A mis amados hijos

INTENSIFICATION OF WHITE SHRIMP *LITOPENAEUS VANNAMEI* (BOONE) LARVICULTURE

Intensifiëring van de larvicultuur van de witte garnaal *Litopenaeus vannamei* (Boone)

MARÍA DE LOURDES COBO

Promoters: Prof. Dr. Patrick Sorgeloos
Department of Animal Production
Faculty of Bioscience Engineering, Ghent University, Belgium
patrick.sorgeloos@ugent.be

Dr. ir. Roeland Wouters
INVE Technologies
r.wouters@inveaquaculture.com

Members of the Examination and Reading Committee:

Prof. Dr. Colin Janssen - Chairman
Department of Applied ecology and Environmental biology
Faculty of Bioscience Engineering, Ghent University, Belgium
colin.janssen@ugent.be

Prof. Dr. ir. Guy Smagghe - Secretary
Department of Crop Protection
Faculty of Bioscience Engineering, Ghent University, Belgium
guy.smagghe@ugent.be

Prof. Dr. ir. Peter Bossier
Department of Animal Production
Faculty of Bioscience Engineering, Ghent University, Belgium
peter.bossier@ugent.be

Prof. Dr. Jorge Calderón
Faculty of Research and Postgraduate Studies
Escuela Superior Politécnica del Litoral (ESPOL), Ecuador
jcaldero@espol.edu.ec

Prof. Dr. Ronaldo Cavalli
Professor of Aquaculture
Federal Rural University of Pernambuco, Brazil
ronaldocavalli@gmail.com

Dr. Karin van de Braak
Sustainable Aquaculture Solutions
karin@sasnet.nl

Prof. Dr. Hans Nauwynck
Department of Virology, Parasitology and Immunology
Faculty of Veterinary Medicine, Ghent University, Belgium
hans.nauwynck@ugent.be

Dean: Prof. Dr. ir. Guido Van Huylenbroeck
Rector: Prof. Dr. Paul Van Cauwenberge

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MARÍA DE LOURDES COBO

Escuela Superior Politécnica del Litoral (ESPOL)

Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM)

Ecuador

Promoters:

Prof. Dr. Patrick Sorgeloos, Department of Animal Production, Laboratory of Aquaculture & Artemia Reference Center, Rozier 44, 9000 Gent

Dr. ir. Roeland Wouters, INVE Technology, Hoogveld 93, 9200 Dendermonde, Belgium

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
APHA	American Public Health Association
AWWA	American Waterworks Association
bp	Base pairs
CENAIM	Centro Nacional de Acuicultura e Investigaciones Marinas
CFU	Colony forming units
cm	Centimeter
CT	Culture tank
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DO	Dissolved Oxygen
DW	Dry weight
ER	Enriched rotifers
ESPOL	Escuela Superior Politécnica del Litoral
FAN	Frozen *Artemia* nauplii
FAO	Food and Agriculture Organization of the United Nations
FUA	Frozen Umbrella-stage Artemia
g	Gravity
g	Gram
h	Hour
Ha	Hectare
kg	Kilogram
L	Liter
LC_{50}	Median Lethal Concentration
LSI	Larval Stage Index
LT_{50}	Median Lethal Time
M	Molar
M1	Mysis 1
M2	Mysis 2
M3	Mysis 3
m^2	Square meter
m^3	Cubic meter
MA	Marine agar
mg	Miligram

min	Minute
mL	Mililiter
mm	Milimeter
mM	Millimolar
µL	Microliter
µm	Micrometer
µM	Micromolar
N	Nauplii
N5	Nauplii 5
Nd	Not determined
NH3-N	Un-ionized ammonia - Nitrogen
NH4-N	Ionized ammonia - Nitrogen
NO_2-N	Nitrite-Nitrogen
NO_3-N	Nitrate-Nitrogen
NS	Not significant
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PL	Postlarvae
PL1	Postlarvae 1
PL2	Postlarvae 2
PL4	Postlarvae 4
PL10	Postlarvae 10
PL12	Postlarvae 12
PL15	Postlarvae 15
RAPD	Random Amplified Polymorphic DNA
RAS	Recirculating aquaculture system
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
SE	Standard error
SEM	Standard error of the media
SGR	Specific growth rate
TAN	Total ammonia nitrogen
Taq	Thermus aquaticus
TAS	Traditional aquaculture system
TCBS	Thiosulphate-Citrate-Bile Salt-Sucrose
TSS	Total suspended solids

UPGMA	Unweighted Pair Group Method with Arithmetic Averages
USA	United States of America
UV	Ultra-violet
V	Volt
VNR	Volumetric Nitrification Rate
VLIR	Vlaamse Interuniversitaire Raad (Flemish Inter -University Council)
w/v	Weight per volume
WPCF	Water Pollution Control Federation
WSSV	White spot syndrome virus
Z1	Zoea 1
Z2	Zoea 2
Z3	Zoea 3
16S rRNA	Subunit of 16S ribosomal RNA

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CHAPTER I

GENERAL INTRODUCTION AND THESIS OUTLINE

GENERAL INTRODUCTION AND THESIS OUTLINE

Aquaculture continues to be the fastest growing food-producing sector. With a production of less than 1 million tonnes per year in the early 1950s, aquaculture production in 2010 was reported to be 60 million tonnes with a value of US$119 billion (excluding aquatic plants and non-food products), showing an average annual increase of 8.8 percent (FAO, 2012). Moreover, aquaculture contribution per capita fish food supply has increased from 1.1 kg in 1980 to 8.7 kg in 2010, accounting for 47% of the world's fish food supply (FAO, 2012).

Marine shrimp make up a significant part of crustacean aquaculture. Shrimp are also the most important commodity by value in the world seafood trade. In 2000, in terms of global aquaculture production, *Penaeus monodon* was the dominant species with a production value of US$ 4 billion (FAO, 2002). As of 2005, the shrimp and prawns formed the second most important species group within aquactic organisms with a value of US $18.2 billion. Within this group the highest value for a single species of shrimp was reported for *Litopenaeus vannamei* with US$5.9 billion (Lowther, 2007). Values reported for *L. vannamei* in 2006 and 2008 amounted to US$ 7.8 and $8.9 billion respectively (FAO, 2010). Today the production of marine species is still dominated by the white leg shrimp, *L. vannamei* with a reported value of US$11.3 billion, whereas the giant tiger prawn, *P. monodon*, has lost importance in the last decade (FAO, 2012). The reasons this species became the main penaeid shrimp produced worldwide is mainly due to breakthroughs in broodstock domestication and concomitant strain selection through broodstock improvement programmes, its tolerance to environmental conditions and higher disease resistance, development of disease management strategies and lower production costs during grow-out culture.

The demand for aquaculture products is expected to increase while catches of marine fish and shellfish is declining or stagnating at best, therefore stimulating the expansion of world aquaculture. This has led not only to increased production via conventional farming systems, but also to a strong shift toward more intensive farming practices. With the

expected expansion of shrimp production, high numbers of good quality larvae will be required from commercial hatcheries. Generally, further development of a sustainable shrimp aquaculture is largely dependent on improvements of larviculture practices. Despite the progress made, larval rearing is still a bottleneck in the production cycle. The unstable larval production and larval quality are mainly due to suboptimal husbandry techniques and feeding regimes, deterioration of the culture environment, and occurrence of diseases associated with traditional culture practices. High-density culture systems which maximize output per unit investment and integrate more environmentally friendly technologies need to be assessed for larval production in order to achieve higher production of good quality shrimp larvae. Recirculating Aquaculture Systems (RAS) offer good prospects to respond to this need. In RAS, the water contaminated with toxic compounds is treated internally which reduces the amount of water use and discharge from aquaculture operations. Moreover, adopting RAS for larval rearing is also driven by biosecurity concerns, since water recirculation reduces the possibility of pathogen introduction.

For shrimp larviculture, however, the use of RAS is a relatively new field, while it could be a powerful tool to improve the larval output. Although only a limited amount of studies addresses the use of RAS in shrimp larviculture, there is evidence of the advantages these systems impair in survival, growth and larval quality (Gandy, 2004; Thach *et al*., 2005; Ghanekar, 2005; Truong *et al*., 2009). Besides, the development of recirculating systems for larval rearing is also a prerequisite for the intensification of larviculture (allowing higher stocking densities) and for rendering hatchery production more profitable.

The general objective of this thesis was to develop new culture techniques for the intensive production of *L. vannamei* larvae through the use of a recirculating system. To reach this objective our research pursued three consecutive steps:

- In a first step, the feasibility to intensively produce *L. vannamei* larvae through the implementation of a recirculating system was evaluated. In an experimental recirculating system, the effects of stocking density, feeding strategy and water

exchange rate on the survival and growth of *L.vannamei* larvae were investigated.

- In a second step, the possible application of the intensive culture of *L. vannamei* in a recirculating system was examined at pilot scale.
- In a third step, the intensive culture in the recirculating system was further optimised through improving the feeding regime. Special attention was given to evaluate the quality of the larvae produced intensively in the recirculation system under nursery and grow-out culture conditions.

Thesis outline

In *Chapter II* (Literature review), the biology of *L. vannamei* and the existing culture techniques of penaeid shrimps are highlighted based on a literature study.

In *Chapter III* (Ammonia Tolerance of *L. vannamei* larvae), the acute toxicity of ammonia in the larval stages of *L. vannamei* was determined contributing to the knowledge of tolerance limits of the species regarding water quality.

In *Chapter IV* (Super-intensive larviculture of *Litopenaeus vannamei* in a recirculating system: effects of stocking density, water recirculation rate and feeding strategy) the feasibility to intensively produce *L. vannamei* larvae in an experimental recirculating system were studied with special attention to the stocking density, feeding regime and recirculation rate. The achievements obtained in this study are the baseline for the next chapters in this thesis.

Chapter V (High density production of *Litopenaeus vannamei larvae* in a recirculating system), evaluates the possibility for further application of the intensive production of *L. vannamei* larvae trough the implementation of a recirculating system on pilot scale. The advantages of this culture system compared to the traditional culture system are discussed thoroughly.

In *Chapter VI* (Evaluation of the use of umbrella-stage *Artemia* as a food source for rearing *Litopenaeus vannamei* larvae), the use of umbrella-stage *Artemia* was evaluated as a

food source for larval rearing of *L. vannamei* under traditional culture conditions in order to replace rotifers while simplifying and improving the feeding regime.

Chapter VII (Intensive culture of *Litopenaeus vannamei* in a recirculating system with the use of umbrella stage *Artemia* as a food source), assesses the use of umbrella-stage *Artemia* in a high density *L. vannamei* culture in a recirculating system. The study focuses also on the reliability of intensive larvae production in the recirculating system and its economical feasibility.

The overall results of this thesis are then further discussed and the main conclusions as well as further research needs are summarized in *Chapter VIII.*

The last chapter, *Chapter IX* (References) contains the bibliographic references cited in this study.

