ppm to 5 ppm, 10 ppm, 20 ppm and 50 ppm

respectively in subsequent days. At last, fully grown bacterial strains in 50 ppm rifampicin was mixed with 40% glycerol (1:1 v/v) and stored at -80^{o}C.

3.1.3. AHL degradation assay

a. Culture of AHL reporter strain

Chromobacterium violaceum CV026 was used as a reporter strain (Natrah *et al*., 2011a) which cannot produce AHL but can be able to detect AHL molecules with acyl-side chain of 4 to 8 carbon atoms. In presence of exogenous hexanoyl homoserine lactones (HHL), it produces purple pigment called violacein. From the stock of *C. violaceum* CV026, 10 µl of inoculum was added into 5 ml of LB_{12} broth. The inoculated broth was incubated at 28^{o}C for 24h under constant agitation (100 min^{-1}).

b. HHL preparation

A vial containing 10 mg of N-hexanoyl-L-homoserine lactone (HHL; Sigma-Aldrich) was mixed with 10 ml of autoclaved distilled water and 1000 ppm of stock solution was prepared. It was then wrapped with aluminum foil and stored at -20^{o}C for further use.

c. Preparation of AHL degradation assay

The optical density of an overnight grown culture of AHL degraders in LB_{12} broth was measured and cell density was calculated. AHL degrading strains NFMI-T and NFMI-C (10^{7} $CFU.ml^{-1}$) was inoculated into 20 ml of fresh LB_{12} broth with MOPS (200 $mg.l^{-1}$) and HHL (10 ppm). LB_{12} broth with MOPS and HHL was considered as negative control. Cultures were grown until 48h at 28^{o}C with constant agitation (100 min^{-1}). MOPS was used to stabilize the pH of the culture medium to 6.5.

d. Confirmation test

At 24h and 48h, fully grown cultures along with negative control were filtered using 0.2 µm filter. 100 µl of an overnight grown reporter strain CV026 was spread evenly on LB_{12} agar plates, and subsequently 10 µl of the cell-free supernatant was dropped to the center of the LB_{12} plates. The plates were then incubated at 28^{o}C for 24h, and purple violacein pigment was observed.

3.1.4. Coculture of algae and QS signal degrading bacteria

Starter inoculum of *Tetraselmis suecica* was obtained from the algal stock room of Artemia Reference Center, Ghent University, Belgium. Autoclaved seawater of 12 ppt (UV treated) and walne media was used for microalgae culture. Walne media was used as a growth medium

for microalgae. Initially, the cell density of microalgae was counted using Burker haemocytometer, and then 10^5 cells.ml^{-1} was added into cultured water containing walne media. Subsequently, natural rifampicin resistant mutants of AHL degraders (10^5 CFU.ml^{-1}) were also added into the culture. The composition of the walne media and quantities added are provided in **Table 3.2**. The culture was kept at 20-22^{o}C under constant illumination and filtered air was allowed to enter in the bottle (**Figure 3.1**). For determining the growth of microalgae, daily sampling was performed until culture reached the stationary phase and the density was determined using Burker haemocytometer. On 1st, 7th and 14th day, the algal samples were collected and plated on LB_{12} agar plates containing 50 ppm rifampicin to enumerate the amount of AHL degrader bacteria present in the coculture.

Table 3.2. Composition of Walne media and quantities added for microalgae culture.

Reagents	Quantity
Solution A	1ml/l water
Na_2EDTA, H_3BO_3, $NaNO_3$ (KNO_3), $NaH_2PO_4.H_2O$, $MnCl_2.4H_2O$, $FeCl_3.6H_2O$	
Solution B	Solution B mixed with solution A (1:10)
$ZnCl_2$, $CoCl_2.6H_2O$, $(NH_4)_6Mo_7O_{24}.4H_2O$, $CuSO_4.5H_2O$	
Vitamin mixture	0.1ml/l water
Thiamin chlorhydrate, Cynocobalamin	

Please note: Detailed information are provided in the Annexure

Figure 3.1. AHL degraders-microalgae (*Tetraselmis suecica*) coculture experimental setup.

3.1.5. Determination of the impact of AHL degraders and their supernatants on growth of *V. campbellii* BB120.

a. Impact of cell-free supernatant of the AHL degraders

For this assay, *V. campbellii* BB120 strain was cultured until 10^9 CFU.ml^{-1} (OD_{600} = 1) and AHL degrader strains were grown overnight. Further, BB120 strain was diluted 100 times to get 10^7 CFU.ml^{-1}. An overnight grown AHL degrader strains (NFMI-T and NFMI-C) were filtered by whatman 0.2 µm filter and their cell-free supernatant was extracted. The cell-free extract of different AHL degraders was taken as negative control. Based on treatments, BB120 and cell-free supernatant of AHL degraders were mixed with different volume ratio (**Table 3.3**). 200 µl of the mixed culture was pipetted and transferred in each well (96 well plates, transparent type). Further, culture was grown in microaerobic atmosphere for 24h at 28^{o}C and turbidity at 600 nm was monitored every hour using a multi-reader machine (Infinite® 200-TECAN, Austria). The experiment was performed in twelve replicates.

Table 3.3. Experimental design for growth assay of *V. campbellii* BB120 with cell-free supernatants of degrader bacteria.

Treatments	Cell density of BB120	Volume ratio
LB_{12} (negative control)	-	1
$[NFMI\text{-}T]_{supernatant}$	-	1
$[NFMI\text{-}C]_{supernatant}$	-	1
$[NFMI\text{-}T+C]_{supernatant}$	-	50:50
*BB120 + $[NFMI\text{-}T]_{supernatant}$	10^7	50:50
*BB120 + $[NFMI\text{-}C]_{supernatant}$	10^7	50:50
*BB120 + $[NFMI\text{-}T+C]_{supernatant}$	10^7	50:(25:25)

*1ml of BB120 (10^7 CFU.ml^{-1}) was pipetted in eppendorf tubes and centrifuge at 5000 rpm for 10 min. 500 ml of the supernatant was removed slowly and then 500ml cell-free extracts of AHL degraders were added to make equal volume mixture.

b. Coculture with AHL degraders

The growth was measured based on the bioluminescence of *V. campbellii* BB120 strain. The BB120 strains and other AHL degraders were mixed in equal v/v ratio (**Table 3.4**). 200 µl of the mixed culture was pipetted and transferred in each well (96 well plates, black type). Further, culture was grown in microaerobic atmosphere for 12h at 28^{o}C and bioluminescence was monitored every hour using a multi-reader machine (Infinite® 200-TECAN, Austria). A non-luminescent strain JAF548 was added with BB120 and considered as positive control. The experiment was performed in six replicates.

c. Coculture with microalgae and AHL degraders

In this experiment, additionally 10^4 cells.ml^{-1} of *Tetraselmis suecica* was added in each treatment, displayed in **Table 3.4**. The bioluminescence was monitored every hour using a multi-reader machine (Infinite® 200-TECAN, Austria). The experiment was performed in six replicates.

Table 3.4. Experimental design for growth assay of BB120 with AHL degrader isolates.

Treatments	Cell density	Volume ratio
LB_{12} (negative control)	-	1
*BB120 + JAF548#	10^7	50:50#
*BB120 + NFMI-T	10^7	50:50
*BB120 + NFMI-C	10^7	50:50
*BB120 + [NFMI-T+C]	10^7	50:50

* 1ml of BB120 (10^7 CFU.ml^{-1}) and 1ml of AHL degrader isolates (10^7 CFU.ml^{-1}) was pipetted in eppendorf tubes and centrifuge at 5000 rpm for 10 min. From all tubes, 500 ml of the supernatant was removed slowly and then BB120 and AHL degraders were mixed together. For NFMI-T and NFMI-C, 1ml of strains with a cell density of 0.5×10^7 CFU/ml was centrifuged separately, then 500ml of the supernatant was removed, mixed together to make 1ml volume, centrifuged, and again 500ml of supernatant was removed. Later, NFMI-T+C mixture was mixed with BB120.

JAF548 was not taken into consideration when coculture of microalgae and AHL degraders was performed.
BB120 (10^7 CFU.ml^{-1}) supplemented with microalgae (10^4 CFU.ml^{-1}) were taken as positive control.
The microalgae cells was counted and concentrated at high density. The volume ratio is 1 in this case.

3.1.6. Virulence factors assays

Virulence factor assay was performed with AHL degraders and their cell-free supernatant. The culture of AHL degraders was filtered with Whatman 0.2 µm filters to get cell-free extract. All assays were performed in triplicate.

a. Swimming motility

For performing motility test with AHL degraders, soft LB_{12} agar (0.3% agar), sterilized at 121^{o}C for 20 minutes and plates was prepared. Mixtures of BB120 and AHL degraders were prepared (**Table 3.5)**. Then, 5 µl of mixed culture was spotted on each plate. Plates spotted with BB120 were considered as control.

Swimming motility assay with cell-free supernatant was performed by mixing soft LB_{12} agar plates (0.3% agar) with 10% v/v cell-free extract of AHL degraders. Soft LB_{12} plates without cell-free supernatant were used as control. 5 µl of well grown BB120 strain (OD_{600} = 1) was spotted at the center of each plate.

