In vivo effects of single or combined N-acyl homoserine lactone quorum sensing signals on the performance of *Macrobrachium rosenbergii* larvae

Kartik Baruah\(^a\), Dang T.V. Cam\(^a\), Kristof Dierckens\(^a\), Mathieu Wille\(^a\), Tom Defoirdt\(^a,b\), Patrick Sorgeloos\(^a\), Peter Bossier\(^a,⁎\)

\(^a\) Laboratory of Aquaculture and Artemia Reference Center, Ghent University, Rozier 44, 9000 Ghent, Belgium
\(^b\) Laboratory of Microbial Ecology and Technology (LabMET), Ghent University, Coupure Links 653, 9000 Gent, Belgium

**Abstract**

Three experiments, each of 8-days, were conducted to evaluate the effects of (5) N-acyl homoserine lactone (AHL) either alone or in combination on the development and survival of *Macrobrachium rosenbergii* larvae. Experiment 1 was carried out as preliminary trial to confirm whether AHL mixture added daily at 1 mg/L concentration could have any negative effect on the larval performance. In experiment 2, it was verified whether the negative effect on the larvae was due to individual AHL or their interactions and experiment 3 was carried out to determine the critical threshold dose of AHL mixture. Results revealed that AHL mixture added daily at 1 mg/L concentration significantly \((P<0.01)\) reduced the development and survival of the larvae. Except for C4-HST, there was no significant effect \((P>0.05)\) of any of the AHL molecules added individually at 1 mg/L concentration on the development and survival of the prawn larvae. The AHL molecule C4-HST significantly \((P<0.01)\) reduced the larval stage index (LSI) and survival of prawn larvae by about 9.1 and 8.7%, respectively. In contrast, the AHL mixture added daily at 1 mg/L concentration significantly \((P<0.01)\) reduced the LSI and survival of the larvae by 12 and 41%, respectively. Thus the poor development and low survival of the larvae is probably due to the interaction of different AHL molecules which could have instigated the production of various virulence factors in micro-organisms associated with the larvae. Using the AHL mixture, the dose response relationship with respect to the performance of the larvae showed that the development of the larvae was already negatively affected by adding the mixture to a final concentration of 0.125 mg/L while at that concentration, the survival was still unaffected. This indicate that the standing microbial communities associated with the prawn larvae can become virulent when a quorum or critical threshold level is reached through the daily addition of the AHL mixture at a concentration of 0.125 mg/L.

© 2008 Elsevier B.V. All rights reserved.