CHAPTER II

LITERATURE REVIEW

LITERATURE REVIEW

Biology of the species under study

Taxonomy

The taxonomic classification of the white shrimp *L. vannamei* (Boone) (Pérez-Farfante and Kensley, 1997) is as follows:

Kingdom	Animalia
Phylum	Arthropoda
Subphylum	Crustacea
Class	Malacostraca
Subclass	Eumalacostraca
Superorder	Eucarida
Order	Decapoda
Suborder	Dendrobranchiata
Superfamily	Penaeoidea
Family	Penaeidae
Genus	*Litopenaeus*
Species	*Litopenaeus vannamei* (Boone, 1931)
English name	Whiteleg shrimp

Distribution

The whiteleg shrimp is native to the Eastern Pacific coast from Sonora, Mexico in the North, through Central and South America as far south as Tumbes in Peru (Dore and Fridmodt, 1987). This open thelycum shrimp is found in depths between 0 and 72 m in areas where water temperatures are normally above 20 °C throughout the year (Dore and Fridmodt, 1987). This species is relatively easy to culture and therefore stocks of *L. vannamei* have been shipped all over the world.

Habitat, biology and life cycle

The life cycle of *L. vannamei* includes several distinct stages found in a variety of habitats. Adults live and spawn in the open ocean (Dall *et al*., 1990). The natural age that females become mature can be from 4 months onwards (Harrison, 1990), but usually is around 8 to 10 months (Bray and Lawrence, 1992). This corresponds to sizes of 30 - 40 g in *L. vannamei* females and somewhat less in males (Bray and Lawrence, 1992). Females of *L. vannamei* weighing 30 - 45 g will spawn 100 000 - 250 000 eggs. Hatching occurs about 16 hours after spawning and fertilization. Larval stages inhabit plankton rich surfaces water off-shore, with and on-shore migration as they develop (Dall *et al*., 1990).The first stage larvae, termed nauplii, swim intermittently and are positively phototactic. Nauplii do not feed, but live on their yolk reserves (Dall *et al.,* 1990). The next larval stages (zoea, mysis and early postlarvae) remain planktonic for some time, eat phytoplankton and zooplankton, and are carried towards the shore by tidal currents (Dall *et al*., 1990). The postlarvae (PL) change their planktonic habit about 5 days after moulting into PL, move inshore and begin feeding on benthic detritus, worms, bivalves and crustaceans. Juveniles often prefer brackish water of estuaries, coastal wetlands or mangroves areas (*Dall et al.,* 1990) (Figure 1).

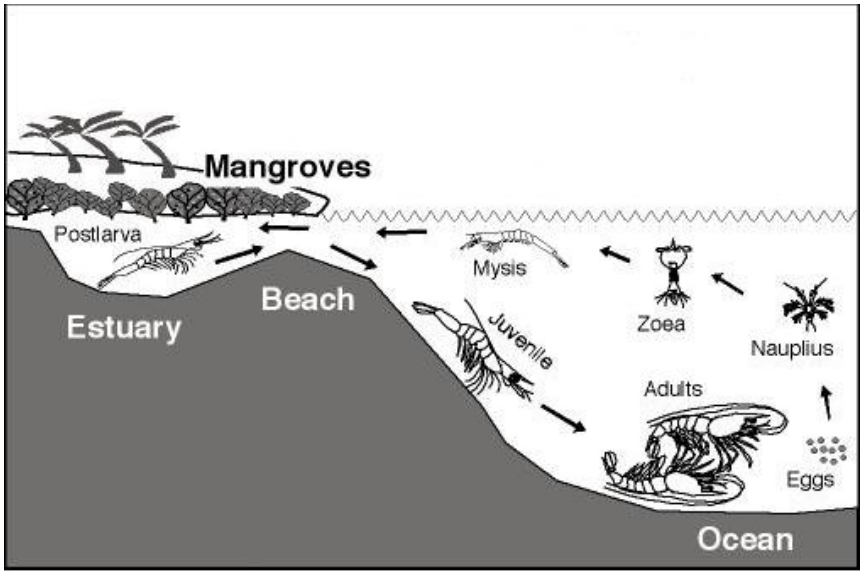

Figure 1. The penaeid shrimp life cycle (source: http//oceanworld.tamu.edu)

Global Status of *Litopenaeus vannamei* Production

In 1973, the first nauplii spawned from a wild-caught *L. vannamei* mated female was shipped from Panama to Florida. By the early 1980s, the development of intensive breeding and rearing techniques permitted its culture in Hawaii, the United States of America (USA) and especially in Central and South America. The commercial culture of this species increase rapidly in Latin America. FAO statistics show that the total farmed production of *L. vannamei* increased steadily from 8 000 tonnes in 1980 to 194 000 tonnes in 1998. After a small decline in 1999 and a more significant decline in 2000 (143 000 tonnes) due to the arrival of White spot syndrome virus (WSSV) in Latin America, data show a rapid increase in production to over 1 300 000 tonnes in 2004, due to the spread of this species to Asia (Figure 2).

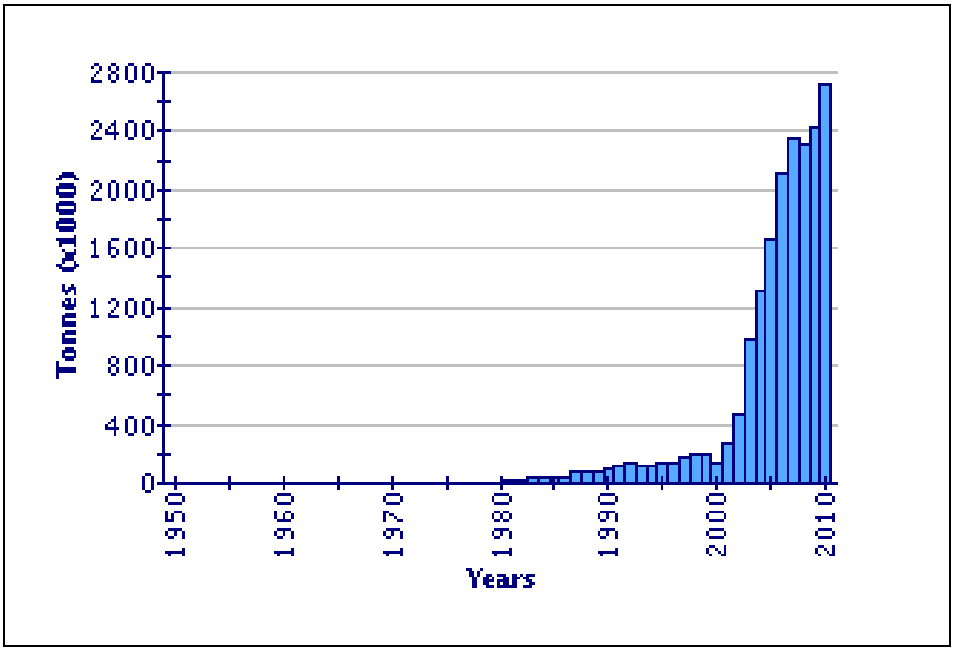

Figure 2. Total farmed production of *Litopenaeus vannamei* during the years.

The replacement of *P. monodon* by L. vannamei in Asia was due to a number of favorable characteristics, such as the resistant and hardy nature of *L.vannamei* and the lower production costs during grow-out culture. Shrimp farming production continued to grow. According to the FAO Fishery Statistics (2012) the main producer countries in 2010 were

China (1 062 000 tonnes), Thailand (501 000 tonnes), Indonesia (208 000 tonnes), Ecuador (150 000 tonnes), Mexico (130 000 tonnes), Brazil (70 000 tonnes), Vietnam (38 000 tonnes) and Malaysia (36 000 tonnes). Currently, *L. vannamei* is the dominating cultured marine shrimp species (Figure 3) exceeding a production of 2.5 million tonnes with a reported value of more than US$11 billion (FAO, 2012).

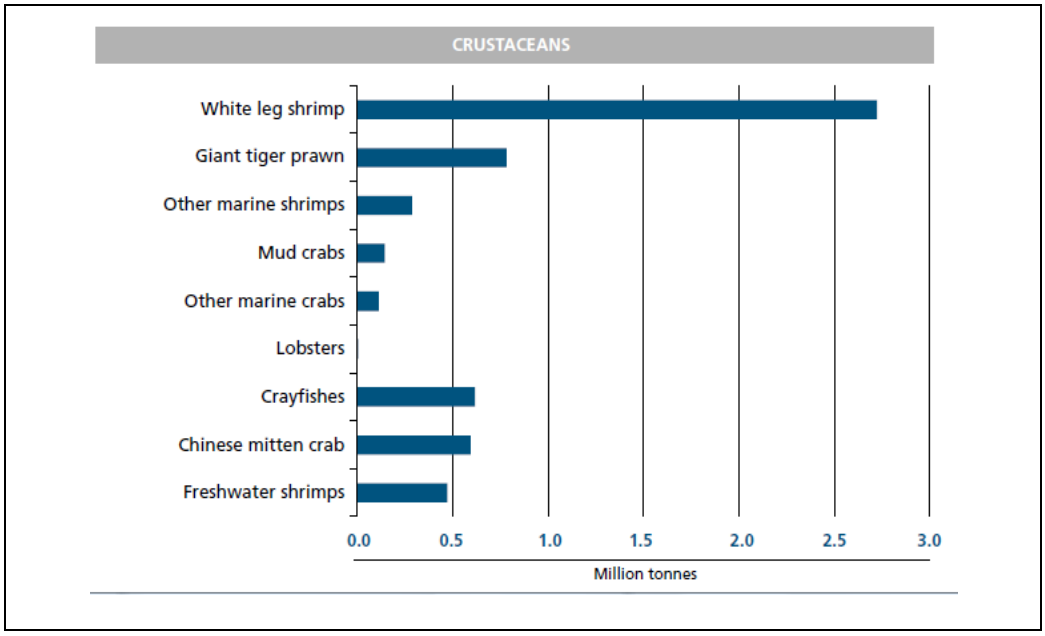

Figure 3. Production of major crustacean species or species groups from aquaculture in 2010 (FAO Fishery Statistics, 2012)

The rapid expansion of *L. vannamei* culture in recent years, particularly in Asia, has led to a drop in price of harvested shrimp. Therefore, producers with high production costs may not be able to survive within the industry. Shrimp farmers are also aware of the growing need to adopt more responsible, traceable, and low impact practices which can enhance biosecurity and help protect the environment, while producing shrimp in a cost efficiently way. The intensive and super-intensive system approach incorporating techniques which can increase biosecurity (*e.g.* zero-exchange, re-use or recirculating systems) may potentially address all of these concerns and should be studied thoroughly.

Overview of Larval Culture Systems

Shrimp production is carried out in two major steps: seed production and (grow-out) farming. Seed production, also known as hatchery rearing, is performed under controlled conditions in large laboratory type facilities and may be divided in two phases: maturation and larviculture. Maturation facilities include all activities related to the production of nauplii, including broodstock maturation, spawning and hatching. Larviculture includes the development of the different shrimp larval stages until they are ready to be collected and transferred to the shrimp grow-out farms (Treece, 2000).