After 24h of incubation at 28^{o}C, the diameter of halo was measured in both cases.

b. Caseinase

For performing virulence assay with AHL degrader isolates, plates were prepared by mixing double strength LB_{12} with 4% skim milk powder suspension (Fluka). They were sterilized separately at 121^{o}C for 20 minutes. Mixtures of BB120 and AHL degrader isolates were prepared (**Table 3.5)**. Then, 5 µl of mixed culture was spotted on each plate. Plates spotted with BB120 were considered as control.

For virulence assay with cell-free supernatants, autoclaved double strength LB_{12} and skim milk powder suspension (Fluka) was mixed properly and distributed into 50 ml of falcon tube. Then, 10% v/v cell-free extract of AHL degraders was added in the agar and plates were prepared. The LB_{12} plates without cell-free supernatant were used as control. 5 µl of well grown BB120 strain (OD_{600} = 1) was spotted at the center of each plate.

After 48h of incubation at 28^{o}C, the colony diameter and clearing zone were measured in both cases.

c. Hemolytic activity

Hemolytic assay was performed only with cell-free supernatant. For this assay, plates were prepared by supplementing LB_{12} with 5% defibrinated sheep blood (Oxoid, UK) and 10% v/v cell-free extract of different AHL degraders. LB_{12} plates mixed with sheep blood were used as control. 5 µl of well grown BB120 strain (OD_{600} = 1) was spotted at the center of each plate. After 48h of incubation at 28^{o}C, the colony diameter and clearing zone was measured.

Table 3.5. Experimental design for virulence factors assay such as caseinase and swimming motility with AHL degrader isolates.

Treatments	Cell density	Volume ratio
BB120	5×10^{8}	1
*BB120 + NFMI-T	10^{9}	50:50
*BB120 + NFMI-C	10^{9}	50:50
*BB120 + [NFMI-T+C]	10^{9}	50:50

* 1ml of BB120 (0.5×10^{9} CFU.ml^{-1}) and 1ml of AHL degrader isolates (0.5×10^{9} CFU.ml^{-1}) was pipetted in eppendorf tubes and centrifuge at 5000 rpm for 10 min. From all tubes, 500 ml of the supernatant was removed slowly and then BB120 and AHL degraders were mixed together. For NFMI-T and NFMI-C, 1ml of strains with a cell density of 0.25×10^{9} CFU.ml^{-1}was centrifuged separately, then 500ml of the supernatant was removed, mixed together to make 1ml volume, centrifuged, and again 500ml of supernatant was removed. Later, NFMI-T+C mixture was mixed with BB120.

3.2. *In vivo* experiments

3.2.1. Preparation of *Macrobrachium rosenbergii* broodstock

Giant freshwater prawn was reared in a recirculation system at Laboratory of Aquaculture, Artemia Reference Center. Freshwater prawn broodstock maintenance was performed according to Cavalli *et al*. (2001); water quality parameter was adjusted according to New (2002); breeding strategies was followed according to (Baruah *et al*., 2009) and spawning, hatching and other maintenance procedure were performed as suggested by Pande *et al*. (2013a).

3.2.2. Preparation of larvae for challenge test

The newly hatched larvae with yolk sac were left for 24h in the hatching tank containing slightly brackishwater (6 ppt). The next day, larvae with absorbed yolk were distributed in 200 ml glass tubes containing 100 ml of 12 ppt autoclaved brackishwater (Instant ocean synthetic sea salt, Aquarium System Inc., Sarrebourg, France). Each glass tube containing 25 larvae were provided with mild aeration, and was placed in a water bath maintained at 26-27^{o}C (**Figure 3.2**). The larvae were acclimatized to the experimental conditions for 24h and were fed daily with 2 Artemia nauplii/larvae. During the experiment the water quality parameter was kept at minimum 5 mg.l^{-1} dissolve oxygen, maximum 0.5 mg.l^{-1} ammonia-N and maximum 0.05 mg.l^{-1} nitrite-N.

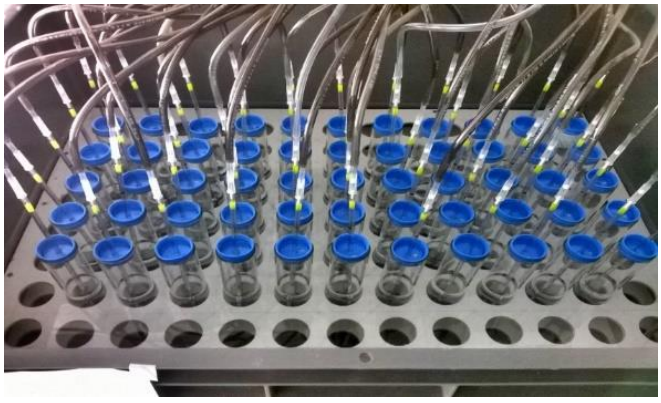


Figure 3.2. *In vivo* experimental setup.

3.2.3. Preparation of natural rifampicin resistant mutants

10 µl of natural rifampicin resistance stocks of *V. campbellii* BB120 and AHL degraders (NFMI-T and NFMI-C) from -80^{o}C was inoculated directly into 5 ml of LB_{12} broth containing 50 ppm rifampicin. The culture was incubated at 28^{o}C under constant agitation (100 min^{-1}). The strains were cultured continuously to five subsequent days by refreshing media daily. Everyday 10 µl of previous grown culture was inoculated in fresh 5 ml media.

3.2.4. Preparation of axenic *Artemia*

Decapsulation and hatching of axenic *Artemia* processes were performed as described by Marques *et al*. (2004). 200 mg of *Artemia* cyst, stored at 4ºC (INVE, Ocean Nutrition Europe, Essen, Belgium) was hydrated in 18 ml of distilled water for 1h. After hydration, decapsulation of *Artemia* cyst was achieved by adding 660 µl NaOH (32%), 10 ml NaOCl (50%) and 14 ml of $Na_2S_2O_4$ (added after 2 minutes).The decapsulation process was carried out under a laminar flow hood and filtered aeration was provide throughout the process to maintain axenic condition. The decapsulated cysts were then washed over a 100 µm sterile sieve with 12 ppt autoclaved brackishwater (Instant Ocean synthetic sea salts) and transferred to a sterile falcon tubes. A small quantity of decapsulated cyst was transferred to another falcon tube containing 30 ml of sterile seawater (35 ppt). The falcon tube was incubated at 28ºC for 24h on a rotor under constant light source. Rest of the decapsulated cyst was preserved at 4ºC for further use.

3.2.5. *M. rosenbergii* larvae challenge study

M. rosenbergii larvae challenge study were performed with and without microalgae. The xenic *Tetraselmis suecica* was collected from the Algal culture lab of Artemia Reference Center. The experimental designs of larval challenge study are summarized in given **Table 3.6** and **Table 3.7**. The day after first feeding of *Artemia*, prawn larvae were challenged with *V. campbellii* BB120 by adding strains at 10^6 CFU.ml^{-1} into the culture water. AHL degraders (NFMI-T and NFMI-C) were also added at the same time after challenging with *V. campbellii* BB120. Survival of *M. rosenbergii* larvae in the positive control (challenged with wild type *V. campbellii* BB120) was counted daily and stopped when the mortality reaches 50%. At this point larval survival was determined in all treatments by considering that only those larvae presenting movement of appendages were alive. The challenge study was performed in five replicates.

3.2.6. Larval Stage Index (LSI) measurement

At the final day of the experiment, LSI was measured according to Maddox and Manzi (1976) by sampling randomly two larvae from each replicate of treatments. LSI was then calculated and recorded based on the description by Uno and Kwon (1969).

$$\text{LSI} = \sum \frac{\text{Si}}{\text{N}}$$

Where, Si is the stage of the larvae (i = 1 to 12); N is the number of larvae examined.

Table 3.6. Experimental design for *M. rosenbergii* larvae challenge study without microalgae.

Treatments	Relevant information
Control (prawn larvae only)	
BB120	
BB120 + NFMI-T	*V. campbellii* BB120 (10^6 CFU.ml^{-1}),
BB120 + [NFMI-T]$_{autoclaved}$	*Pseudomonas* sp. NFMI-T (10^5 CFU.ml^{-1}),
BB120 + NFMI-C	*Bacillus* sp. NFMI-C (10^5 CFU.ml^{-1}) were
BB120 + [NFMI-C]$_{autoclaved}$	added according to treatments.
BB120 + [NFMI-T+C]	
BB120 + [NFMI-T+C]$_{autoclaved}$	

Table 3.7. Experimental design for *M. rosenbergii* larvae challenge study with microalgae.