**Keywords:** *Macrobrachium rosenbergii*, N-acyl homoserine lactone, Virulence, Artemia

1. Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii* has great aquaculture potential due to its large size attainment, tolerance to water quality changes, ability to cope with handling stress, and to feed on unconventional feeds (El Sayed, 1997). Additionally, it has considerable export value. Because of these potentials, freshwater prawn culture is expanding rapidly in many countries. Augmentation of grow-out systems of freshwater prawn demanded hatchery production of healthy seed. Presently, several hatcheries are under operation across the world to cater for the need of the hour. Nevertheless, the farmers are encountering shortage of healthy seed. One of the major factors that are hampering the quality of hatchery-reared seed, culminating in low yield because of mass mortality and their subsequent culture in grow-out systems is diseases. Diseases are often reported as a limiting factor in hatcheries (Nayak and Mukherjee, 1997; Jayaprakash et al., 2006). Several authors reported the occurrence of various pathogenic or opportunistic bacteria such as *Vibrio, Aeromonas, Alcaligenes, Acinetobacter, Pseudomonas, Moraxella, Plesiomonas, Chryseomonas* spp., *Cellulomonas*, *Pasteurella* and members of the Enterobacteriaceae family in *M. rosenbergii* larvae (Karunasagar et al., 1994; Al-Harbi and Uddin, 2004; Kennedy et al., 2006). These pathogenic or opportunistic bacteria cause diseases not only in prawn hatcheries but also in the intensive rearing of fish and molluscs, causing great losses, even up to 100% (Soto-Rodriguez et al., 2003). Hitherto, different strategies had been developed to overcome this problem in prawn hatcheries. These include the use of specific-pathogen-free broodstock, optimization of feed, use of greenhouse system, improvement of husbandry techniques and good sanitation. In conjunction with good health management, probiotics and immunostimulants are also used for disease control in prawn hatcheries (Smith et al., 2003; Keysami et al., 2007). The addition of substantial amounts of antibiotics an chemotherapeutics remains the method of choice for disease control in many prawn industries. But, its frequent use has led to the development of multiple-resistant bacteria...
feeding, AHLs was added to each glass cone. A control was maintained fed twice daily at the density of 5 prey organisms (Eberl, 1999). Examples of the phenotypes regulated by AHL-based QS breakdown of AHL. The glass cones were placed in a rectangular tank containing water maintained at 28±1 °C using a thermostatic heater. Biodegradation of AHL is mediated, among others, by N-acyl-L-homoserine lactones, AHLs (Eberl, 1999). Hence it is of interest to investigate if the AHL molecules (individually or mixture) affect the performance (development and virulence factor expression in several (aquatic) pathogens. Prawn larvae potentially contain several micro-organisms (Kennedy et al., 2006) which are able to release several AHL molecules (Swift et al., 2004) in vivo. Thus, the above cited conventional approaches did not seem to be able to solve every problem alone and hence the issue of disease still remains unresolved. Therefore, before developing any alternative control strategies, it has become imperative to understand more deeply how the various micro-organisms behave in the natural environment, become virulent, and infect the host.

In recent years it has become clear that diverse bacterial pathogens (including aquatic pathogens, Defoirdt et al., 2004) employ signal molecules to regulate the production of virulence factors. In Gram-negative bacteria, this type of regulation (termed quorum sensing, QS) is mediated, among others, by N-acyl-L-homoserine lactones, AHLs (Eberl, 1999). Examples of the phenotypes regulated by AHL-based QS include luminescence, conjugation, nodulation, swarming, sporeulation, biocorrosion, antibiotic production, biofilm formation, production of lytic enzymes, toxins, siderophores, and adhesion molecules (Defoirdt et al., 2004). This indicates that there is a link between QS and virulence factor expression in several (aquatic) pathogens. Prawn larvae potentially contain several micro-organisms (Kennedy et al., 2006) which are able to release several AHL molecules (Swift et al., 2004). Hence it is of interest to investigate if the AHL molecules (individually or mixture) affect the performance (development and survival) of Macrobrachium larvae and in this way try to understand the importance of the presence of these types of molecules in vivo. So, the present study was conducted with the objective of determining the effects of individual AHL molecules or a mixture on M. rosenbergii performance. Understanding this QS-based interaction could help in developing effective strategies for pathogen control in hatcheries.

2. Materials and methods

2.1. Experimental animals

This study was conducted at the Laboratory of Aquaculture and Artemia Reference Centre, Ghent University, Belgium, in a controlled temperature room. Adult M. rosenbergii obtained from this lab and maintained in two separate freshwater recirculation units were used as brooders. The larvae for the experiment were obtained from a single ovigerous female breeder. Matured female which had just completed its pre-mating moult was mated with a hard-shelled male. Spawning usually occurred within 24 h after mating. The female with concentrated eggs was then maintained for a period of 20 to 25 days to undergo embryonic development. When fully ripe (indicated by dark grey eggs), female was transferred to the hatching tank (30 L) containing slightly brackish water (6 g/L). Water temperature, pH and salinity over a 150 μm pore size sterile net and transferred to two sterile 500 mL screw cap bottles, each containing 200 mL FABW. Their caps were fitted with 0.2 μm filters to prevent clogging and sedimentation of the cysts. Cysts were exposed to constant incandescent light (2000 lx) and temperature maintained at 28 °C for 20–24 h.