Larviculture systems are mainly categorized in western and oriental-style rearing systems. The more traditional methods were originally developed in Japan and Taiwan (Liao, 1985) and are recognized as less intensive community systems. Later, the Galveston system was developed in the USA, which involves a more intensive and highly controlled approach (Mock and Neal, 1974; Lawrence *et al*., 1985; Treece and Fox, 1993).

The **Japanese System**, known as community culture, is characterized by low stocking densities of around 10-30 larvae L^{-1} in large tanks of up to 200 m^3. The tanks are stocked with wild-caught gravid females. Water is pumped directly from the sea and fertilized to promote growth of diatom microalgae and zooplankton organisms, which serve as food for the larvae. Water exchange rates are low (20%). This system produces about 15 000 PL m^{-3}, i.e. 50% survival (Mock and Neal, 1974; Treece and Fox, 1993). This method is mostly used in temperate conditions. The use of such large-volume tanks is difficult to control in tropical conditions, and whenever a disease occurs it is almost impossible to apply curative treatments in such large volumes.

The **Taiwanese System** uses a mean density of 30-100 larvae L^{-1} and medium size tanks of 30-50 m^3. Tanks are kept in the dark to promote a microbial community dominated by bacteria rather than microalgae. Fresh diatoms, traditionally including *Skeletonema*, are grown separately and supplied to the culture tanks daily (Fegan, 1992; Liao, 1985).

The **Galveston System** known as “clear water system” is characterized by high larval densities of 100 larvae L^{-1} stocked in small tanks in the range of 1-10 m^3 (Treece and Fox, 1993). Nauplii are produced from gravid females in a separate system. Microalgae and

Artemia are cultured in independent systems and are supplied as live food in predetermined quantities. Water quality is maintained with high daily water exchange rates (50-200%). Larvae remain inside the culture tank until they reach PL 5-8 (Mock and Neal, 1974). This method allows accurate control of the water quality, food quantity and quality, and the possibility to apply preventive and curative disease treatment (Treece and Fox, 1993).

Currently a wide range of systems is used all evolving from these basic concepts. They incorporate the best of these technologies and have blurred the differences between the clear water and the community systems.

Larval Rearing: Development of Larval Stages

Larval development of *L. vannamei* includes nauplii, zoea (protozoea), mysis and postlarvae stages. Fertilized eggs undergo a complex process of embryonic development which ends in free-swimming nauplius larvae 14-16 hours after spawning at 28°C (Morales, 1993).

Nauplii

Nauplii are the first larval stage of penaeid shrimp which is common among all crustaceans. *L. vannamei* has 5 nauplii sub-stages with a total duration of 40-50 hours. The main features at this stage are: undifferentiated body, one medium size ocellus or nauplii eye, and the head appendages (antennules and antennae) are used for swimming (Dall *et al.*, 1990) (Figure 4). Nauplii do not feed but rely on their internal yolk reserves (Dall *et al.*, 1990; Edemar *et al*., 1996; Arellano, 1990; Morales, 1993).

The total body length reached at the end of the stage is 430 µm and 220 µm for the body width at 28-30°C. Nauplii swim intermittently and exhibit phototactic behavior, which is used in maturation facilities to harvest and select them (Arellano, 1990; Morales, 1993).

Zoea

Nauplii molt into the zoea stage, referred to also as protozoea stage. The zoea stage has three sub-stages with a total duration of 4 to 6 days. This stage is characterized by differentiation of the body into a cephalothorax and abdomen. The zoea 1 sub-stage (Z1) has

compound eyes, present as a single entity, but they do not extend beyond the cephalothorax. Average length is 1 mm. The digestive tract is completely formed and functional, displaying peristaltic movements from the oesophagus. Zoeae swim continuously in the water column while feeding vigorously immediately after molting. The zoea 2 sub-stage (Z2) has a rostrum and prominent stalked compound eyes and forked supra-orbital spines. An elongation of the abdomen is clearly noticeable in this sub-stage. Length is 1.3 to 2.1 mm. The zoea 3 sub-stage (Z3) is characterized by the presence of biramous uropods and spines on the dorsal and lateral sides of the abdominal segments. Length is 2.1 to 2.7 mm (Arellano, 1990; Morales, 1993). Figure 4 shows the zoeal larval sub-stages of *L. vannamei.*

Mysis

Zoeae molt into a mysis stage which has three sub-stages. There is a drastic change in appearance and behavior. The body is bent in the abdominal region. Mysis swim head-down with vigorous movements of the tail. This stage lasts approximately 4 to 6 days. The mysis 1 sub-stage (M1) has rudimentary pleopod buds on the ventral side of the first abdominal segment. A telson which is bifurcated is present. Length is between 2.5 to 3.5 mm. In the mysis 2 sub-stage (M2) the pleopods elongate and remain unsegmented. Length is between 3.0 to 4.0 mm. At the mysis 3 sub-stage (M3), the pleopods become segmented and are used to create a current which directs food and water towards the pereopods and mouth (Figure 4). Healthy and strong M3 tend to swim towards the water surface. Length is between 3.6 to 4.4 mm (Arellano, 1990; Morales, 1993).

Postlarvae

The postlarvae (PL) begin to swim forward using the pleopods which are longer and have setae. The larvae now resemble an adult shrimp and also become progressively benthic and tend to swim against water currents. Length after reaching the first postlarva stage can vary from 3.5 to 4.3 mm (Arellano, 1990; Morales, 1993) (Figure 4).

It is common to refer to postlarvae by the number of days after they have metamorphosed, *i.e.* PL1 for one day old postlarvae (Figure 4), PL2 for two day old postlarvae, etc. As postlarvae continue to grow, they develop complex gill structures which allow for more efficient gas exchange and osmotic regulation (Dall *et al*., 1990).

Larval Rearing: Zootechnical and Nutritional Aspects

Water Quality parameters

The maintenance of water quality is probably the most important and critical aspect in the larviculture of penaeids (Treece, 1985; Juarez *et al*., 2010). Table 1 indicates the general water quality requirements for shrimp hatcheries.

Table 1. General water quality requirements for shrimp hatcheries

Variable	Acceptable range
Temperature	27 - 29 °C
Salinity	30 - 34 g L^{-1}
pH	7.60 - 7.80
Dissolved oxygen	> 5 mg L^{-1}
NH_3-N	< 0.01 mg L^{-1}
NO_2-N	< 0.10 mg L^{-1}

Source: Juarez *et al.*, 2010

To reduce the time required to grow postlarvae, some hatcheries use a high temperature regime of 29 - 34 °C for larval rearing (Fegan, 1992; Juarez *et al*., 2010). However, elevated temperatures may also increase the rate of bacterial growth.

Water treatment

Hatcheries can use a well point system, which consist of finely slotted well pipes buried in the sand or direct water intake from the open ocean. After a first filtration, a combination of settling reservoirs, sand, gravel, cartridge, bag, diatomaceous earth, and granular activated carbon can be used (Juarez *et al*., 2010). Water for larval rearing should be filtered up to 5 µm in order to assure low concentrations of total suspended solids.

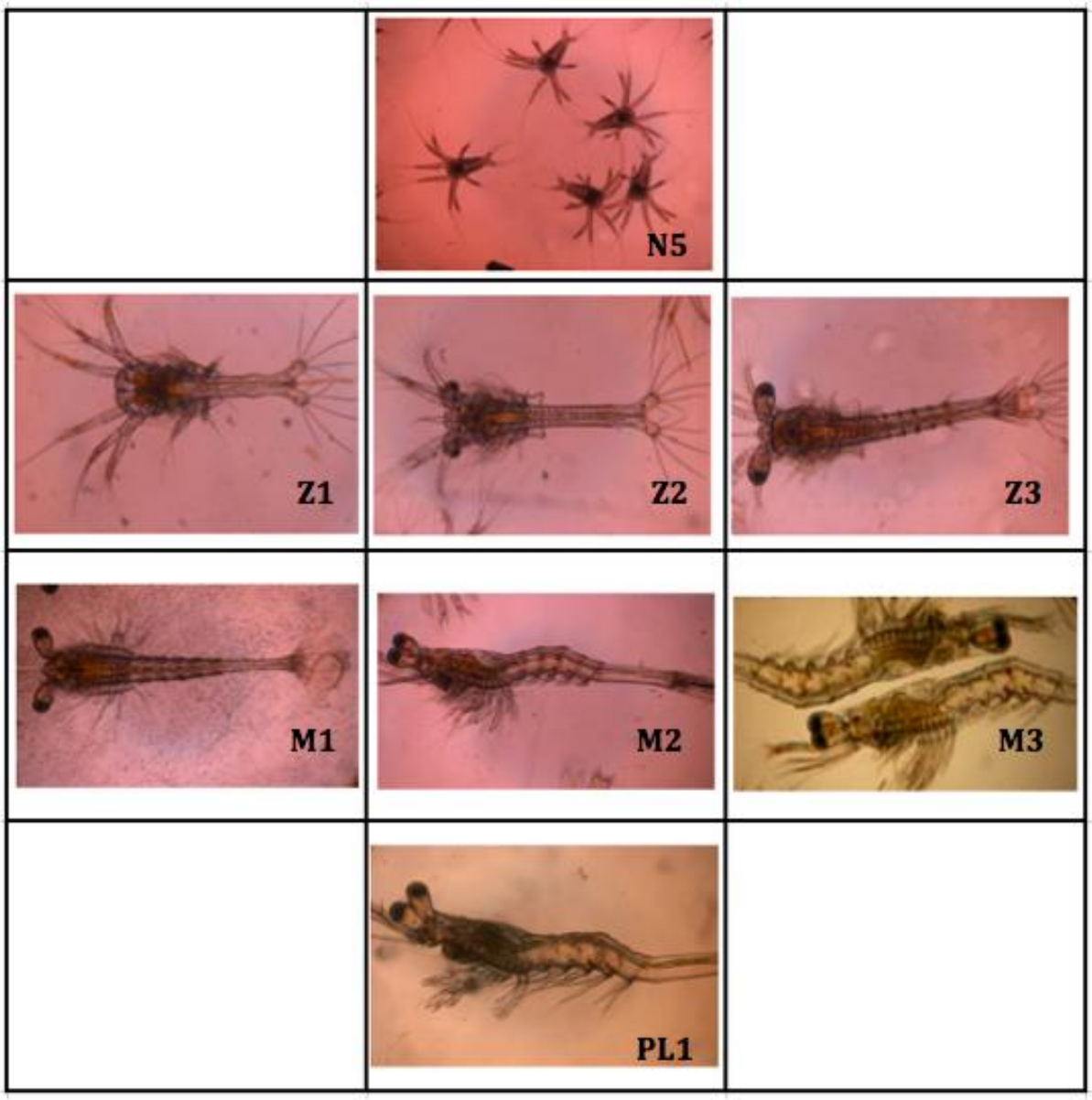

Figure 4. *Litopenaeus vannamei* larval stages and sub-stages; Nauplii 5 (N5), zoea 1 (Z1), zoea 2 (Z2), zoea 3 (Z3), mysis 1 (M1), mysis 2 (M2), mysis 3 (M3) and postlarvae 1 (PL1).