Treatments	Relevant information
Control (prawn larvae only)	
BB120	
BB120 + *Tetraselmis*	
BB120 + [*Tetraselmis*]$_{autoclaved}$	*V. campbellii* BB120 (10^6 CFU.ml^{-1}),
BB120 + [*Tetraselmis* + NFMI-T]	*Tetraselmis suecica* (10^4 CFU.ml^{-1}),
BB120 + [*Tetraselmis* + NFMI-T]$_{autoclaved}$	*Pseudomonas* sp. NFMI-T (10^5 CFU.ml^{-1}),
BB120 + [*Tetraselmis* + NFMI-C]	*Bacillus* sp. NFMI-C (10^5 CFU.ml^{-1}) were
BB120 + [*Tetraselmis* + NFMI-C]$_{autoclaved}$	added according to treatments
BB120 + [*Tetraselmis* + NFMI-T+C]	
BB120 + [*Tetraselmis* + NFMI-T+C]$_{autoclaved}$	

3.2.7. Enumeration of AHL degrader isolates from cultured water and prawn larvae gut

During the challenge study, water and larvae of the treatments were sampled twice (2nd and last day when mortality reached 50%) in which only AHL degraders was added. From each tube, two larvae were selected randomly and 1ml of larval rearing water was collected. The water sample from each replicate of the treatments were mixed together in a falcon tube and later 1 ml of water was sampled from tubes and mixed with 9 ml of 12 ppt sterile brackishwater.

The larvae were put on sterile 500 µm sieves and washed with 12 ppt of sterile brackishwater. The animal was anesthetized in a 0.1% benzocaine solution (Sigma) for 10 sec. and then surface bacteria was removed by dipping in a 0.1% benzalkonium chloride solution (Sigma) for 10 sec. as described by Nhan *et al*. (2010). After chemical treatments, larvae were rinsed with 12 ppt of sterile brackishwater. Further, larvae were transferred into sterile plastic bag

containing 4 ml of sterile brackishwater and later the volume was made to 10 ml. The larvae were crushed manually between fingers and then homogenized for 60 sec. using a stomacher machine as mentioned by Nhan *et al*. (2010).

Water sample and the homogenized larvae were serially diluted. From different dilution series, 100 µl of sample was spread on LB_{12} agar plates containing 50 ppm rifampicin. The plates were incubated for 24h at 28^{o}C and colony was counted.

3.3. Statistical analysis

Collected data of an *in vitro* study of growth, bioluminescence and virulence factors (motility, caseinase and hemolytic activity) and *in vivo* study of larval survival and larval stage index, with and without microalgae were analyzed using one way ANOVA. Duncan's post hoc test was performed to measure significant differences. The impact of coculture of AHL degraders with and without microalgae on bioluminescence of *V. campbellii* was analyzed using independent sample student's *t*-test. Significant differences among treatments were tested at the 0.05 significant level. All data analysis was carried out using Statistic's Package, SPSS version 20.0.

4. RESULTS

4.1. *In vitro* experiments

4.1.1. AHL degradation by the AHL degraders isolated from microalgae

This experiment aimed to confirm whether the AHL degrader bacteria *Pseudomonas sp.* NFMI-T (isolated from the microalga *Tetraselmis suecica*) and *Bacillus sp.* NFMI-C (isolated from the microalga *Chaetoceros muelleri*) indeed are able to degrade the AHL N-hexanoyl-homoserine lactone (HHL). *Chromobacterium violaceum* CV026, that produces a purple pigment (violacein) in the presence of AHLs, was used as a reporter strain for HHL (Natrah *et al.*, 2011a). Quorum sensing degrader strains were tested for their AHL degradation activity for 48h. Supernatants of cultures of the strains grown in LB medium supplemented with HHL were spotted on plates covered with CV026, and the pigment production of the reporter was determined. After 24h, the induction zone was smaller for supernatants of both strains when compared to the control (**Figure 4.1**), indicating that partial degradation of the HHL had occurred. For supernatants taken after 48h, no purple colour was detected, indicating that complete degradation of the HHL had occurred.

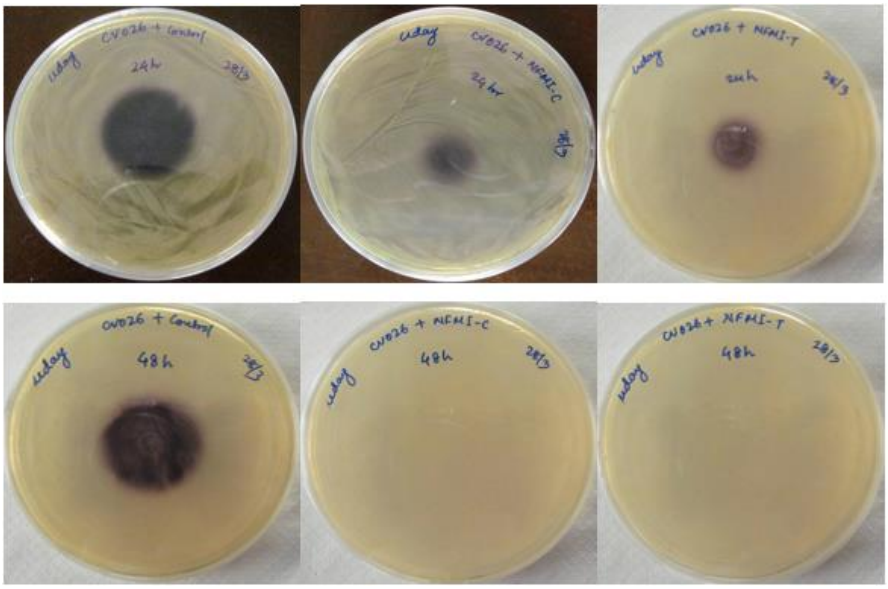


Figure 4.1. Induction of the AHL reporter strain *Chromobacterium violaceum* by cell-free supernatants of strains NFMI-C (2nd column) and NFMI-T (3rd column) grown in LB medium supplemented with HHL. Samples were taken after 24h (upper row) and 48h (lower row). The first column shows the result for sterile medium supplemented with HHL.

4.1.2. Interaction between microalgae and AHL degrader bacteria

a. Algal growth dynamics

To study the interaction between algae and bacteria, a coculture experiment of conventionally reared *Tetraselmis suecica* with and without the 2 different AHL degrader isolates NFMI-T and NFMI-C, was performed. The alga was cultured until stationary phase (reached after 8 days). When compared to the control, the growth of algae cocultured with NFMI-T, NFMI-C and NFMI-T+C was significantly lower on the first 3 days of the culture. At the 9th day of the culture, there is no difference in algal cell density between cultures with and those without the isolates (**Figure 4.2**). From this a conclusion may be derived that the isolates have no effect on growth of microalgae.

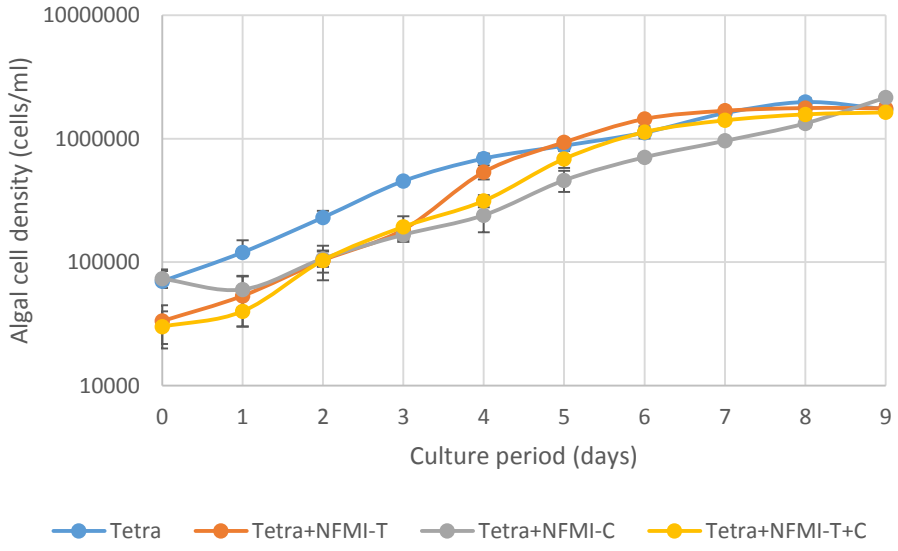


Figure 4.2. Cell density of *Tetraselmis suecica* with and without the AHL degrader bacteria NFMI-T and/or NFMI-C. Error bars represent the standard deviation of 3 replicates.

b. Growth of the isolates in algal culture

Natural rifampicin resistant mutants of the AHL degrader bacteria were used in this experiment in order to be able to distinguish them from the bacteria that are naturally present in the *Tetraselmis* culture. The strains were added to the microalgae at a concentration of 10^5 CFU.ml^{-1}. Sampling of the cultures was carried out on the 1st, 7th and 14th day of the culture period and the cell density of the isolates was determined by plating on agar containing rifampicin. In general, the density of the isolates decreased in time. The strains were still present after 7 days. However, on the 14th day, the densities were below detection limit. Please note that the ratio of bacteria added in the treatment “Tetra + NFMI-T+C” is 1:1 (v/v), both at 10^5 CFU.ml^{-1}.

Table 4.1. Density of natural rifampicin resistant mutants of the AHL degraders (CFU/ml) in open cultures of *Tetraselmis suecica.* Rifampicin resistant mutants of the isolates were inoculated at 10^5 CFU/ml and cell densities were determined by plating on agar containing 50 ppm rifampicin.