2.2. Experimental design

After hatching, the newly hatched larvae were immediately distributed to glass cones containing 400 mL autoclaved brackish water (12 g/L salinity), at a density of 60 larvae per cone and were reared for 8 days. The pH of the brackish water was previously adjusted to 7.3±0.1 by using MOPS (Janssen Chimica, Belgium) to minimise the chemical breakdown of AHL. The glass cones were placed in a rectangular tank containing water maintained at 28±1 °C using a thermostatic heater. The cones were covered with perforated plastic lids so as to insert the aeration pipes. The larvae were fed from day 2 post hatch onwards with fresh axenically hatched Artemia nauplii (mostly instar II) till day 7. They were fed twice daily at the density of 5–10 nauplii/mL/day. Immediately after feeding, AHL(s) was added to each glass cone. A control was maintained without the addition of AHL(s). Everyday, water (30–50%) along with prey organisms (Artemia) was filtered out from all the larval rearing cones. The glass cones were then refilled with pre-heated autoclaved water (salinity: 12 g/L; pH: 7.3±0.1). This operation was carried out with great care to avoid the loss of larvae.

In total, three experiments were conducted to verify the effects of five AHLs either as individual compound or as mixtures on the performance of M. rosenbergii larvae. In experiment 1, a daily addition of an AHL mixture (final concentration 1 mg/L) was verified for its effects on M. rosenbergii larvae performance. In experiment 2, it was verified which AHL molecule had a negative effect on the larval survival. In experiment 3, a dose response relationship was determined by looking at larval performance in the presence of an increasing amount of added AHL mixture, allowing establishing a threshold addition. In all the experiments, 6 replicates were kept for each treatment. In order to investigate the reproducibility of the effects, the addition of a mixture of AHL molecules (at a daily rate of 1 mg/L) was included in all the 3 experiments as a positive control.

2.3. Artemia and axenic hatching

Artemia franciscana originating from the Great Salt Lake, Utah, USA (EG® Type, batch 21452, INVE Aquaculture, Baasrode, Belgium) was used as food for M. rosenbergii larvae throughout the experiment. The amount of Artemia cysts used for hatching axenically varied with the experiments (0.5–1.8 g). They were hydrated in 45–89 mL distilled water for 1 h. Sterile cysts and nauplii were obtained via decapsulation using 1.7–3.3 mL NaOH (32%) and 25–50 mL NaOCl (50%), adapted from the protocol described by Marques et al. (2004). During the reaction, 0.22 μm filtered aeration was provided. All manipulations were carried out under a laminar flow hood and all necessary tools were previously autoclaved at 121 °C for 20 min. The decapsulation was stopped after about 2 min by adding 25–50 mL Na2S2O3 (10 g/L). The aeration was then stopped and the decapsulated cysts were washed with filtered autoclaved brackish water (FABW) of 12 g/L salinity over a 150 μm pore size sterile net and transferred to two sterile 500 mL screw cap bottles, each containing 200 mL FABW. Their caps were fitted with 0.2 μm filters to prevent clogging and sedimentation of the cysts. Cysts were exposed to constant incandescent light (2000 lx) and temperature maintained at 28 °C for 20–24 h.

2.4. Methods used to verify axenicity of Artemia

After hatching, the axenicity of the Artemia nauplii was verified by spreading 100 μL of the hatching water on Marine agar (Difco, Detroit, USA) followed by incubating at 28 °C for 5 days (Yeong et al., 2007).

2.5. Preparation of AHLs and their mixture

Five AHLs (Fluka, Table 1) and their mixture were used in this experiment. Stock solution of each individual AHL (1000 mg/L) was prepared as follows: AHL (25 mg) was first dissolved in 200 μL of ethanol (95%) in a sterilized eppendorf tube. The solution was then transferred to an autoclaved vial (50 mL) and the volume was made to 25 mL by adding autoclaved distilled water. A stock mixture of 5 AHL molecules was prepared by mixing equal volumes. The whole procedure was carried out under laminar flow.