In order to minimize introduction or spread of pathogens, competitors and/or vectors, intake water should be disinfected. A simple method used for disinfection is the addition of chlorine, which is afterwards neutralized with sodium thiosulfate, ascorbic acid or with heavy aeration to make chlorine volatilize. The use of ozone to disinfect the water is another method commonly used in hatcheries, but control in dose must be taken care of in order to avoid production of residuals (Juarez *et al*., 2010).

Water management or water renewal

Water quality water is important to maintain health and have good larval performance. High quality can be maintained by water exchange at different intervals through screens of different mesh sizes depending on larval stages. This approach is known as static rearing system with discontinuous water renewal, with one or two renewals day^{-1}. Water exchange may vary between 50 to 100% day^{-1} (Vinatea and Andreatta, 1997). High water exchange rates although promoting healthy larvae, are wasteful with regard to feed, water and heat. Therefore with respect to food, sufficient quantities should be administered to compensate food consumed by the larvae and food flushed with water exchange (Juarez *et al*., 2010). Also high water exchange rates can destabilize the microbial communities giving the opportunity for new niches for pathogenic bacteria to develop (Browdy, 1998). A different strategy to maintain high water quality in larval cultures is continuous water renewal with rates up to 300% per day (Vinatea and Andreata, 1997).

Recirculating technology and intensification of larviculture

There are few studies carried out either on high densities culture of penaeid shrimp larvae or on water renewal strategies. Intensification causes drastic changes in the quality of the water through the build-up of toxic metabolites, especially ammonia and nitrite, which have harmful effects on the reared species, such as a decrease in growth or survival (Millamena *et al*., 1991). Maintenance of water quality is probably the most important and critical aspect in the larviculture of penaeids (Treece, 1993).

Cook and Murphy (1969) when studying different penaeid species and different stocking densities obtained an average survival of 50% for three penaeid species in densities

up to 266 larvae L^{-1}. Jones *et al.* (1979) obtained survival of 53-86% for *Marsupenaeus japonicus* using densities of 240-260 larvae L^{-1}. Higher stocking densities up to 400 larvae L^{-1} were evaluated by Beard *et al.* (1977) who obtained survival between 42-83% for *Fenneropenaeus merguiensis*. In a study with *Fenneropenaeus indicus* with stocking densities of 35 to 200 larvae L^{-1}, Emmerson and Andrews (1981) established maximum limits for zoea up to the postlarval stages of 100 larvae L^{-1}, and then suggested thinning to 50 larvae L^{-1} for later postlarval stages. Wang and Ma (1990) described that the production at large scale of *Fenneropenaeus chinensis* in China used stocking densities up to 300 postlarvae L^{-1}. The water renewal strategy used for most of the aforementioned studies was a discontinuous renewal strategy (static rearing system with one or two water renewals day^{-1}, at rates of 30-100% day^{-1}). There is scarce information concerning the larviculture of penaeid larvae applying a continuous water renewal strategy, which would probably maintain better water quality in cultures with high stocking densities. Vinatea and Andreatta (1997) evaluated two high stocking densities of 200 and 350 larvae L^{-1} with two water renewal strategies (continuous and discontinuous), each with three water renewal rates (100, 200 and 300% day^{-1}). They observed that biological parameters were significantly higher using a discontinuous renewal at an initial stocking density of 200 larvae L^{-1} and a renewal rate of 100%.

Recirculating Aquaculture Systems (RAS) are increasingly being used in fish hatcheries, not only to save heating costs, but especially to guarantee more stable water quality conditions, which apparently cause less stress on the animals and results in better larviculture performance (van Rijn, 1996; Blancheton, 2000; Faulk and Holt, 2005; Faulk *et al.,* 2007). For shrimp larviculture however, the use of RAS is a relatively new practice, although some trials have been carried out at experimental level.

Where in the typical traditional aquaculture systems (TAS), water quality can rapidly degrade leading to increased mortalities and a higher incidence of diseases, RAS might help to achieve stable environmental conditions and might improve biosecurity. In addition, the development of RAS for larval rearing is thought to be a prerequisite for the intensification of larviculture (higher stocking densities) and for rendering hatchery production more profitable and more environmentally friendly.

In Ecuador at the start of the penaeid shrimp larviculture during the years 1989-90, the commercial hatchery INBIOSA located in Punta Blanca, Santa Elena Province operated a RAS for *L. vannamei* larvae. The system was composed of a biofilter containing crushed coral rock as substrate connected to the larval rearing tank. The stocking density used was 100 larvae L^{-1}. Recirculation started at Z3 until the postlarvae were ready for transfer to outdoor raceways. Larvae produced with the RAS were in good condition. Based on similar principles, Ocean Boy, an intensive shrimp project in Florida operated a RAS for *L. vannamei* producing good quality postlarvae (Juarez *et al.*, 2010).

Gandy (2004) evaluated RAS for the production of *F. aztecus* PL. The stocking density used in the rearing trials was 100 Z1 L^{-1}. Water was re-used during six larval rearing cycles, and in the latest trials – after implementing a biofilter to exert better nitrite reduction – the PL survival in RAS was similar or higher than that in control tanks. Also length, weight and stress tolerance were similar to the control. The author recommended complete cleaning and sterilization of the larval rearing system and start-up of a new biofilter for each larval cycle.

Ghanekar (2005) reported preliminary results with RAS for *P. monodon* larviculture in India. The survival in the larval phase (N6-PL4) was 66% using initial stocking density of 300 larvae L^{-1}, and survival in the PL phase (PL4-PL15) was 86% using an initial stocking density of 66 larvae L^{-1}. The larval development rate however, was reduced, and the system therefore required further improvements. Thach *et al.* (2005) also applied a RAS for commercial-scale *P. monodon* larviculture in Vietnam, with stocking densities of 250 larvae L^{-1}. The first eight days after stocking, a static culture system was applied. From day 9 onwards, a recirculation rate of 300% day^{-1} was applied, which was increased to 400% day^{-1} when decreased water quality was observed. PL12 survival ranged from 20% to 80%, with half of the batches yielding 60% or more. Truong *et al.* (2009) evaluated six stocking densities from 150 up to 900 nauplii L^{-1} for *P. monodon* in an improved RAS composed of a biofilter, protein skimmer and ozone generator at laboratory scale. They found the best performance at a density of 450 N L^{-1} with a survival of 56% and a yield of 252 larvae L^{-1}. However, all the aforementioned studies using RAS have used conventional to moderately high stocking densities, but so far there are no studies attempting the use of super-high stocking densities.

A novel approach with high stocking densities, using probiotics and no water exchange has recently been applied by commercial hatcheries in Brazil and Mexico (Juarez *et al*., 2010). Stocking densities of 300 - 500 nauplii L^{-1}, being three to five times higher than the traditional standard of 100 nauplii L^{-1}, were used. Probiotics and nitrifying bacteria were incubated separately and were inoculated daily to the larval culture in order to maintain pathogenic bacteria and toxic nitrogenous compounds within safe levels. Full water capacity of the larval rearing tanks was used from the beginning of the culture until the early postlarval stages when water was exchanged. Results indicated that survival was lower than with traditional culture systems, but that production is increased and production costs were reduced due to savings in heating, feeding and water pumping (Juarez *et al*., 2010)..

Larval nutrition and feeding

Feeding habits

Shrimp larvae are temporary members of the zooplankton community and therefore they obtain their nutrition within the planktonic ecosystem. Their phylogenetic characteristic, such as the exoskeleton, which restricts body size, ensures that feeding strategies are directed towards the lower trophic levels. Thus, shrimp larvae use either phytoplankton or a combination of phyto- and zooplankton as their major food source (Jones *et al*., 1997).

As shrimp larvae develop through different larval stages, these stages require different prey sizes and their feeding behaviour changes from herbivorous filter-feeders to carnivorous hunters (Lavens and Sorgeloos, 1996). Shrimp larvae employ either filter or raptorial feeding methods depending on their trophic feeding level. Early herbivorous penaeid zoea require high concentrations of algal cells (50 000 - 120 000 cells mL^{-1}) (Wouters and Van Horenbeeck, 2003). The stage at which the change from filter to raptorial feeding occurs differs between penaeid species, but all are capable of utilizing both methods over a number of larval stages (Kurmaly *et al.*, 1989). The particle size for filtration during zoea stages is within the ranges of 1-30 µm diameter, while zoea can also capture prey with size of up to 100 µm raptorially. For penaeid larvae the particle size of food offered has important nutritional implications. Kurmaly *et al.* (1989) demonstrated an increase in ingested energy for later larval stage M1 and M2 at which a change in foraging behaviour

from filter to raptorial feeding produces a distinct energetic advantage. Although initial contact with food particles is by chance encounter in all decapod larvae (Moller, 1977; Langdon *et al.,* 1985; Kurmaly *et al.,* 1990), both mechano- and chemoreception are involved in food selection once contact has been made.

Food type: live or artificial

Zoea stages are herbivorous and therefore require microalgae, whereas mysis and postlarvae are carnivorous and require zooplankton. Several species of microalgae such as the diatoms *Chaetoceros* spp., *Thalassiosira* spp. and *Skeletonema* spp. have been successfully used in rearing penaeid larvae (Cook and Murphy, 1966; Mock *et al*., 1980; Emmerson, 1980; Martins *et al*., 2006; Soares *et al*., 2006). Phytoflagellates such as *Isochrysis* spp. and *Tetraselmis* spp. have also been successfully used to culture penaeid larvae. Kuban *et al.* (1985) observed similar survival when comparing feeding with diatoms versus feeding with phytoflagellates in four different species of penaeid larvae. However, larvae from all species fed diatoms metamorphosed significantly faster ($p < 0.05$) to the first mysis stage than those fed phytoflagellates. Similar findings were observed by D'Souza and Lorenalgan (1999). Piña *et al.* (2005) showed that a mono-algal diet based on *C. muelleri* is an adequate option for feeding early larvae of *L. vannamei*. Feeding a combination of microalgae may however result in better larval nutrition and survival than feeding a mono-algal diet, as has been demonstrated by Gallardo *et al*. (1995); Ronquillo *et al.* (1997) and Jaime-Ceballos *et al.* (2006). In general, freshly collected microalgae are used to feed first larval stages, but alternatives such as the use of concentrates of microalgae (centrifuged or flocculated) have been investigated by D'Souza *et al.* (2000). These concentrates have the advantage of enabling storage during periods of low hatchery demand or high algae production. Concentrations of microalgae supplied to early larval stages can vary from 40-120 $x10^3$ cells mL^{-1} for diatoms (Kuban *et al*., 1985; Gallardo *et al*., 1995; Martins *et al*., 2006; Soares *et al*., 2006; Juarez *et al*., 2010) while for phytoflagellates it may fluctuate between 2-100 $x10^3$ cells mL^{-1} (Kuban *et al.*, 1985; Gallardo *et al*., 1999; D' Souza and Lorenagan, 1999; Juarez *et al*., 2010).