Culture period	Cell density (CFU/ml)		
	Tetra + NFMI-T	Tetra + NFMI-C	Tetra + NFMI-T+C
Day 1	$(2.9 \pm 0.1) \times 10^5$	$(2.2 \pm 0.1) \times 10^5$	$(6.9 \pm 0.5) \times 10^5$
Day 7	$(3.0 \pm 0.3) \times 10^4$	$(2.1 \pm 0.3) \times 10^4$	$(3.9 \pm 0.4) \times 10^4$
Day 14	$<3.0 \times 10^2$	$<3.0 \times 10^2$	$<3.0 \times 10^2$

4.1.3. Impact of cell-free supernatant of the AHL degraders on growth and virulence factors of *Vibrio campbellii* BB120.

a. Effect of cell-free supernatant on growth of *Vibrio campbellii* BB120.

This experiment was performed to investigate the effect of cell-free supernatant of the AHL degraders on growth performance of *Vibrio campbellii* BB120. Cell-free supernatants were prepared by filtering overnight grown cultures of the degrader bacteria over a 0.2 µm filter. The supernatants were mixed with a suspension of BB120 in a 50:50 (v/v) ratio. The mixtures were incubated at 28°C for 24 h in a microaerobic atmosphere. The turbidity at 600 nm was monitored every hour using a multi-reader machine (Infinite® 200-TECAN, Austria). The cell-free supernatants of the AHL degrader bacteria had no clear effect on growth of *V. campbellii* (**Figure 4.3**). LB_{12} and non-inoculated cell-free supernatant of the AHL degraders were used as negative controls for confirming that no contamination had occurred.

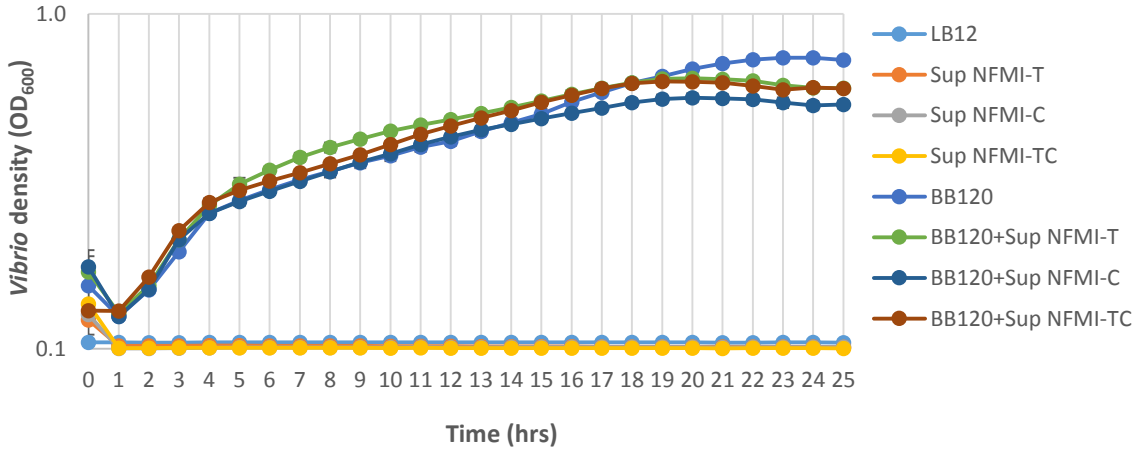


Figure 4.3. *V. campbellii* BB120 growth with and without addition of cell-free supernatants of the AHL degraders NFMI-C and/or NFMI-T. Error bars (mostly too small to be visible) represent the standard deviation of 12 replicates.

b. Effect of cell-free supernatant on swimming motility

In this experiment, the impact of cell-free supernatant on the motility of *V. campbellii* BB120 was investigated. The strain was spotted on soft agar (0.3%) with and without inclusion of cell-free supernatant of the isolates, and zones of motility were measured after 24h of incubation (**Figure 4.4**). The result of the motility assay revealed that the cell-free supernatants have no significant effect on swimming motility (**Table 4.2**).

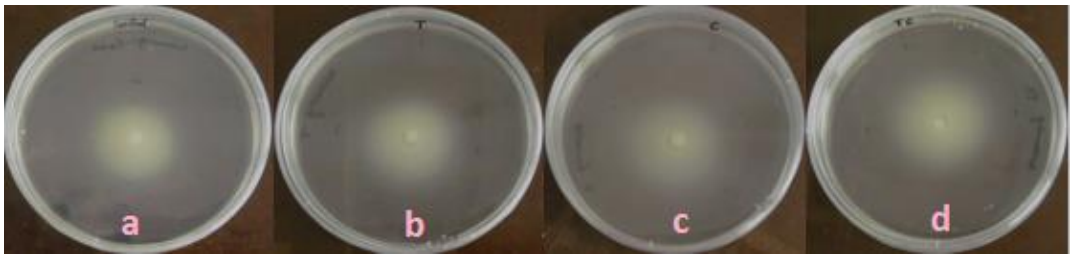


Figure 4.4. Motility of *V. campbellii* BB120 after 24h without (a) and with cell-free supernatant of NFMI-T, NFMI-C and NFMI-T+C (b, c and d, respectively).

Table 4.2. The swimming motility halo (mm) of *V. campbellii* BB120 after 24h of incubation, with and without AHL degrader supernatant 10% v/v (average ± standard deviation of 3 replicates).

Treatments	Swimming motility halo (mm)
Control (BB120)	17 ± 1[a]
BB120 + [NFMI-T] supernatant	17 ± 1[a]
BB120 + [NFMI-C] supernatant	15 ± 3[a]
BB120 + [NFMI-T+C] supernatant	16 ± 1[a]

Values in the same column with different superscript letters are significantly different (p<0.05)

c. Effect of cell-free supernatant on caseinase activity

Caseinase activity of *V. campbellii* BB120 was studied to examine the effect of cell free supernatant of the isolates on protease production. BB120 was spotted on agar containing skimmed milk with and without inclusion of cell-free supernatant of the isolates and colony diameters and clearing zones were measured after 48h of incubation at 28^{o}C (**Figure 4.5**). It was noticed that the caseinases activities were not affected (p>0.05) by the addition of the cell-free supernatants (**Table 4.3**).

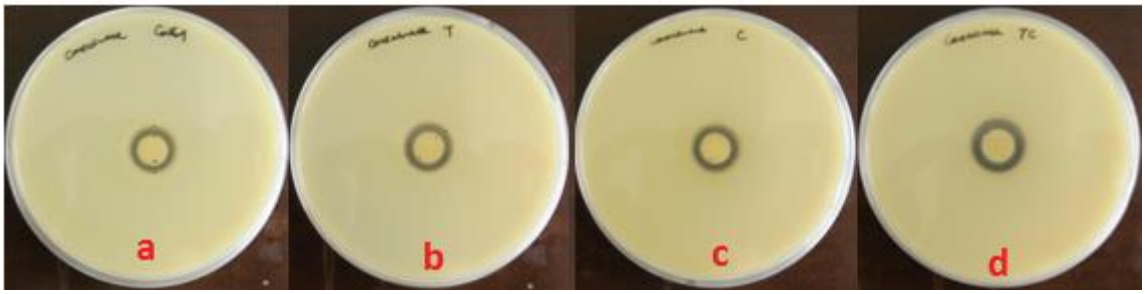


Figure 4.5. Caseinase activity of *V.campbellii* BB120 after 48h without (a) and with cell-free supernatant of NFMI-T, NFMI-C and NFMI-T+C (b, c and d, respectively).

Table 4.3. Clearing zone diameter, colony diameter, and ratio between clearing zone and colony diameter of *V.campbellii* BB120 on 4% skimmed milk agar after 48 hours of incubation at 28ºC, with and without AHL degrader supernatant 10% v/v (average ± standard deviation of 3 replicates).

Treatments	Clearing zone (mm)	Colony diameter (mm)	Ratio
Control (BB120)	16 ± 1^{a}	8 ± 1	1.9 ± 0.1^{A}
BB120 + [NFMI-T] supernatant	16 ± 1^{a}	8 ± 1	1.9 ± 0.1^{A}
BB120 + [NFMI-C] supernatant	16 ± 2^{a}	8 ± 1	1.9 ± 0.1^{A}
BB120 + [NFMI-T+C] supernatant	17 ± 1^{a}	9 ± 0	1.9 ± 0.1^{A}

Values in the same column with different superscript letters are significantly different (p<0.05)

d. Effect of cell-free supernatant on hemolytic activity

In this experiment, we determined the effect of addition of cell-free supernatant on hemolytic activity of *V.campbellii* BB120. The strain was spotted on agar supplemented with 5% sheep blood. The colony diameters and clearing zones were measured after 48h of incubation at 28ºC (**Figure 4.6**). The results shows not much variation; statistical analysis was performed, and differences was found insignificant (p>0.05; **Table 4.4**).