### Table 1

<table>
<thead>
<tr>
<th>Different N-acyl-homoserine-lactone (AHL) used in these experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AHL molecules</strong></td>
</tr>
<tr>
<td>N-Butyryl-dl-homoserine lactone</td>
</tr>
<tr>
<td>N-Butyryl-dl-homocysteine thiolactone</td>
</tr>
<tr>
<td>N-Hexanoyl-dl-homoserine lactone</td>
</tr>
<tr>
<td>N-Heptanoyl-dl-homoserine lactone</td>
</tr>
<tr>
<td>N-Octanoyl-dl-homoserine lactone</td>
</tr>
</tbody>
</table>
### 2.6.1. Residual AHL concentration in larval rearing water

Every day water samples (just before water exchange and before adding daily dose of AHLs) from each treatment were collected in sterile eppendorf tubes for determining the residual AHL concentration (RAC). Chromobacterium violaceum strain CV026, a mini-Tn5 mutant derived from the C. violaceum strain ATCC31532 (McClean et al., 1997) was used as AHL-reporter for measuring the RAC in larval rearing water. This strain cannot produce AHL, but can detect and respond to a range of AHLs by inducing the synthesis of purple pigment violacein. An overnight-grown culture of CV026 strain was filtered using a 100 µm sieve (to retain nauplii) and collected in a sterile 50 mL falcon tubes. Groups of 50 nauplii were then transferred from the cone to an autoclaved conical flask. From the flask, 25 mL of the water was filtered using a 100 µm sieve (to retain Artemia) and collected in a sterile 50 mL Falcon tubes. Groups of 50 Artemia nauplii were then transferred from the flask to the Falcon tubes. After the transfer, the Falcon tubes were put on the rotor (4 rotations/min) at 28 °C with constant illumination (approximately 2000 lx). After 24 h, survival was calculated in each tube using the following formula:

\[
\text{Survival} = \frac{\text{Total number of live larvae on day 8}}{\text{Initial number of larvae stocked}} \times 100
\]

### 2.6.2. Larval survival study

The number of surviving larvae in all the three experiments was counted on the final day (day 8). Only those larvae presenting movement of appendages and responding to mechanical stimulus were considered alive. The survival was recorded as follows:

Survival data (%) = \( \frac{\text{Total number of live larvae on day 8}}{\text{Initial number of larvae stocked}} \times 100 \)

### 2.6.3. Larval stage index (LSI)

A sample of 60 larvae from each treatment was preserved in lugol solution on day 8 for determination of larval stage. The LSI was then estimated according to Maddox and Manzi (1976) as:

\[
\text{LSI} = \frac{\sum_i S_i}{N} \quad \text{[Si is the stage of the larvae (i = 1 to 12); } \times N \text{ is the number of larvae examined]}
\]

### 2.6.4. Artemia survival

On day 5, larval rearing water along with Artemia was siphoned from each cone to an autoclaved conical flask. From the flask, 25 mL of the water was filtered using a 100 µm sieve (to retain Artemia) and collected in a sterile 50 mL Falcon tubes. Groups of 50 Artemia nauplii were then transferred from the flask to the Falcon tubes. After the transfer, the Falcon tubes were put on the rotor (4 rotations/min) at 28 °C with constant illumination (approximately 2000 lx). After 24 h, survival was calculated in each tube using the following formula:

Artemia survival (%) = \( \frac{\text{Total number of live Artemia after 24 h}}{\text{Initial number of Artemia}} \times 100 \)

### 2.7. Statistical analysis

Survival data (%) are arcsin transformed to satisfy normal distribution and homocedasticity requirements as necessary. Data of experiment 1 were analysed separately by the Student’s t-test using the statistical software Statistical Package for the Social Sciences (SPSS) version 14.0. Data of experiment 2 and 3 were subjected to one-way analysis of variance (ANOVA). Data are represented as mean±SE, n=6 for survival and n=60 for LSI. Different superscripts in the same column represent significance difference (P<0.05). Control — no AHL mixture addition; AHLmix1— AHL mixture added daily at 1 mg/L.
**Table 3**