Newly-hatched *Artemia* nauplii are the most traditionally used food item for mysis stages in penaeid hatcheries. *Artemia* are usually administered alive, but in order to offer

them at earlier stages, different treated forms, such as frozen, heat-killed and blended *Artemia*, have been used as well (Wilkenfeld *et al*., 1984; Wouters and Van Horenbeeck, 2003; Juarez *et al.,* 2010). An additional advantage of using inactive *Artemia* is that it overcomes problems associated with ongrowing of uneaten *Artemia* in the larval rearing tanks (Sorgeloos *et al.*, 1998). However, these treatments may result in nutrient losses affecting the nutritional quality and may deteriorate rearing water quality. García-Ortega *et al.* (1995) studied the effect of heat treatment on the nutritional quality of decapsulated *Artemia* cysts, observing that already at 60°C around 30% of the protein was denaturalized and more than 50% of the enzymatic activity was decreased. In Vietnam, umbrella-stage *Artemia*, harvested from *Artemia* hatching tanks after only 12 h, are sometimes fed to *P. monodon* from M1 to PL 2 (Wouters and Van Horenbeeck, 2003; Wouters *et al*., 2009). Umbrella-stage *Artemia* are smaller than *Artemia* nauplii and have higher energy content (Lavens and Sorgeloos, 1996). *Artemia* can also be enriched with different dietary components and can be offered as a bigger prey to postlarvae. Also decapsulated cysts have been reported to be an excellent feed for postlarvae and can replace partially or totally the use of *Artemia* nauplii (Wouters *et al.,* 2009) demonstrating the versatility of this food item.

Numerous studies have focused on the optimal stage for the introduction of an animal food source in penaeid larviculture. Emmerson (1984) demonstrated that *F. indicus* larvae consume rotifers as early as Z1, while Yúfera *et al.* (1984) and Hirata *et al.* (1985) showed rotifer consumption from Z2-Z3 sub-stages for *Melicertus kerathurus* and *M. japonicus*, respectively. Naessens *et al.* (1995) observed that rotifers were ingested by *L. vannamei* from Z2, accelerating the larval development. As a result, the metamorphosis to the postlarval stage took place almost one day earlier than in a control group. It has also been demonstrated by several studies that when introducing *Artemia* nauplii in the feeding regime of penaeid larvae at Z2 stage, the mysis stage is reached earlier than when fed only microalgae (Wilkenfeld, 1992; Kuban *et al*., 1985; Juarez *et al*., 2010).

Due to instability in supply and increase in the cost of *Artemia* cysts, there is a continuous interest in the search of alternative feeds for shrimp larviculture. Candidate organisms to supplement or replace *Artemia* nauplii include nematodes, *Panagrellus redivivus* (Wilkenfeld *et al.*, 1984; Biedenbach *et al.*, 1989; Focken *et al*., 2006), rotifers (Emmerson, 1984; Samocha *et al.*, 1989; Naessens *et al.*, 1995) and copepods (D'Abramo *et*

al., 2006). Growth and survival of *L. vannamei* larvae fed nematodes and algae equalled or exceeded those on diets of *Artemia* and algae (Biedenbach *et al.*, 1989). Hirata *et al.* (1985) found little difference in survival, growth or total length of *M. japonicus* larvae fed rotifers, *Artemia* or a combination of the two. However, energy intake from predation on rotifers was shown to be much lower than in case of *Artemia* predation for *F. indicus* larvae (Emmerson, 1984). Samocha *et al*. (1989) reported a significant reduction in *P. semisulcatus* postlarval dry weight when *Artemia* were supplemented with rotifers and a decrease in growth and survival when *Artemia* were completely replaced with rotifers. On the contrary, Liu (1991) and Naessens *et al.* (1995) observed a better survival and a marked advance in larval development for *F. chinensis* and *L. vannamei* larvae, respectively.

Formulated diets are used in shrimp larval rearing as a supplement to live feeds. There is variety of brands of micro-particulate and micro-encapsulated larval feeds used to incorporate specific nutrients and/or to reduce the amount and expense of *Artemia* cysts (Wouters *et al*., 2009). Most hatchery operators use them as supplement and prefer to mix different artificial feeds rather than use a single diet. The daily rations generally are divided into at least four feedings per day and some even feed every one or two hours (Velasco *et al*., 1999). Frequent feeding promotes ingestion of fresh food and avoids deterioration of water quality (Juarez *et al*., 2010).

Larval Diseases

L. vannamei culture has been plagued with disease problems during the hatchery phase. Larvae can be affected by diseases of viral, bacterial, fungal, protozoan and toxic etiologies. *Baculovirus penaei* (BP), a viral disease, mainly affects larvae in the zoea, mysis and early postlarval stages (Couch, 1974) often resulting in high mortality. Currently, BP is not considered as a major pathogen in shrimp hatcheries anymore, due to improved sanitary management.

Bacterial diseases are considered to be the major cause of mortality in larviculture of *L. vannamei* (Garriquez and Arevalo, 1995; Griffith, 1995; Gomez-Gil *et al*., 2000; Juarez *et al*., 2010). Their control is an important concern because of their impact on production and economics of both the hatchery and farm. Luminescent bacteria can affect larvae and can

cause heavy mortalities. Larvae infected with this bacteria glow blue-green when observed in complete darkness. Non-luminous bacterial infection of larvae can also occur, provoking high mortalities within few days. Another disease affecting larval rearing in hatcheries is the disease known as "bolitas", referring to the spherical bodies found in the hepatopancreas of infected larvae (Griffith, 1995; Vandenberghe *et al.,* 1999). This disease can cause up to 90% mortality in a period of 12 h (Griffith, 1995). During the development of a control program for the "bolitas" disease, Griffith (1995) observed that there were two distinguished bacterial groups involved: innocuous species (*Vibrio alginolyticus, Aeromonas* spp., *Flexibacter* spp.) and possibly pathogenic species (*V. parahaemolyticus, V. vulnificus* and *Corynebacteria* spp.). The author observed that the occurrence of "bolitas" disease was preceded by a decrease in *V. alginolyticus* and an increase in *V. parahaemolyticus*.

Vanderberghe *et al.* (1999) performed bacteriological surveys at different hatcheries in Ecuador, taking samples of routine productions of healthy and diseased *L. vannamei* larvae and postlarvae. They found that *V. alginolyticus* was the dominant *Vibrio* species in both healthy and diseased larvae. An association of *V. alginolyticus* was found with healthy *L. vannamei* larvae during the nauplii and zoea stages, while during postlarval stages, *V. alginolyticus* was isolated frequently form diseased larvae. Also, *V. harveyi* was predominantly isolated from diseased postlarvae but not recovered from nauplii and zoea stages. In 60% of the disease outbreaks, *V. harveyi* was isolated as the predominant bacterium.

Zoea 2 syndrome is another common disease, characterized by cessation of feeding, lethargy, inflammation of the digestion tract, atrophy of the hepatopancreas and a rapid intestine evacuation (Juarez, 1997). The cause of Z2 syndrome is not fully established, but Vandenberghe *et al.* (1999) found that *V. alginolyticus* strains were associated with larvae affected with Z2 syndrome.

Mysis molt syndrome, also called exuvial entrapment, is characterized by incomplete molting which makes the molts getting stuck to different parts of the larvae's body by deformities, cessation of feeding and finally heavy mortalities. The cause of the syndrome is not clear, but poor feed quality and quantity and/or bacterial disease have been implicated. Vandenberghe *et al.* (1999) identified *Vibrio alginolyticus* strains associated with the molt syndrome. For the prevention and cure of bacterial larval diseases conventional approaches

have been used which include the use of disinfectants and antibiotics. But, several alternative strategies for the use of antibiotics have been proposed and have already been applied successfully. The use of antibiotics in *L. vannamei* larviculture has dropped drastically, while the production has increased (Griffith, 1995). This drastic reduction of antibiotics use was mainly due to an improvement of rearing conditions, but also to a great extent to the use of probiotics (Garriques and Arevalo, 1992; Griffith, 1995; Gomez Gil, 2000; Zhertmant *et al.*, 1997; Vandenberghe *et al*., 1999; Rodríguez *et al.*, 2007). Probiotics are considered as beneficial microflora, which help in the exclusion of pathogenic bacteria and may improve animal health through different mechanisms: enhancement of digestion, nutrition and the stimulation of the immune system (Verschuere *et al*., 2000; Moriarty, 1999).

Filamentous bacteria can be detrimental to shrimp larvae, fouling the gills, setae, appendages and body surface of shrimp in all life stages. Infestations can provoke problems in molting, oxygen uptake and can lead to death. *Leucothrix mucor, Flexibacter* sp., *Cytophaga* spp., and *Thiothrix* sp. have been implicated in this bacterial infestation (Aguirre and Ascencio, 2000). These filamentous bacteria can be introduced through the live food. Increasing water exchange and the use of chemicals have been suggested to help or reduce the problem (Juarez *et al*., 2010).

Fungal infections are considered as another significant cause of mortality of young larvae in hatcheries. The most common fungus infecting larvae is *Lagenidium callinectes* (Lightner, 1996). *Lagenidium* infects all life stages, but nauplii and zoea are more sensitive. Measures for prevention are good sanitation practice, especially within maturation facilities and disinfecting nauplii with different chemicals.

Ciliated protozoans can also cause infestations in shrimp larvae, the most common being *Vorticella* spp., *Epistylis* spp. and *Zoothamnium* spp. The presence of these protozoans is an indication of excessive organic matter load in the culture water or of suboptimal conditions inhibiting the normal molting of the larvae. Formalin has been recommended at doses of 10-12 mg L^{-1} followed by water exchanges (Juarez *et al.*, 2010).

Whatever the cause of the disease, it can be stated that major disease problems affecting *L. vannamei* larvae generally occur as secondary effect of poor treatment of the

intake water, deterioration of rearing water quality, poor husbandry, larval nutrition deficiencies, overcrowding and poor sanitation.

Larval Quality

A variety of larval quality indicators and consequent assessment methods have been described and reviewed by Fegan (1992), Wilkenfeld (1992), Samocha *et al*. (1998) and Racotta *et al.* (2003). They include various observations and refer to physiological condition, performance (survival and growth), behavior, microscopic examinations and resistance to stress or challenge tests (*e.g.* salinity, chemicals, resistance to pathogens). Morphological characteristics such as *e.g.* muscle size in the sixth abdominal segment, gill development, overall pigmentation are also claimed as valid criteria for the evaluation of larval quality (Fegan, 1992; Bauman and Scura, 1990). Observation of larval behavior such as swimming activity is one of the simplest methods to evaluate larval quality. Postlarvae are submitted to centripetal water that is manually created in a bucket. It is believed that good quality postlarvae have the strength to swim against the current and move towards the wall of the bucket (Naessens *et al*., 1995).