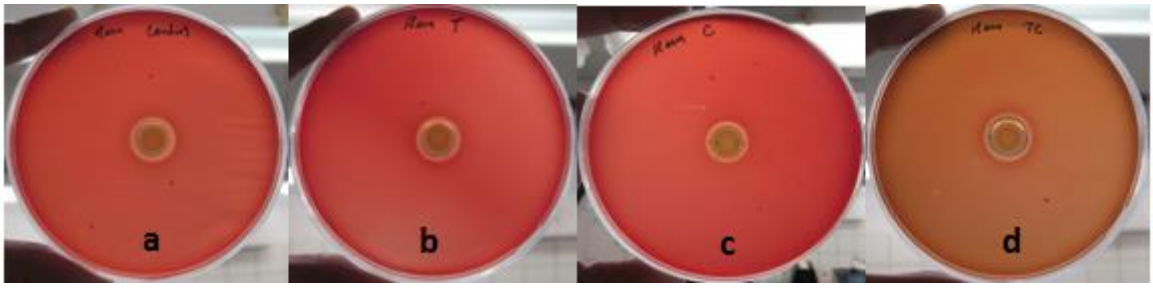


Figure 4.6. Hemolytic activity of *V.campbellii* BB120 after 48h without (a) and with cell-free supernatant of NFMI-T, NFMI-C and NFMI-T+C (b, c and d, respectively).

Table 4.4. Clearing zone diameter, colony diameter, and ratio between clearing zone and colony diameter of *V.campbellii* BB120 on LB12 supplemented with 5% sheep blood after 48 hours of incubation at 28ºC, with and without AHL degrader supernatant 10% v/v (average ± standard deviation of 3 replicates).

Treatments	Clearing zone (mm)	Colony diameter (mm)	Ratio
Control	11 ± 1[a]	6 ± 0	1.8 ± 0.1[A]
BB120 + [NFMI-T] supernatant	11 ± 1[a]	6 ± 0	1.8 ± 0.1[A]
BB120 + [NFMI-C] supernatant	11 ± 1[a]	6 ± 1	1.7 ± 0.1[A]
BB120 + [NFMI-T+C] supernatant	11 ± 0[a]	6 ± 0	1.8 ± 0.0[A]

Values in the same column with different superscript letters are significantly different (p<0.05)

4.1.4. Effect of AHL degrader bacteria and their cell-free supernatants on quorum sensing activity of *V. campbellii* BB120

Because bioluminescence is one of the phenotypes that are regulated by quorum sensing in *V. campbellii*, we determined the effect of the AHL degrader bacteria and their cell-free supernatants on bioluminescence of BB120 as a read-out to determine the effect on quorum sensing. BB120 and AHL degraders were mixed at a density of about 10^5 CFU.ml^{-1} each. In order to compensate for the lower cell density of BB120 in coculture, we mixed BB120 with its mutant JAF548 as a control. JAF548 has a completely inactive quorum sensing system (and therefore is not luminescent). The results revealed that isolate NFMI-C, but not NFMI-T, inhibited the luminescence of *V. campbellii* BB120 in coculture (**Figure 4.7**). However, there were no such effects when BB120 was grown in the presence of cell-free supernatants of the strains (**Figure 4.8**).

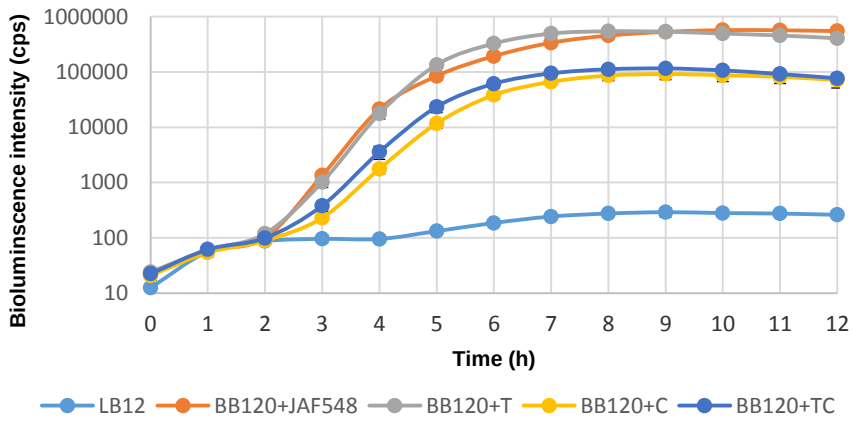


Figure 4.7. Bioluminescence of *V. campbellii* BB120 in coculture with the AHL degraders NFMI-C (C) and/or NFMI-T (T). A coculture with the dark mutant of BB120, JAF548, served as control. Error bars represent the standard deviation of 6 replicates.

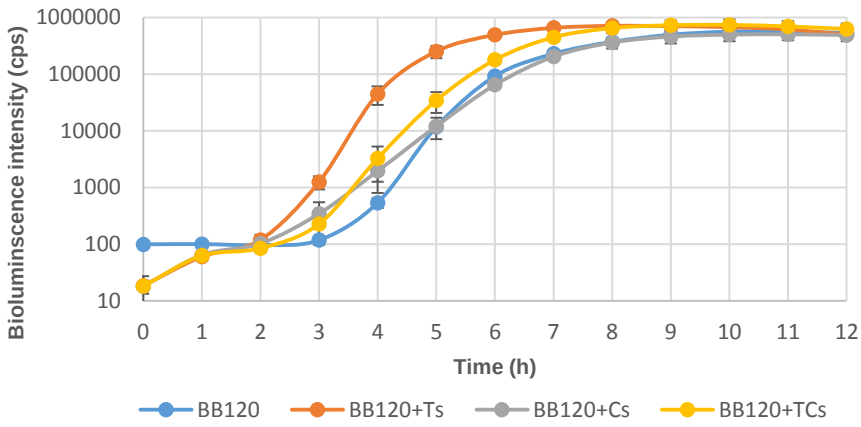


Figure 4.8. Bioluminescence of *V. campbellii* BB120 with and without supernatants (50:50 v/v) of the AHL degraders NFMI-C (Cs) and/or NFMI-T (Ts). Error bars represent the standard deviation of 6 replicates.

A further experiment was performed with the addition of microalgae in coculture of AHL degraders and *V. campbellii* BB120 to measure the effect of microalgae on bioluminescence. The result showed that the addition of microalgae in coculture have no significant impact on bioluminescence of *V. campbellii* ($p>0.05$; **Figure 4.9**).

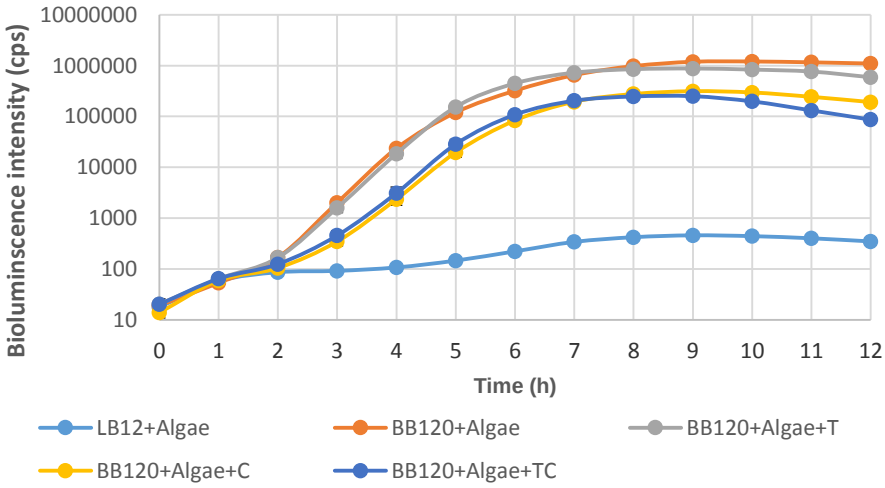


Figure 4.9. Bioluminescence of *V. campbellii* BB120 with coculture of algae and degraders NFMI-C (C) and/or NFMI-T (T). Error bars represent the standard deviation of 6 replicates.

4.2. *In vivo* experiments

4.2.1. Impact of the AHL degraders on survival of giant river prawn larvae challenged with *V. campbellii* BB120

The larvae were challenged with BB120 with or without live or autoclaved AHL degraders. The larvae showed lower survival when treated with BB120, with or without NFMI-T or NFMI-C as compared to the other treatments ($p<0.05$; **Table 4.5**). The addition of the mixture of the isolates or of autoclaved isolates increased the survival of challenged larvae when compared to the BB120 treatment.

Table 4.5. Percentage survival of *M. rosenbergii* larvae after 6 days of challenge with *V. campbellii* BB120, with addition of live or autoclaved AHL degraders (average ± standard deviation of 3 replicates).

Treatments	Percentage survival (%)
Control (larvae only)	47 ± 10^{b}
BB120	33 ± 8^{c}
BB120 + [NFMI-T]	35 ± 2^{c}
BB120 + $[NFMI\text{-}T]_{autoclaved}$	48 ± 4^{b}
BB120 + [NFMI-C]	35 ± 2^{c}
BB120 + $[NFMI\text{-}C]_{autoclaved}$	51 ± 10^{b}
BB120 + [NFMI-T+C]	$65 \pm 5a^{a}$
BB120 + $[NFMI\text{-}T+C]_{autoclaved}$	56 ± 0^{ab}

Values in the same column with different superscript letters are significantly different ($p<0.05$)

The larval stage index (LSI) was used as a specific indicator to assess larval growth and development. None of the treatments had any influence on the larval growth performance. ($p>0.05$; **Figure 4.10**). The larval stages of zoea IV and zoea V were observed.