Effect of different individual AHLs and their mixture (1 mg/L) on the survival (%) and larval stage index (LSI) of *M. rosenbergii* and on the survival of *Artemia* (Experiment 2)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Survival of larvae</th>
<th>LSI</th>
<th>Survival of Artemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>92.5±2.6</td>
<td>5.4±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62.7±2.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C4-HSL  (positive control)</td>
<td>94.1±2.5</td>
<td>5.1±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47.4±2.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C4-HST</td>
<td>92.5±2.5</td>
<td>4.9±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47.4±2.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C6-HSL</td>
<td>88.1±2.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.9±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>55.0±5.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C8-HSL</td>
<td>89.5±2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.1±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47.4±2.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are represented as mean±SE, n=6 for survival and n=60 for LSI. Different superscripts in the same column represent significant difference (P<0.05). Survival of *Artemia* was estimated after 24 h exposure to residual concentration of individual AHLs and their mixture. Negative control — no AHL mixture addition; *AHLmix* — AHL mixture added daily at 1 mg/L; C4-HSL — N-Butyryl-cit-homoserine lactone; C4-HST — N-Hexanoyl-cit-homoserine lactone; C6-HSL — N-Heptanoyl-cit-homoserine lactone; C8-HSL — N-Octanoyl-cit-homoserine lactone.

ANOVA followed by Duncan’s multiple range tests to determine significant differences among treatments. Significance level was set at P=0.05.

**3. Results**

**3.1. Experiment 1**

The AHL concentration in the control was below the detection limit (0.1 mg/L) of CV026 strain throughout the rearing period as there was no induction of violaean production. The cumulative AHL concentration in the AHL treatment was also below the detection limit up to day 4, reaching 1.6 mg/L on day 5 and further increasing, reaching 3.4 mg/L on day 8 (Fig. 1).

The addition of the AHL mixture significantly (P<0.05) affected the survival of larvae on day 8 (Table 2). Survival in the AHL treatment dropped to 49% on day 8. In contrast, the survival in control was 70%. In the AHL treatment *Macrobrachium* larvae grew significantly slower (by about 7.5%, P<0.001) with respect to the control (Table 2).

**3.2. Experiment 2**

The residual AHL was detected by CV026 in treatment *AHLmix*1, C4-HST, C6-HSL and C8-HSL from day 4 onwards (Fig. 2). The RAC in control, treatments C4-HSL and C7-HSL were below the detection limit throughout the experimental period. In treatments *AHLmix*1, C4-HST, C6-HSL and C8-HSL, the cumulative RAC reached 1.8, 1.9, 1.5 and 1.1 mg/L, respectively on day 5, increasing gradually, finally reaching 3.9, 4.6, 2.7 and 3.3 mg/L, respectively on day 8. The QS molecules significantly (P<0.001) reduced the survival of larvae when added as mixture (*AHLmix*) at a concentration of 1 mg/L (Table 3). The survival was reduced by about 41% as compared to the negative control (no AHL addition). Treatment C4-HST did not differ significantly (P>0.05) from the other individual AHL molecule treatments, however, differed significantly (P<0.001) from both negative and positive controls. The survival of the larvae treated with C4-HST reduced by about 8.6% as compared to negative control. Different individual AHL molecules and their mixture had significantly (P<0.001) influenced the LSI (Table 3). Highest and lowest LSI was observed in negative and positive controls, respectively. Treatment *AHLmix*1 did not differ significantly (P>0.05) from treatment C4-HST and C7-HSL. There were also no significant differences (P>0.05) among the treatments C4-HSL, C4-HST, C6-HSL, C7-HSL and C8-HSL. However, they differed significantly from the negative control.

Data on the survival (%) of *Artemia* after 24 h exposure to different residual concentration of individual AHLs and their mixture are presented in Table 3. The highest survival of *Artemia* was recorded in negative control and the lowest in the *AHLmix*1 treatment, which however, was not significantly different (P>0.05) from the treatments C4-HST and C7-HSL. No significant differences (P>0.05) were also recorded among negative control, treatments C4-HSL, C6-HSL and C8-HSL.