Common practice is to evaluate the physiological condition of the larvae by determining their ability to withstand stressing conditions induced by temperature, salinity shocks, certain chemicals or combination of these (Tackaert *et al*., 1989; Bauman and Jamandre, 1990; Samocha *et al.*, 1998; Racotta *et al.*, 2004). The salinity stress test is the most commonly used test (Villalón, 1991; Fegan, 1992; Samocha *et al*., 1998; Racotta *et al.*, 2003). It is agreed that these stress tests provide information on the animal's quality or condition at the time of determination, but there is no reported evidence that there is a good correlation with performance during the subsequent grow-out phase (Samocha *et al*., 1998; Racotta *et al*., 2003). These stress tests are generally performed on advanced PL stages (PL10 onwards) and few studies have focused on early PL stages. It has been observed that salinity tolerance depends on increased osmoregulatory capacity through PL development (Charmantier *et al*., 1988; Samocha *et al.*, 1998).

Another approach to test larval quality at different larval stages is an *in vivo* infection or "challenge test" to determine larval resistance to pathogens. The usefulness of this

method has been demonstrated primarily in the evaluation of virulence of *Vibrio* isolates associated with mortalities, to test prophylactic, curative treatments and dietary treatments, and in the study of the host-factors influencing the expression of bacterial virulence (Roque *et al.*, 1998; Alabi *et al.*, 1999; Saulnier *et al.*, 2000; Chang *et al.*, 2003; Rodríguez *et al.*, 2007).

CHAPTER III

AMMONIA TOLERANCE OF *Litopenaeus vannamei* (Boone) LARVAE

María de Lourdes Cobo[1], Stanislaus Sonnenholzner[1], Mathieu Wille[2], Patrick Sorgeloos[2]

[1] Escuela Superior Politécnica del Litoral (ESPOL), Campus Gustavo Galindo Velasco, Km 30.5 vía Perimetral, P.O. Box 09-01-5863, Guayaquil, Ecuador. Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM).

[2] Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University, Rozier 44, B-9000 Ghent, Belgium

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AMMONIA TOLERANCE OF *Litopenaeus vannamei* (Boone) LARVAE

Abstract

The tolerance of *Litopenaeus vannamei* larvae to increasing concentrations of total ammonia nitrogen (TAN) using a short-term static renewal method at 26 °C, 34 g L^{-1} salinity and pH 8.5 was assessed. The median lethal concentration (24h LC_{50}) for TAN in zoea (1 - 2 - 3), mysis (1 - 2 - 3) and postlarvae 1 were respectively, 4.2 - 9.9 - 16.0; 19.0 - 17.3 - 17.5 and 13.2 mg L^{-1} TAN (0.6 - 1.5 - 2.4; 2.8 - 2.5 - 2.6 and 1.9 mg L^{-1} NH_3-N). The LC_{50} values obtained in this study suggest that zoeal and postlarval stages are more sensitive to 24h ammonia exposure than the mysis stage of *L. vannamei* larvae. Based on the ammonia toxicity level (24h LC_{50}) at zoea 1, we recommend that this level does not exceed 0.42 mg L^{-1} TAN - equivalent to 0.06 mg L^{-1} NH_3-N - to reduce ammonia toxicity during the rearing of *L. vannamei* larvae.

Introduction

Currently, the Pacific whiteleg shrimp *Litopenaeus vannamei* is the most important cultivated penaeid shrimp species in the world. Semi-intensive and intensive shrimp culture systems are a common activity in many countries. But these culture practices usually result in degradation of the culture water by uneaten food and waste products of the shrimps. Thus, water quality management and knowledge of water quality requirements are essential to any culture system.

One of the most important limiting factors in intensive culture systems is the build-up of toxic nitrogenous waste. Ammonia is the main end-product of protein catabolism in crustaceans and can account for 60-70% of nitrogen excretion with only small amounts of amino acids, urea and uric acid (Chen and Kou, 1996 a, b). Of the nitrogenous compounds, ammonia is the most toxic with nitrite and nitrate being less toxic to shrimp (Chen *et al.*, 1990). In water, ammonia is present in both ionized (NH_4^+) and un-ionized (NH_3) state, with NH_3 as the toxic form due to its ability to diffuse across cell membranes (Fromm and Gillete, 1968; Emerson *et al*., 1975). The relative proportions of NH_3 and NH_4^+ depend on pH, temperature and to a lesser extent on salinity (Bower and Bidwell, 1978).

In penaeid shrimp, high concentrations of ammonia may affect growth rates and survival, and can in extreme cases cause mortality (Wickins, 1976; Zin and Chu, 1991; Chen and Lin, 1992). Ammonia damages the gills and reduces the ability of hemolymph to transport oxygen while increasing oxygen consumption by tissues (Chien, 1992; Racotta and Hernández-Herrera, 2000). Osmoregulatory capacity decreases with increasing ammonia concentration and exposure time (Lin *et al*., 1993). Ammonia may also increase the moulting frequency of shrimps (Chen and Kou, 1992).

Several studies have been carried out to determine toxicity levels of ammonia in different life stages of penaeid shrimp, such as *Penaeus monodon* (Fabricius), *Marsupenaeus japonicus* (Bate), *Farfantepenaeus paulensis* (Peréz-Farfante), *Fenneropenaeus chinensis* (Osbeck) and *Litopenaeus setiferus* (Burkenroad) (Chin and Chen, 1987; Chen and Lin, 1991 a, b; Lin *et al.*, 1993; Ostrensky and Wasielesky, 1995; Alcaraz *et al*., 1999). In *L. vannamei* studies on the acute toxicity of ammonia have been conducted on juveniles (Frías-Espericueta *et al*., 1999; Lin and Chen, 2001) and on postlarval stages (Magallón-Barajas *et al*., 2006), but so far not on larval stages. However, larviculture is a process of primary

importance from which qualified larvae must be produced to obtain maximum yields during postlarval, nursery and grow-out culture. In traditional larviculture systems, water quality can rapidly degrade leading to increased mortality and a higher incidence of diseases, particularly in high density cultures. Therefore, the objective of this study was to determine the acute toxicity of ammonia in the larval stages of *L. vannamei*, contributing to the optimization of water quality management.

Materials and methods

Experimental animals and larval rearing conditions

Nauplii 5 (N5) of *L. vannamei* were obtained from EGIDIOSA hatchery (San Pablo, Santa Elena Province, Ecuador) and transported to the research facilities of the Escuela Politécnica del Litoral (ESPOL) – Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM) in San Pedro de Manglaralto (Santa Elena Province, Ecuador). The nauplii were disinfected with 100 mg L^{-1} of Argentyne® (Argent, Redmond, WA, USA) for 1 min and acclimatized to the experimental conditions. N5 were stocked at 200 N5 L^{-1} in 4 500 L round-shaped fibreglass tanks filled with sand-filtered and UV-treated seawater of 34 g L^{-1} and a pH of 8.1. Temperature was maintained at 28 ± 1°C. Dissolved oxygen concentration was kept above 4 mg L^{-1}. Larvae were fed with the microalgae *Chaetoceros gracilis* from N5 until PL1 (one day postlarvae). From Z2 until PL1, the larvae were fed enriched rotifers *Brachionus* sp. Cayman (Dooms *et al*., 2007), frozen and live *Artemia* nauplii and artificial food Epifeed-LHF® (Epicore, Eastampton, NJ, USA), FRIPPAK® (INVE Aquaculture NV, Dendermonde, Belgium) and LARVAL Z PLUS® (Zeigler, Gardners, PA, USA).

Experimental design and toxicity tests

Short-term toxicity tests (24h) were carried out to determine the acute total ammonia toxicity levels according to the method described by APHA-AWWA-WPCF (1992). Test solutions of ammonia were prepared by dissolving 9.5518 g of ammonium chloride (NH_4Cl) in 250 mL distilled water to make 10 000 mg L^{-1} stock solution and then diluting it to the desired concentration. Ammonia stock solution was added with an automatic pipette (1-10 mL) directly to a series of 2 L glass beakers filled with 1 L of UV treated seawater of 34 g L^{-1} salinity. Flasks were placed into a container filled halfway with water. The water bath was heated to 26 ± 1 °C. Before the start of the actual toxicity tests, range-finding tests were carried out to define concentrations for the final toxicity test. The total ammonia nitrogen (TAN) nominal concentrations used for every larval sub-stage are presented in Table 1.

Table 1. Nominal concentrations (mg L^{-1}) of total ammonia nitrogen and ammonia NH_3-N used for toxicity tests of *Litopenaeus vannamei* larval stages

Larval stages	Nominal Concentration of Total Ammonia Nitrogen (mg L^{-1})	Nominal Concentration of Ammonia NH_3-N (mg L^{-1})
Z1, Z2, Z3	5.0, 7.5, 10.0, 12.5, 15.0, 20.0	0.7, 1.1, 1.5, 1.8, 2.2, 2.9
M1, M2, M3 and PL 1	5.0, 10.0, 12.5, 15.0, 20.0, 25.0, 30.0	0.7, 1.5, 1.8, 2.2, 2.9, 3.7, 4.4

Water from two test flasks was sampled to determine actual concentration of the testing solutions. Ammonia analysis was performed according to the method described by Solórzano (1969). For every bio-assay pH of the seawater was adjusted to 8.5 with a 2N sodium hydroxide (NaOH) solution. Groups of 20 larvae at each larval stage were taken randomly from the culture tank and transferred to the experimental units, once acclimatized for temperature. All treatment concentrations had 5 replicates. Larvae were not fed and the water was not renewed during the 24 hour test, following the procedure of Buikema *et al.* (1982). Mortality was checked after 24h of exposure. Death was assumed when the larvae became immobile and showed no response. Based on the mortality the 24 h Median Lethal Concentration (LC_{50}) and its 95% confidence intervals were calculated using a Logistic regression with a JMP Statistical Package Program. The toxicity of un-ionized ammonia-N (NH_3-N) was calculated according to the equations proposed by Whitfield (1974), based on a

salinity of 34 g L^{-1}, a temperature of 26 ± 1 °C and a pH of 8.5. The safe levels to rear *L. vannamei* larvae were calculated as one tenth of the 24 h LC50 values.

Results

Survival of *L. vannamei* larvae exposed to increasing concentrations of TAN at Z1 to PL1 stages for 24 h are presented in Figure 1. High survival (80-100%) was observed in the control group. Z1 showed lower survival than the control from the lowest concentration tested (5 mg L^{-1} TAN), while an increased tolerance to ammonia was observed for Z2 and zoea 3 (Z3) sub-stages. Survival above 50% was obtained for TAN concentrations of 10 and 15 mg L^{-1} for Z2 and Z3, respectively. Ammonia tolerance increased with larval developmental stages, particularly at mysis, which displayed the highest tolerance with survival up to 70% for 15 mg L^{-1} TAN. No variation was observed within mysis sub-stages. The increased ammonia resistance observed with larval development did not extend to the PL1 stage, which showed a survival of 60% at 12.5 mg L^{-1} TAN.