4.2.2. Impact of algal cultures supplemented with the AHL degraders on survival of giant river prawn larvae challenged with *V. campbellii.*

A second challenge experiment was conducted with the addition of live and autoclaved algal cultures supplemented with the AHL degraders to see whether algal cultures inoculated with the AHL degrader bacteria are effective in controlling infection caused by *V. campbellii*. The larvae challenged with *V. campbellii* without addition of algae showed lower survival when compared to all other treatments ($p<0.05$; **Table 4.6**). Addition of algae (both live and autoclaved) increased the survival of challenged larvae to the same level as non-challenged larvae, and the presence of AHL degraders did not further increase this effect.

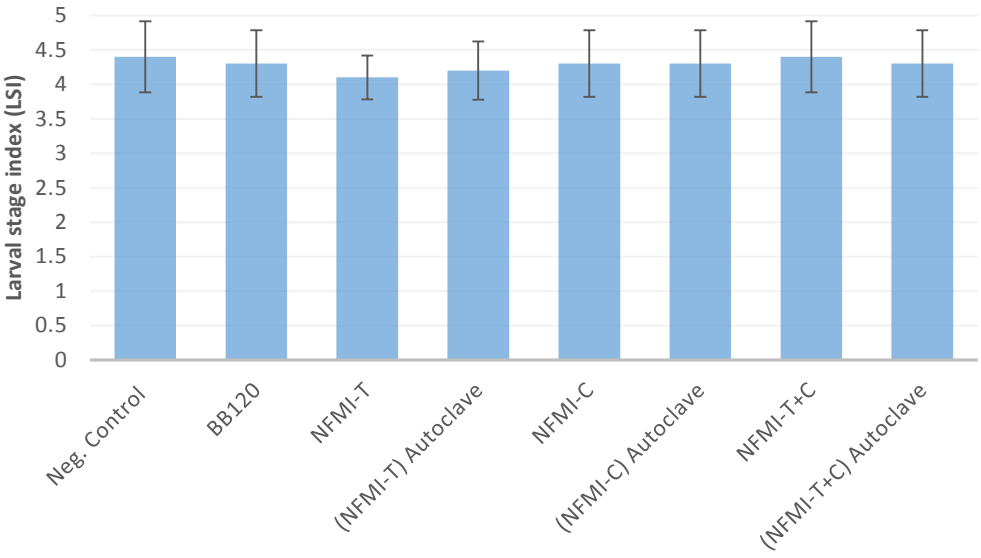


Figure 4.10. Larval stage index of *M. rosenbergii* larvae after 6 days of challenge with *V.campbellii* BB120, with live and autoclaved AHL degraders without microalgae. Error bars show the standard deviation of 10 larvae/treatment.

Table 4.6. Percentage survival of *M. rosenbergii* larvae after 4 days of challenge with *V. campbellii* BB120, with addition of live and autoclaved AHL degrader supplemented with algae (average ± standard deviation of 5 replicates).

Treatments	**Percentage survival (%)**
Control (larvae only)	70 ± 5^{a}
BB120	42 ± 10^{b}
BB120 + Tetra	61 ± 13^{a}
BB120 + $[Tetra]_{autoclaved}$	60 ± 18^{a}
BB120 + [Tetra + NFMI-T]	70 ± 9^{a}
BB120 + $[Tetra + NFMI\text{-}T]_{autoclaved}$	60 ± 10^{a}
BB120 + [Tetra + NFMI-C]	68 ± 6^{a}
BB120 + $[Tetra + NFMI\text{-}C]_{autoclaved}$	64 ± 8^{a}
BB120 + [Tetra + NFMI-T+C]	75 ± 9^{a}
BB120 + $[Tetra + NFMI\text{-}T+C]_{autoclaved}$	66 ± 13^{a}

Values in the same column with different superscript letters are significantly different ($p<0.05$)

None of the treatments had any influence on the larval growth performance. ($p>0.05$; **Figure 4.11**). The larval stages of zoea III and zoea IV were observed.

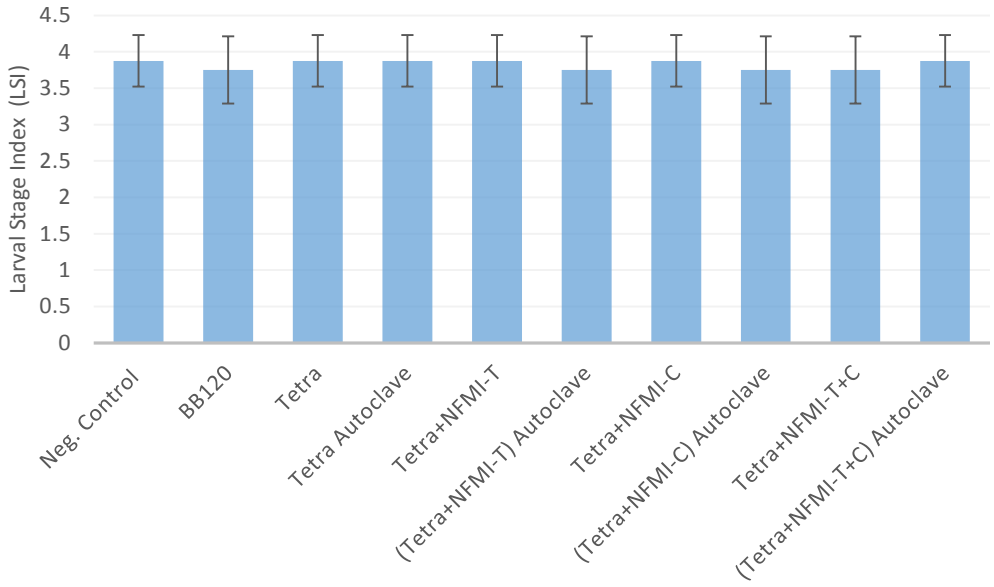


Figure 4.11. Larval stage index of *M. rosenbergii* larvae after 4 days of challenge with *V.campbellii* BB120, with live and autoclaved algal cultures supplemented with the AHL degraders. Error bars show the standard deviation of 8 larvae/treatment.

4.2.3. Enumeration of bacteria from the culture water and gut of the larvae.

In the challenge tests, natural rifampicin resistant mutants of the AHL degrader bacteria were used and the density of the degraders in the larval rearing water and gut was studied by plating on agar containing 50 ppm rifampicin. In the challenge test without microalgae, the degraders were not detected anymore at the day after the challenge test, whereas they were still detected in the challenge test with microalgae (**Table 4.7**). The AHL degraders could not be detected in samples taken from the larval gut.

Table 4.7. Total plate count of AHL degraders from the culture water (with and without microalgae) at the day after challenged test (average ± standard deviation of 3 replicates).

Treatment	Cell density (CFU.ml^{-1})	
	without microalgae	with microalgae
BB120 + [NFMI-T]	Below detection limit	(2.1 ± 0.3) × 10^4
BB120 + [NFMI-C]	Below detection limit	(2.2 ± 0.3) × 10^4
BB120 + [NFMI-T+C]	(1.3 ± 0.2)× 10^2	(5.8 ± 0.4) × 10^4

5. DISCUSSION

5.1. *In vitro* experiments

5.1.1. AHL degradation activity of the isolates

In this study, *Chromobacterium violaceum* CV026, a mini-Tn5 mutant of wild-type strain ATCC31532 deficient in AHL synthase (Chernin *et al*., 1998; McClean *et al*., 1997) was used as a reporter strain. In the presence of exogenous HHL, *C. violaceum* produces violacein pigment, a water-soluble purple pigment with antibacterial activity. It can detect a small range of AHL molecules, usually C_4 to C_8 with N-acyl side chain. A finding by McClean *et al*. (1997), suggested that pigment production in *C. violaceum* is regulated via quorum sensing and can be used as a potential biosensor for detection of AHLs.

In this study, we used natural rifampicin resistant mutants of the strains NFMI-C (*Bacillus* sp.) and NFMI-T (*Pseudomonas* sp.) that had previously been isolated from the microalgae *Tetraselmis suecica* and *Chaetoceros muelleri*. In a first test, we verified that the mutants were able to degrade AHLs. The confirmation test was done by culturing the isolates in LB_{12} medium supplemented with 10 ppm of HHL. We reported that CV026 plates spotted with degrader cell-free supernatant exhibited no induction of purple pigment after 48h. This verified that these isolates NFMI-T and NFMI-C are efficient in degrading AHL signals.

HHL degradation by these isolates confirmed that they have a specific enzyme to inactivate HHL molecules. The enzyme produced by the *Bacillus* sp. and *Pseudomonas* sp. as well as the mechanism to degrade AHL molecule has been described by several authors. *Bacillus* spp. are known to produce AHL lactonase enzymes (AiiA) which hydrolyzes the lactone ring of AHLs (Dong *et al*., 2000; 2001; Wang *et al*., 2004; Czajkowski and Jafra, 2009; Park *et al*., 2008; Kaufmann *et al*., 2005; Defoirdt 2011). However, *Pseudomonas* spp. are known to produce AHL acylase enzymes, which cleave off the acyl side chain (Huang *et al*., 2003; Sio *et al*., 2006; Tinh *et al*., 2007; Jimenez *et al*., 2010). .

Thus, it is possible that NFMI-C and NFMI-T could be used as potential quenchers of quorum-sensing-regulated functions in pathogenic bacteria and *in vivo* challenge test was performed for further confirmation.