**3.3. Experiment 3**

In the control and two other AHL treatments (daily addition to a final concentration of 0.125 and 0.25 mg/ml), the RAC was below the detection limit of CV026 throughout the experiment (Fig. 3). *AHLmix* was undetectable in the treatments *AHLmix*0.125 and *AHLmix*0.25 up to days 5 and 4, respectively. On day 8, the final AHL concentration was 1.8 and 3.6 mg/L, respectively.

On the last day of the experiment, only in the treatment with the highest concentration of AHL addition (*AHLmix*1), a significant decrease in larval survival was noticed (P<0.001, Table 4). The survival in this treatment was 58% whereas in control it was 94%. The development of the larvae (LSI) treated with different AHL mixture concentrations decreased significantly (P<0.001) as compared to the untreated ones (Table 4). The highest LSI was recorded in control and the lowest in the *AHLmix*1 treatment. In addition, no significant difference (P>0.05) between treatment *AHLmix*0.125 and *AHLmix*0.25 were recorded.

The effect of different residual concentrations of AHL mixture on the survival of *Artemia* is shown in Table 4. The survival of *Artemia* in *AHLmix*1 treatment was significantly (P<0.01) lower (51%) than the control ones (67%). No significant differences (P>0.05) in survival of *Artemia* were noted between the treatments *AHLmix*0.125, *AHLmix*0.25 and *AHLmix*0.50 and the control.

Table 4

Effect of different concentrations of AHL mixture (1 mg/L) on the survival (%) and larval stage index (LSI) of *M. rosenbergii* and on the survival of *Artemia* (Experiment 3)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Survival of larvae</th>
<th>LSI</th>
<th>Survival of Artemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>94.2±1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.6±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67.3±3.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>AHLmix</em>0.125</td>
<td>91.1±1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.5±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66.7±3.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>AHLmix</em>0.25</td>
<td>90.0±2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.2±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66.3±2.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>AHLmix</em>0.50</td>
<td>88.1±2.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.3±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>62.3±2.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>AHLmix</em>1</td>
<td>57.8±3.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.9±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51.0±4.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are represented as mean±SE, n=6 for survival and n=60 for LSI. Different superscripts in the same column represent significant difference (P<0.05). Survival of *Artemia* was estimated after 24 h exposure to residual concentration of AHL mixture. Control — no AHL mixture addition; *AHLmix*0.125, *AHLmix*0.25, *AHLmix*0.50 and *AHLmix*1 are AHL mixtures added daily at concentration of 0.125, 0.25, 0.50 and 1 mg/L, respectively.
4. Discussion

Most Gram-negative bacteria use QS systems for modulating a variety of physiological processes including the production of virulence determinants (Dunmy and Winans, 1999; Swift et al., 1999). Some bacteria among them employ individual signal molecules while others employ more than one (Buch et al., 2003). Defoirdt et al. (2005) demonstrated that autoinducer-2 is necessary for virulence of V. harveyi towards Artemia. Tinh et al. (2007) reported that both harveyi like autoinducer-1 and autoinducer-2 signals need to be inactivated to neutralize the negative effects of V. harveyi towards gnotobiotic rotifers. These observations raise questions concerning the importance of the QS molecules in the aquaculture environment, characterised by high microbial densities. Several Gram-negative bacteria were associated with the giant freshwater prawn larvae in the hatchery system (Kennedy et al., 2006) and these bacteria release several AHL molecules (Swift et al., 1999). Yet data on the effect of AHLs on the host are scarce. The present study was therefore conducted to elucidate the effect of AHLs on M. rosenbergii larval performance.