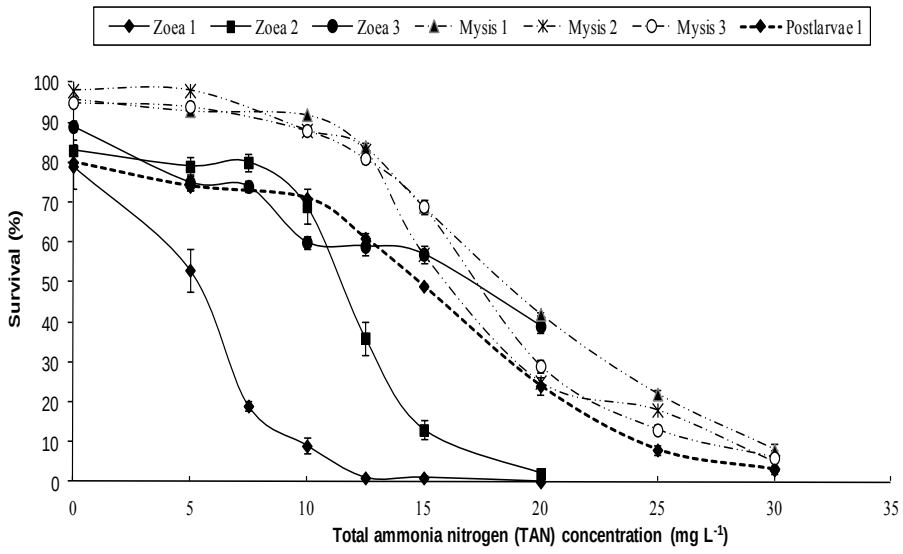

Figure 1. Survival of *Litopenaeus vannamei* larval stages from zoea 1 to postlarvae 1 exposed for 24 h to increasing concentrations (mg L^{-1}) of total ammonia nitrogen (TAN).

LC_{50} values for TAN and their 95% confidence intervals for *L. vannamei* larval stages are shown in Table 2. The 24 h-LC_{50} for Z1 was 4.2 mg L^{-1} TAN (0.6 mg L^{-1} NH_3-N); this

increased with larval development to 9.9 and 16.0 mg L^{-1} TAN (1.5 and 2.4 mg L^{-1} NH_3-N) for Z2 and Z3, respectively. Higher TAN LC_{50} were obtained for mysis sub-stages, being 19.0, 17.3 and 17.5 mg L^{-1} TAN equivalent to 2.8, 2.5 and 2.6 mg L^{-1} of un-ionized NH_3-N for M1, M2 and M3, respectively. The 24 h-LC_{50} for PL1 was 13.2 mg L^{-1} TAN and 1.9 mg L^{-1} NH_3-N. The safe levels for rearing zoea stages were estimated to be between 0.42 to 1.6 mg L^{-1} TAN (0.06 to 0.24 mg L^{-1} NH_3-N). Mysis safe levels were estimated to be from 1.73 to 1.90 mg L^{-1} TAN (0.25 to 0.28 mg L^{-1} NH_3-N) and for PL1, values of 1.32 and 0.19 mg L^{-1} for TAN and NH_3-N, were respectively estimated.

Table 2. The 24h-LC_{50} values (mg L^{-1}) of total ammonia nitrogen (NH_3-N + NH_4^+-N) and NH_3-N for *Litopenaeus vannamei* larval stages and sub-stages. 95% Confidence intervals are shown between parentheses

Larval stages	24 h - LC_{50} Total Ammonia Nitrogen (mg L^{-1})	24 h - LC_{50} Un-ionized Ammonia (mg L^{-1})
Z1	4.2 (3.5 - 4.8)	0.6 (0.5 - 0.7)
Z2	9.9 (9.2 - 10.7)	1.5 (1.4 - 1.6)
Z3	16.0 (14.3 - 18.4)	2.4 (2.1 - 2.7)
M1	19.0 (18.1 - 20.0)	2.8 (2.7 - 2.9)
M2	17.3 (16.5 - 18.1)	2.5 (2.4 - 2.7)
M3	17.5 (16.7 - 18.4)	2.6 (2.5 - 2.7)
PL1	13.2 (12.1 - 14.2)	1.9 (1.8 - 2.1)

Discussion

Several researchers have examined ammonia toxicity in various species of penaeid shrimp and at different developmental stages, especially for juveniles. Chen and Lei (1990) determined that for *P. monodon* juvenile toxicity of ammonia decreased with exposure time. Chen and Lin (1992) observed an increased susceptibility to ammonia as salinity decreased from 30 to 10 g L^{-1} in *F. chinensis* juveniles. For *Penaeus semisulcatus* juveniles exposed to different concentrations of ammonia-N in a series of acute toxicity tests at four different water temperatures, Kir *et al.* (2004) found that lower temperatures clearly increased tolerance of the shrimp to ammonia. Growth rates of *M. japonicus* juveniles exposed to

different ammonia concentrations were investigated by Chen and Kou (1992). The authors concluded that ammonia had a stronger effect on weight rather than length. Studies in *L. vannamei* juveniles have been conducted to evaluate acute toxicity levels of ammonia (Frías-Espericueta *et al*., 1999) and at salinity levels of 15 - 35 g L^{-1} (Lin and Chen, 2001). Racotta and Hernández-Herrera (2000) evaluated the metabolic responses of ammonia exposure in *L. vannamei* juveniles, while Magallón-Barajas *et al*. (2004) determined the daily variations in short-term ammonia toxicity in *L. vannamei* postlarvae (1-30 days old).

Compared to juveniles, scarce information is available about acute toxicity levels of ammonia in larval stages of penaeid shrimp. In table 3, the 24 h LC_{50} of un-ionized ammonia on several penaeid species at larval stages are given.

Table 3. The 24 h LC_{50} (mg L^{-1}) of un-ionized ammonia (NH_3-N) in several penaeid species for early larval stages

	Larval stages			Test conditions			
Penaeid species	Zoea	Mysis	Postlarvae (PL days old)	pH	Temperature (° C)	Salinity (g L^{-1})	Reference
Penaeus monodon	0.76	2.17	4.70 (PL6)	8.2	29.5	34	Chin and Chen (1987)
Marsupenaeus japonicus	0.97	1.08	1.98 (PL1)	7.9	25.0	36	Lin *et al*. (1993)
Fenneropenaeus chinensis	0.65	0.90	1.30 (PL4)	8.2	*	34	Chen and Lin (1991)
Farfantepenaeus paulensis	1.79	2.91	1.40 (PL4)	**	25.0	28	Ostrensky and Wasielesky (1995)
Litopenaeus vannamei	1.47	2.63	1.94 (PL1)	8.5	26.0	34	This study

*temperature for each larval stage: zoea (21 °C); mysis (23 °C); post-larvae (25 °C)

** pH for each larval stage: zoea (8.2); mysis (7.9); postlarvae (8.1)

A progressive tolerance to ammonia with larval development was observed for *P. monodon* (Chin and Chen, 1987), *M. japonicus* (Lin *et al.*, 1993) and *F. chinensis* (Chen and Lin, 1991 b), concluding that zoea stages were the least tolerant and postlarvae the most tolerant to ammonia. In contrast, our results from short-term toxicity tests in *L. vannamei* revealed that mysis stage was considerably more resistant to ammonia than the other stages, coinciding with the findings of Ostrensky and Wasielesky (1995) for *F. paulensis* larvae.

The LC_{50} values for un-ionized ammonia in *L.vannamei* zoea and mysis stages were higher than those reported for *P. monodon, M. japonicus and F. chinensis* (Chin and Chen, 1987; Lin *et al.*, 1993; Chen and Lin, 1991b, respectively) suggesting that *L. vannamei* is more resistant to ammonia at these stages. Similar 24 h LC_{50} values for ammonia through the

larval stages were obtained in this study compared to those reported for *F. paulensis* larvae (Ostrensky and Wasielesky, 1995). Moreover, the same pronounced decrease in ammonia tolerance in the postlarval stage was also observed. During the postlarval stage the final and most important metamorphosis occurs where the larvae become juvenile shrimp. This could explain the lower ammonia tolerance.

Increase in pH levels favor the formation of the more toxic un-ionized form of ammonia or enhance the toxic effects (Colt and Armstrong, 1981). Chen and Sheu (1990 a,b) reported that if the pH level gets higher than 8.2, increasing pH in a given ammonia solution could increase ammonia toxicity to *P. monodon* M2 and *M. japonicus* postlarvae. Similar findings were observed by Chen and Chin (1989), when studying the effect of ammonia at different pH levels in *P. monodon* postlarvae. The median lethal time (LT_{50}) values showed that ammonia toxicity to *P. monodon* postlarvae increased as pH increased. Bower and Bidwell (1978) stated that the fraction of NH_3 does not only depend on pH but also on temperature and to a lesser extent salinity. As pH and temperature rises, proportion of NH_3 to NH_4^+ increases and the toxicity of ammonia to animals increases. Magallón-Barajas *et al.* (2006) investigated the daily variations in short-term (4h) toxicity of ammonia to *L. vannamei* postlarvae (1-30 days old) exposed to several ammonia concentrations in two different scenarios, scenario 1 (pH 8, 26 °C) and scenario 2 (pH 9, 30 °C). In scenario 1, ammonia concentrations up to 18 mg L^{-1} did not provoke mortality while for scenario 2, mortality was recorded at all postlarval ages. In this study the pH was higher than scenario 1 resulting in a higher survival, this suggests that *L. vannamei* larvae are resistant to ammonia under this water conditions.

The mean safe levels calculated in this study for rearing *L. vannamei* larvae were 0.15, 0.26 and 0.19 mg L^{-1} NH_3-N for zoea, mysis and postlarvae stages, respectively, based on a pH 8.5, a temperature of 26 °C and salinity 34 g L^{-1}. A similar value was observed by Chin and Chen (1987) for the "safe level" (0.10 mg L^{-1} NH_3-N) for *P. monodon* larvae; however, they calculated this value from the 96 h LC_{50} value for postlarvae with the application factor of 0.1 (Sprague, 1971), and based on pH 8.2, temperature 29.5 °C and salinity of 34 g L^{-1}. Ostrensky and Wasielesky (1995) stated that the safe level for rearing *F. paulensis* in the hatchery was estimated to be 0.03 mg L^{-1} NH_3-N, a value 5 to almost 10 times lower than the one obtained in our study. They calculated the safe level using the 24 h LC_{50} of embryos

(0.30 mg L^{-1} NH_3-N) for rearing larvae, based on pH 8.1, temperature of 25 °C and salinity of 28 g L^{-1}. A comparison of safe levels between penaeid species becomes difficult since different methodologies, test conditions, time exposure and larval stages were used among related studies.

In conclusion, our results suggest that at larval stages *L. vannamei* is a resistant species to ammonia. The results obtained in this study could be used as a baseline for future studies and would be helpful in water quality management protocols of *L. vannamei* hatcheries.