5.1.2. Interaction between microalgae and AHL degraders

We hypothesized that the AHL degraders may have an impact on the growth of microalgae and vice versa. To understand this interaction coculture of the AHL-degraders and *Tetraselmis suecica* were performed in an open system. The coculture with AHL degraders was performed till the alga reached the stationary phase, and no effect on the growth performance of the

microalgae was noticed. Sampling of microalgae was also carried out on 1st, 7th and 14th day of the culture period and the densities of AHL degraders were determined. We found that the concentration of AHL degraders decreased over time.

The impact of addition of bacteria on the growth of axenic microalgae was studied by several authors. Grossart, (1999) reported that the addition of bacteria can either stimulate or even inhibit algal growth, depending on the physiological status of the algae. An experiment performed by Fukami *et al*. (1997) on the growth of the *Chaetoceros ceratosporum* upon addition of bacterial isolates, resulted in a stimulatory growth effect with constant cell yield. A plant growth promoting bacterium, *Bacillis pumilus,* was able to promote the growth of *Chlorella vulgaris* (Hernandez *et al*., 2009). A *Flavobacterium* sp. was found to promote growth of a marine diatom *Chaetoceros gracilis* in gnotobiotic conditions (Suminto and Hirayama, 1997). All these studies provide evidence that bacteria can improve the growth of axenic microalgae. In our study, coculture of non-axenic microalgae and degraders isolates showed no such stimulatory effect. This might be the cause suggested by Qu *et al*. (2014) that variation in chemical composition of microalgae in the presence of bacterial community changes the stoichiometry of microalgae and inhibit its growth. It was also suggested that some *Pseudomonas* spp. and *Bacillus* spp. secretes toxic substance into the culture medium which inhibit the growth of *C. vulgaris* by breaking respiratory chain or inhibiting cell wall synthesis (Qu *et al*., 2014).

Furthermore, in coculture, we also observed that the density of isolates decreased in time. In general, the bacterial-microalgae interaction can be positive or negative. Microalgae produces secondary metabolites (extracellular exudates) for the bacteria, and in favor bacteria re-mineralise nutrients and vitamins for the growth of microalgae (Grossart and Simon, 2007; Bi *et al*., 2012). On the other side, microalgae also produces antibacterial compound such as hexane or methanol that inhibit the growth of gram-positive bacteria (Natrah *et al*., 2014). However, it is suggested by (Paver *et al*., 2013) that nutrient limitation can also alter algal and bacterial interaction. In the open algal culture, bacteria coexist within the phycosphere, and upon addition of the AHL degrader, they compete for nutrients and space. Thus, competition for inorganic matters, space, surface and production of antibacterial compound might be the possible cause towards significant reduction of the AHL degraders in coculture.

5.1.3. Impact of cell-free supernatant of AHL degraders on growth of *Vibrio campbellii* BB120*.*

In this study, we speculate that the cell-free extract of NFMI-T and NFMI-C may contain extracellular enzymes, which have an impact on the growth of *V. campbellii*. To assess the growth, mixed culture of BB120 and cell-free extract (50:50 v/v) were grown under

microaerobic condition and turbidity was measured at OD_{600}. We observed that the cell-free extract of NFMI-T and NFMI-C did not inhibit the growth of the pathogen. This indicates that the inhibitory compounds are either intracellular or their concentration in the supernatant is too low to control the growth.

5.1.4. Effect of AHL degraders and their cell-free supernatants on quorum sensing activity and virulence factor production of *V. campbellii* BB120.

The light emitting *vibrios* provide an excellent material to study the interaction of cellular communication because bioluminescent is a sensitive marker for the quorum sensing activity (Nackerdien *et al*., 2008). Bioluminescence in *Vibrio* sp. does not persist for long and at higher cell density, it decreases rapidly due to the limitation of oxygen and nutrients in the medium (Nakayama *et al*., 2005). The bioluminescence of *V. campbellii* was measured until 12h in microaerobic condition and the impact of AHL degraders, and its cell-free supernatants was analyzed.

The results showed that the use of cell-free extracts of AHL degraders has no impact, whereas coculture with NFMI-C but not NFMI-T had a significant impact on the quorum sensing regulated bioluminescence activity. This might be due to competitive exclusion of pathogenic bacteria or the intracellular production of enzymes that are capable of degrading quorum sensing molecules (AHLs).

The competitive exclusion can occur through an intrinsically higher growth rate and competitive uptake of essential nutrients. These activities has been reported in *Bacillus* sp. and are considered as potential probiotics to treat pathogenic bacteria (Ziaei-Nejad *et al*., 2006; Balcazara *et al*., 2006; Lalloo *et al*., 2010). These bacteria produce an AHL lactonases enzyme which has a nonspecific substrate activity against AHLs (Sakr *et al*., 2013). This enzyme hydrolyze lactone ring of AHL molecules and thus inhibit quorum sensing in pathogenic bacteria (Defoirdt *et al*., 2011; Chen *et al*., 2013). Cao *et al*. (2012) detected AHL lactonase activity only in intracellular proteins of *Bacillus* sp. strain AI96 and identified to degrade a wide spectrum of AHLs including C4-HSL, C6-HSL, C7-HSL, C8-HSL, C10-HSL, C12-HSL, C14-HSL 3-oxo-C8-HSL, 3-oxo-C6-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL, 3-oxo-C14-HSL, 3-hydroxy-C8-HSL, and 3-hydroxy-C14-HSL.

However, we also found out that *Pseudomonas* sp. (NFMI-T) and its supernatant were not so effective towards AHL degradation that are produced by *V. campbellii*. This may be because the *Pseudomonas* produces acylases enzyme and are capable of degrading a narrow spectrum of AHLs molecules. This statement is supported by Bokhove *et al*. (2010), shown that an AHL acylase enzyme PvdQ was characterized from an opportunistic pathogen

Pseudomonas aeruginosa which are capable of degrading long chain AHLs as C_{10} to C_{14}. Interestingly, another acylase enzyme of *P.aeruginosa,* QuiP has preferences to degrade long chain from C_7 to C_{14} (Huang *et al*., 2006). In 2009, Shepherd and Lindow reported two acylase enzyme HacA and HacB from *Pseudomonas syringae* strain B728a. The enzyme HacA has preference to degrade AHLs with eight carbons or more whereas, HacB from C_6 to C_{12}. The common action of all acylase enzymes is to hydrolyze the peptide bond between the acyl chain and HSL core.

In coculture of *V. campbellii* and AHL degrader, the addition of microalgae showed no significant impact on the bioluminescence. Thus, from *in vitro* results we can conclude that the use of *Bacillus* sp. NFMI-C will be much effective in controlling quorum sensing, because of its non-specific substrate selection and ability to degrade a wide spectrum of AHLs.

Furthermore, we investigated the impact of the cell-free extract on the virulence factors of *V. campbellii.* It has been reported by various authors that *Vibrio harveyi* produces several extracellular virulence products, which are responsible for its pathogenesis (Natrah *et al*., 2011; Liu *et al*., 1996; Austin and Zhang, 2006). Thus, we determined swimming motility, caseinase and hemolytic activity.

The swimming motility and caseinase activity are considered to be controlled by quorum sensing activity (Natrah *et al*., 2011; Ruandeepika *et al*., 2011; Yang and Defoirdt, 2014). But, we noticed that the QS activity was not inhibited by these supernatants. Therefore, no effect on production of virulence factors were reported. Finally, we measured the hemolytic activity, which is not regulated by QS. It is a major exotoxin responsible for initiation of a virulence factor (Sun *et al*., 2007). In this case, we also noticed no impact on hemolytic activity.

Since the AHL-degrading activity of isolate NFMI-C towards *V. campbellii* was intracellular, we also studied the impact of the isolates on virulence factor production by *V. campbellii* in cocultures. Unfortunately, this experiment didn't work because the isolates were motile on the assay plates.

5.2. *In vivo* experiments

5.2.1. Impact of the AHL degraders on the survival of giant river prawn larvae challenged with *V. campbellii* BB120.

In *in vivo* experiments, larvae were challenged with *V. campbellii* BB120 in addition with the live and autoclaved AHL degraders. The effectiveness of these AHL degraders against infection caused by *V.campbellii* BB120 was determined. It was observed that the larvae treated with BB120, NFMI-T and NFMI-C showed significantly lower survival as compared to

the larvae treated with dead (autoclaved) bacteria. To understand this situation, we further tried to enumerate the AHL degraders from the culture water on the day after the challenge test. The plating results confirmed that degraders are below the detection limit. As previously mentioned, the degrader bacteria produce lactonase and acylase enzymes that are responsible for controlling quorum sensing regulated activity in *V. campbellii* BB120. The absence of NFMI-T and NFMI-C in the culture water may induce the QS regulation and thus, mortality was detected similar to the larvae treated with only BB120.