There were no references regarding the concentration of AHL needed to induce an effect on prawn larvae. So the first exploratory experiment was carried out using a mixture of five AHL molecules. Results showed that the daily addition of the AHL mixture (1 mg/L) caused a sharp reduction in the survival (from 70% to 49%) and development (LSI, by 7.5%) of the prawn larvae. No supporting evidence is available in the literature on the mode of action of AHL molecules on crustacean larvae. However, the study of Tinh et al. (in press) in fish (turbort) larvae, observing a sharp reduction in turbort larvae survival subsequent to the addition of AHL mixtures to the rearing water, corroborates our findings. They demonstrated that the AHLs are not toxic, per se, but that they have a negative influence on turbort larvae most probably by stimulating the production of virulence factors in microbial communities. A similar mechanism might explain the poor survival and slow development of the Macrobrachium larvae observed in this study, however, the toxic effect of AHL in the prawn (crustacean) larvae cannot be totally excluded. Apart from investigating the effect of AHL molecules on the prawn larvae themselves, attention was also paid to investigate the fate of the daily added AHL molecules in the rearing water during the course of the experiment. AHL molecules undergo auto-degradation at high pH and/or at high temperature (Yates et al., 2002). In addition, there exist microbial communities which are able to grow at the expense of AHL molecules as carbon and nitrogen source. These microbial communities can degrade AHL when present at low concentration, either single or in a background of high nutrient load (Molina et al., 2003; Tinh et al., in press). In our study, the temperature was almost constant (27.5±0.5 °C) throughout the experiment, but the pH increased on days 3 and 4 post hatch (7.71 to 7.83). Interestingly, the cumulative AHL concentration in AHL treated water was also below the detection limit up to day 4 (possibly either due to pH-dependent hydrolysis or microbial degradation), however, reached 1.6 mg/L on day 5, further increased, reaching 3.4 mg/L on day 8. Our results are in line with the findings of Tinh et al. (in press), who observed chemical degradation of AHL molecules added to seawater as a consequence of high pH. The same authors also found a reduction in the concentration of AHL added to the turbort larvae rearing water in the presence of an AHL-degrading enrichment culture (EC5, mixture of microbial communities). Thus, the results of experiment 1 clearly showed that the daily addition of the AHL mixture at 1 mg/L was effective in significantly reducing the development and survival of the larvae.

Next it was investigated whether the negative influence was due to individual AHL molecules or their interaction. Experiment 2 showed that exogenous addition of the AHL mixture (1 mg/L) significantly reduced the development and survival of the prawn larvae by 11 and 41%, respectively, confirming the results of Experiment 1. Among the individual AHL molecules, only C4-HSL significantly reduced the LSI and survival of prawn larvae but by a lesser extent (i.e., by about 9.3% and 8.6%, respectively). Generally, the QS molecules actively regulate the expression of a diverse array of virulence factors in the pathogenic bacteria when the host is infected (or in stress). In our study, the larvae were weaker as indicated by reduced activity (slow movement, based on visual observation), low LSI and poor survival when treated daily with 1 mg/L AHL mixture concentration. We speculate that this low performance of prawn larvae in the presence of AHL mixture could be either due to reduced prey (Artemia) density as Artemia have also been reported to be affected by QS molecules (Defoirdt et al., 2005, 2006) or due to the pathogenicity of the microbial communities associated with the prawn larvae. Results revealed that prey (Artemia) survival in this study reduced from 62.7% (control ones) to about 38.7% when exposed 24 h to a residual concentration of 1.8 mg/L AHL mixture. Generally, there should be about 3–6 Artemia nauplii/mL directly after feeding, depending on the age of the prawn larvae, and 1 Artemia nauplius/mL left in the water just before the next feeding time (New, 2003). In our study, there was more than 1 nauplius/mL at the latter time (data not shown). So, it is most likely that low survival of the prawn was not related to low prey availability. Moreover, water quality parameters (pH, NO3–N and NH4–N) were monitored daily and water exchange was done accordingly to avoid any toxic effect of these parameters on Macrobrachium larvae.