On the other hand, we detected very high survival in case of larvae treated with mixture of NFMI-T&C. This result is supported with the previous finding of Dang *et al*. (2009) and Tinh *et al*. (2007), which suggested that the mixed culture of AHL degrading bacteria results in a strong degradation activity of AHL molecules. Flagan *et al*. (2003) also reported that coculture of *Arthrobacter* strains VAI-A and *V. paradoxus* VAI-C in AHL medium, *Arthrobacter* strains VAI-A apparently utilizes AHL inactivation products and stimulate signal biodegradation by *V. paradoxus* VAI-C. However, the effect was not the same with monoculture strain under same culture condition. Hence, they postulated that microbial consortia may have a synergistic effect towards the mineralization and AHL molecule turnover. A day after the challenge test, we detected the presence of the AHL degrader which might control the pathogenicity of *V. campbellii* BB120.

Moreover, addition of dead bacteria resulted in higher survival. The same findings was reported by Marques *et al*. (2005), which revealed that the addition of dead bacteria can be an important source of nutrient to improve the performance of *Artemia*, especially when administered with poor-quality feed. Additionally, dead bacteria can be used as immunostimulant which enhances the innate immune system of invertebrates such as crustaceans and minimizes the detrimental effect of pathogenic bacteria (Marques *et al*., 2006)

5.2.2. Interaction of *V. campbellii* BB120 with AHL degraders supplemented with microalgae (*Tetraselmis suecica*) and its impact on survival of giant river prawn larvae

The experiment was performed with the coculture of microalgae and AHL degraders challenged with *V. campbellii*. The result showed no significant difference between the live and dead (autoclaved) bacteria, and significant mortality was observed when the larvae were infected with *V. campbellii* BB120.

The prawn larval performance was reported to be good when the microalgae were added in the culture system. This technique has been widely demonstrated to enhance the growth, survival or health status of the marine fish and shrimp larvae (Skiftesvik *et al*., 2003; Van der Meeraon *et al*., 2007; Pan *et al*., 2001; Izquierdo *et al*., 2006). Several hypotheses explained

by various authors towards protection provided by microalgae to larvae, such as direct supply of nutrients that improves the general animal condition (Reitan *et al*., 1997; Suppamattaya *et al*., 2005), stimulation of the digestive abilities of larvae (Cahu *et al*., 1998), production of growth promoting compound or antibacterial substance by *Tetraselmis suecica* (Kellam and Walker, 1989; Austin and Day, 1990; Austin *et al*., 1992), boost the larvae's non-specific immune response against *V. campbellii*. *In vitro* study by Regunathan and Wesley, (2004) suggested that use of *Tetraselmis suecica* as green water has an inhibitory activity against *Vibrios.*

Furthermore, no significant effect was observed among the different treatments with the addition of live and autoclaved AHL degrader bacteria (NFMI-C and NFMI-T) and microalgae. According to Marques *et al*. (2006), dead bacteria as well as several probiotics are claimed to induce and build up protection against a wide range of diseases in vertebrates and invertebrate culture. Chen *et al*. (2013) suggested that the existence of QQ enzymes in QS microbes can attenuate their QS activity, leading to unnecessary gene expression and pathogenic phenotypes. A finding by Vijayan *et al*. (2006) and Ravi *et al*. (2007) proved that *Bacillus cereus* and *Pseudomonas* sp. PS102 act as biocontrol agent against pathogens of various *Vibrio* species.

Bacillus spp., includes *B. subtilis*, *B. cereus*, *B. coagulans*, *B. clausii*, *B. megaterium* and *B. licheniformis* is the most effective probiotics used in aquaculture (Wang *et al*., 2008). They were reported to be more efficient than Gram-negative in transforming organic matter to CO_2 and hence are capable in controlling water quality (Balcazar *et al*., 2006). Kaysami *et al*. (2007) reported that *M. rosenbergii* zoea larvae had a higher survival and faster rate of metamorphosis when fed with *Bacillus subtilis* treated *Artemia* nauplii compared to zoea larvae fed with untreated *Artemia*. Moriarity (1998) measured the impact of *Bacillus* strain, when added in the prawn culture system and confirmed that prawn larvae were protected against vibriosis. Balcazar (2003) demonstrated that the mixture of *Bacillus* and *Vibrio* sp. presented a protective effect against the *V. harveyi* and influenced the growth and survival of juveniles of white shrimp. Furthermore, Several studies reported that AHL lactonase enzyme produced by the *Bacillus* spp. had a non-specific substrate activity against AHLs and therefore, known to degrade a wide range of the AHL molecules that are produced by pathogenic bacteria, by hydrolyzing the lactone ring (Sakr *et al*., 2013; Defoirdt *et al*., 2011; Chen *et al*., 2013; Cao *et al*., 2012). *Bacillus* spp. competes for the nutrients, space and surfaces and thus inhibits other bacteria from growing rapidly. They also secrete a wide range of exoenzymes that degrades biofilms and allows penetrating the slime layer of Gram-negative bacteria (Moriarity, 1996; 1998). An *in vivo* addition of *Bacillus* sp. NFMI-C (AHL degrader isolate) might performed all these activities, which leads to better survival of the prawn larvae.

Pseudomonas sp. PS-102 were also reported as potential antagonistic bacterium against pathogenic *vibrios* in penaeid and non-penaeid rearing systems (Vijayan *et al*., 2006). A marine *Pseudomonas* sp. strain I-2 reported to produce inhibitory compounds against shrimp pathogenic *vibrios* in aquaculture including *Vibrio harveyi, V. fluvialis, V. parahaemolyticus, V. damsela and V. vulnificus* (Chythanya *et al*., 2002; Irianto and Austin, 2002). They are reported to secrete an acylase enzyme that hydrolyze the peptide bond between the acyl chain and HSL core (Lin *et al*., 2003). Thus, *Pseudomonas* sp. NFMI-T may have such action in controlling the QS regulated activity of *V. harveyi.*

In particular, autoclaved AHL degrader bacteria with microalgae had significantly higher survival than larvae treated with *V. campbellii*. This evidence is supported by Marques *et al*. (2005), which confirmed that *Artemia* fed with dead bacteria represented significant increase in total length that non-fed. This suggests that dead bacteria may provide an important source of nutrient to improve the growth performance of *Artemia* nauplii. Moreover, dead bacteria can also be used as an Immunostimulant to enhance the non-specific immune response of invertebrates (Marques *et al*., 2006). It was reported that heat-killed *Pseudomonas aeruginosa* initiate non-specific immune response through Toll-like receptors (TLR) (Erridgea *et al*., 2007). The TLR has been identified to enhance innate immune response in *M. rosenbergii* (Srisuk *et al*., 2014). These could explain the better survival observed in *M. rosenbergii* challenged larvae.

5.2.3. Interaction between AHL degraders and microalgae in the prawn rearing water

The enumeration of AHL degrader isolates was performed to study the interaction of bacteria and microalgae. Significant differences were found between the bacterial counts of plated samples from larval rearing water with microalgae and without microalgae. The AHL degraders were only detected in the treatment in which microalgae was added. It was also observed that larval survival was better when microalgae were added in the larval rearing water as compared to without microalgae. This resulted in understanding that the microalgae is required for the AHL to persist in the larval rearing water and to protect prawn larvae from *V. campbellii*.

Moreover, we also observed the impact of the addition of AHL strains on the growth performance of the prawn larvae. At the last day of the culture period we measured the larval stage index and noticed that there was no significant effect on the growth of the larvae. This finding is supported by Nhan *et al*. (2010), which reported that the addition of AHL degrading bacteria enrichment cultures into rearing water did not affect larval growth and development but significantly improved larval survival and quality.

6. CONCLUSION AND RECOMENDATION

From this study some conclusions could be drawn which are as follows:

a*. In vitro*

The cell-free extract of AHL degraders has no effect on growth and virulence factors of *V. campbellii* BB120*,* which confirmed that they are not effective in controlling pathogenesis. In coculture of *V. campbellii* BB120 and AHL degraders, *Bacillus sp.* NFMI-C (but not NFMI-T) was reported to control QS regulated bioluminescence. This proved that NFMI-C is more efficient in degrading AHL molecules that are produced by *V. campbellii* BB120. We also confirmed that the NFMI-C indeed produces an intracellular enzyme that degrades the AHL signal molecule.

b. *In vivo*

In vivo experiments revealed that the use of AHL degrader isolates (*Bacillus* sp. NFMI-C and *Pseudomonas* sp. NFMI-T) could be an anti-infective strategy to abolish infection caused by *V. campbellii* BB120. Meanwhile, we reported an effective control of *V. campbellii* BB120 when prawn larvae was treated with coculture of microalgae and AHL degrader rather than adding AHL degrader without microalgae in the rearing water. This proved that AHL degraders required microalgae to prove their efficacy. In the absence of microalgae they dissipate from the system. The mixture of AHL degraders and the use of dead bacteria in the rearing water without microalgae were noticed to have higher effect on the survival of the prawn larvae. The AHL degraders (live and autoclaved) with the addition of microalgae have a similar effect on the survival of the prawn larvae.

c. Recommendations for further research

The substrate specificity of the enzymes produced by these AHL degrader isolates should be investigated and hunt for universal QQ enzyme producing bacteria that target a broad range of AHLs for efficient blockage of QS activity. From a biocontrol point of view, the efficacy of these isolates should be tested on different host-pathogen interactions and establish a concrete evidence of the mode of action of these strains towards pathogen. The combination of QQ approach with other treatments, such as antibiotics should be tested for their effects. This could potentially increase the susceptibility of bacteria to antibiotic treatments.

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