The opportunistic bacteria can release one or several AHL molecules (Dunmy and Winans, 1999; Swift et al., 1999) and there is no doubt that many more signalling molecules have still to be discovered. Some pathogenic bacteria among them use single QS system like Ahyi–Ahyb in A. hydrophila, Asal–Asar in A. salmonicida (Swift et al., 1999) for controlling their virulence while other bacteria employs multiple QS system. For example, P. aeruginosa employs Las–LasR and Rhl–RhlR (Winson et al., 1995). V. harveyi utilizes three QS systems: CAI-1–CqsS, HAI-1–LuxN and AI-2–LuxQ (Henke and Bassler, 2004). In P. aeruginosa, N-(3-oxododecanoyl)–l-homoserine lactone (3-oxo-C12-HSL) and N-butyryl-–l-homoserine lactone (C4-HSL) are the two AHLs produced by Las and Rhl QS systems. These two AHLs conjugate with their cognate transcriptional activator proteins (LasR and RhlR) and regulate the expression of various virulent genes (Pearson et al., 1994). Pearson et al. (1997) reported that the R-proteins are not significantly activated by their noncognate AHLs i.e., LasR is not activated by C4-HSL but simultaneously they observed that 3-oxo-C12-HSL is capable of activating RhlR (but only at low-level). In another study, Surette and Bassler (1998) demonstrated that autoinducer molecule (but not AHL) produced by E. coli have the ability to activate the autoinducer 2 (AI-2) of the V. harveyi QS systems. Based on the results of our present study and those of the above mentioned authors, we can assume that the high mortality of prawn larvae treated daily with the AHL mixture (1 mg/L) could be due to the presence of several AHL molecules which besides conjugating with their cognate transcriptional activator proteins and regulating the expression of various virulence factors in one bacterium also might have conjugated with different other non-cognate R-proteins of different bacteria inducing the production of several other virulence factors. The simultaneous production of an array of virulence determinants by several micro-organisms might have instigated a unified attack on the prawn larvae thereby severely affecting their development and survival. Thus, the findings of this first in vivo study are intriguing and suggest that the negative performance of the prawn larvae is due to the interaction of several QS molecules. Several authors had reported that the bacteria initiate a concerted action on the host (defence system) only when the AHL molecules reach a quorum or critical threshold level (corresponding to a particular population density) (Defoirdt et al., 2004; Dunn and Stabb, 2007). In experiment 3, the critical AHL dose using different AHL mixture concentrations was investigated. Results showed that the AHL mixture was not able to cause any significant mortality of prawn...
When larvae were daily added at concentrations up to 0.50 mg/L. However, when larvae were daily added at 1 mg/L, there was a high mortality as in experiments 1 and 2. LSI, on the contrary showed a slightly different trend in comparison to larval survival. The development of the larvae was already negatively affected in the treatment with a daily addition of 0.125 mg/L of AHL mixture, in the absence of detectable residual AHL. Hence, LSI apparently is a much more sensitive parameter than survival. Although AHL molecules can be used as carbon and nitrogen source by microorganisms (Molina et al., 2003; Tinh et al, in press), we assume that the small amount of added AHL molecules is insufficient to steer the microbial community composition directly. However, because of the increased mortality in the A.HL treatment, dead larvae and prey can become substrate for micro-organisms, triggering changes in microbial community composition and activity, eventually, aggravating negative host microbial interactions. This complex interplay of factors will need to be disentangled by more focussed experiments.

In essence, results obtained in our study suggest that the microbial communities associated with the prawn larvae become virulent when a quorum or critical threshold level is reached through the daily addition of an AHL mixture at a concentration of 0.125 mg/L. The impaired development and survival of the prawn larvae is to a great extent due to the interaction of several AHL molecules rather than individual molecules.

The most likely site of action of the microbial communities is the gastrointestinal tract of the host (prawn). Yet at this moment it is not clear to what extent the added AHL molecules contribute to an increase of the AHL concentration in the gastrointestinal tract. Therefore, it will be of importance in the future to develop techniques to measure AHL concentration in the gastrointestinal tract in order to understand better the importance of A.HL. Moreover, more information about the in situ AHL-mediated gene regulation is needed to obtain a full understanding of the importance of AHL.

Acknowledgments

The authors are thankful to the technical staffs of Lab of Aquaculture and Artemia Reference Centre, Ghent University for their assistance in carrying out various analyses during the research work. The financial support given by Vlaamse Interuniversitaire Raad (VLIR), Flemish Interuniversity council, Belgium is also gratefully acknowledged.

References