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CHAPTER IV

SUPER-INTENSIVE LARVICULTURE OF *Litopenaeus vannamei* (Boone) USING A RECIRCULATING SYSTEM: EFFECTS OF STOCKING DENSITY, WATER RECIRCULATION RATE AND FEEDING STRATEGY

María de Lourdes Cobo[1], Olivier Hoste[2], Isabel De Lange[2], Stanislaus Sonnenholzner[1], Mathieu Wille[2], Rubén Román[1], Jorge Calderón[1], Patrick Sorgeloos[2]

[1] Escuela Superior Politécnica del Litoral (ESPOL), Campus Gustavo Galindo Velasco, Km 30.5 vía Perimetral, P.O. Box 09-01-5863, Guayaquil, Ecuador. Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM).

[2] Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University, Rozier 44, B-9000 Ghent, Belgium

SUPER-INTENSIVE LARVICULTURE OF *Litopenaeus vannamei* (Boone) USING A RECIRCULATING SYSTEM: EFFECTS OF STOCKING DENSITY, WATER RECIRCULATION RATE AND FEEDING STRATEGY

Abstract

A series of three experiments was conducted to evaluate the effects of stocking density, feeding strategy and recirculation rate on the survival and growth performance of *Litopenaeus vannamei* larvae. In all three experiments larvae were reared using two culture systems. Initially a static system was applied from Nauplii 5 to Zoea 3 stage; thereafter larvae were transferred to a recirculating aquaculture system to be reared up to postlarvae 2 stage. Four stocking densities (750, 1000, 1500 and 2000 larvae L^{-1}) and four recirculation rates (250, 500, 750 and 1000% day^{-1}) were tested in experiments 1 and 2 with two replicates for each combination. Experiment 2 differed from experiment 1 in the sense that a continuous supply of microalgae was given during the static culture phase and formulated feed ration was gradually increased during the recirculating culture phase. In experiment 3, stocking densities of 1000 and 2000 larvae L^{-1} were tested with either a low or high concentration of microalgae during the static culture. Recirculation rates of 500 and 1000% day^{-1} were applied in this experiment.

Increasing density led to increased mortality, unless feed ration was increased. A considerable increment of more than 20% in survival was obtained for the density of 1000 larvae L^{-1} when artificial diet ration was increased during the recirculating phase. Water recirculation rate had a significant effect ($p<0.05$) on dry weight, length, and biomass, but not on survival when increasing artificial diet ration. In general, increasing the microalgae concentration during the static culture phase significantly ($p<0.05$) higher survival, length and larval development were obtained for both densities, while significantly higher ($p<0.05$) dry weight was obtained only for density of 1000 larvae L^{-1}. At postlarvae stage, the recirculating aquaculture system yielded survival of 60% at 1000 larvae L^{-1} and 39% at 2000 larvae L^{-1} applying water exchange rates of 500% day^{-1}, with and output of 600 and 780 larvae L^{-1}, respectively. The recirculating aquaculture system was able to maintain

acceptable ammonia and nitrite levels. These results demonstrate that larval rearing of *L. vannamei* is feasible at high densities in an experimental recirculating aquaculture system. However the effects of stocking density, feed management and water recirculation rate on survival and growth, need to be further studied and optimized in order to develop an efficient and profitable production system.

Introduction

Critical factors in the industrialization of shrimp farming have been the supply of qualified larvae (Arellano *et al*., 1993; Montaño and Navarro, 1996; Gamboa-Delgado and Le Vay, 2009) and the occurrence of diseases of which bacterial diseases are considered the most problematic in shrimp larviculture (Lightner, 1993; Griffith, 1995; Prayitno and Latchford, 1995; Zherdmant, 1996). Larval mortalities continue to result in significant economic losses. Surprisingly however, larval rearing techniques for shrimp have changed very little over the last decades.

The traditional system for shrimp larviculture uses larval stocking densities of 100 up to 200 larvae L^{-1}, in a static-batch culture from N5 to Z3 sub-stage and with large discontinuous water exchanges (30 - 80% day^{-1}) from Z3 sub-stage onwards. The problem with static culture procedures is that water quality is unstable and degrades rapidly. The impact on disease occurrence has not yet been fully clarified, but it is generally accepted that unfavorable water quality makes larvae more susceptible to disease by negatively affecting the condition of the rearing environment (Suantika, 2001). Recirculating aquaculture systems (RAS) offer a promising approach to manage water quality. A combination of recirculation technology and intensive culture protocols may provide several benefits: limited waste discharge, increased productivity, reduced land and water usage and reduced risk of pathogen introduction.

RAS are considered also to have the potential to intensify culture systems, maximizing biomass output per unit of area, volume or investment (Rosenthal and Black, 1993). Most of the studies investigating recirculating systems deal with fish species. Today around 10 species of fish are produced in RAS (African catfish, eel and trout as major freshwater species and turbot, seabass and sole as major marine species) (Martins *et al*., 2010). Losordo *et al*. (1992) stated that fish may be grown in recirculating, intensive aquaculture facilities at 100 times the density (animal to water ratio) found in aerated ponds. Comparing the use of water per kg of Nile Tilapia produced, Timmons *et al*. (2009) found RAS production can achieve around 1 340 000 kg ha^{-1} $year^{-1}$ using 500 L of water kg^{-1} *vs*. 17 400 kg ha^{-1} $year^{-1}$ using 21 000 L kg^{-1} when raised in earthen ponds. Currently, there is a shift towards RAS technology at hatcheries *e.g.* for production of Atlantic salmon smolts

which has been switched completely from flow-through to RAS farms (Bergheim *et al*., 2008). With RAS technology several benefits have been achieved, for example smolt size has improved to 140-170g compared to 50-70 g obtained in flow-through farms (Joensen, 2008). The number of smolts that can be produced increased up to 85 million (Del campo *et al.*, 2010) and Terjesen *et al.* (2008) suggested an increased smolt quality in terms of growth and survival after sea transfer for RAS-cultured smolts.

The use of RAS for production of other organisms (zooplankton) has also been evaluated. A recirculating system evaluated by Zmora and Shpigel (2006) allowed intensive mass production of *Artemia* with a maximal yield of 47.5 kg m^{-3} wet weight. Also progress in the area of intensive rotifer production has been achieved by using a recirculating system. The combination of a new culture system design and an improved diet enabled rotifer densities up to 23 000 rotifers mL^{-1} (Suantika *et al*., 2000).

Concerning marine shrimp, the intensive culture of *Litopenaeus vannamei* continues to expand. However studies combining intensification with the use of recirculating systems are limited. Davis and Arnold (1998) studied the development of several techniques for the production of marine shrimp in recirculating raceway systems for *L. setiferus* and *L. vannamei*. Initial stocking densities ranged between 582 - 1739 shrimp m^{-3}. These authors obtained final biomass levels as high as 10 kg m^{-3} within 100 - 120 day and 160 - 175 day production cycles, for *L. setiferus* and *L. vannamei*, respectively. Samocha *et al*. (2002) suggested that with current technologies shrimp yields as high as 10 kg m^{-2} $crop^{-1}$ can be achieved in an indoor, super-intensive, closed-recirculation system in which environmental parameters can be controlled. Baron-Sevilla *et al.* (2004) cultivated *L. vannamei* postlarvae for 168 days in a recirculation unit provided with vertical mosquito screens in order to augment tank area. The final crop obtained in relation to tank volume was 9.7 kg m^{-3}. While the above mentioned studies mainly deal with postlarvae and juveniles, the use of recirculating systems or protocols for (super) intensive shrimp larvae rearing is a relatively new field and therefore information for developing an efficient RAS for *L. vannamei* is scarce. Timmons and Losordo (1994) point out that optimum circulation rates may change depending on the larval stage and flows may have to be determined by trial and error. Also, parameters such as nitrogenous waste levels, stocking density, feed that is consumed by the

animal and the frequency of feeding is essential in order to develop a profitable production system (Losordo and Westers, 1994; Teshima *et al*., 2000).

In the present study, the effects of stocking density, water recirculation rate and feeding strategy on the survival and growth of *L. vannamei* larvae were examined with the objective of developing effective techniques for intensive rearing of shrimp larvae in a RAS.

Materials and methods

Three experiments were conducted at the ESPOL (Escuela Politécnica del Litoral) – CENAIM (Centro Nacional de Acuicultura e Investigaciones Marinas) in San Pedro de Manglaralto (Santa Elena Province, Ecuador).

Different larval stocking densities, feeding strategies and water recirculation rates were tested. In each experiment, larval rearing was split up in two phases, which were performed in two different culture systems: From the N5 to the Z3 sub-stage the larvae were reared in a static system; subsequently they were moved to a recirculating system to be reared up to PL2 stage. An overview of the different factors tested in the three experiments is presented in Table 1.

Experimental animals and culture systems

N5 of *L. vannamei* were purchased from a commercial hatchery and transported to the research facilities. N5 were disinfected with 100 mg L^{-1} of Argentine® for 1 min and acclimated to experimental conditions. During the first phase of the experiment, a static culture system was used. N5 were stocked in 500 L round-shaped (115 cm diameter by 75 cm height) transparent poly-ethylene tanks filled with sand-filtered and UV-treated seawater of 34 g L^{-1}. Temperature was maintained at 32 ± 1°C. Aeration was provided through a perforated PVC-pipe placed on the tank bottom, to maintain dissolved oxygen concentration above 4 mg L^{-1} in each tank.

The larvae remained in static culture from N5 until they reached the Z3 sub-stage. Thereafter they were transferred to a recirculating system.

Table 1. Summary of the different factors tested in experiment 1, 2 and 3: Stocking densities (larvae L^{-1}), water recirculation rates (% day $^{-1}$) and feeding strategy used for the larval rearing of *Litopenaeus vannamei* in the static and recirculating culture phase

Experiment	Culture phase	Density (larvae L^{-1})	Water Recirculation Rate (% day $^{-1}$)	Feeding strategy
Experiment 1	Static	750 (D1), 1000 (D2), 1500 (D3), 2000 (D4)		CENAIM 's protocol
	Recirculating	750 (D1), 1000 (D2), 1500 (D3), 2000 (D4)	250 (R1), 500 (R2), 750 (R3), 1000 (R4)	Live food ration increased with different factors to account for increasing stocking densities
Experiment 2	Static	750 (D1), 1000 (D2), 1500 (D3), 2000 (D4)		Continous supply of algae Same concentration of algae irrespective of stocking densities
	Recirculating	750 (D1), 1000 (D2), 1500 (D3), 2000 (D4)	250 (R1), 500 (R2), 750 (R3), 1000 (R4)	Artificial food increased with different factors to account for increasing water recirculation rate
Experiment 3	Static	1000 (D2), 2000 (D4)		Continous supply of algae, either at a low and high concentrations
	Recirculating	1000 (D2), 2000 (D4)	500 (R2), 1000 (R4)	Same as in experiment 2

Two recirculating units were built, both with an identical set-up. The general set-up of the recirculating system is shown in Figure 1.

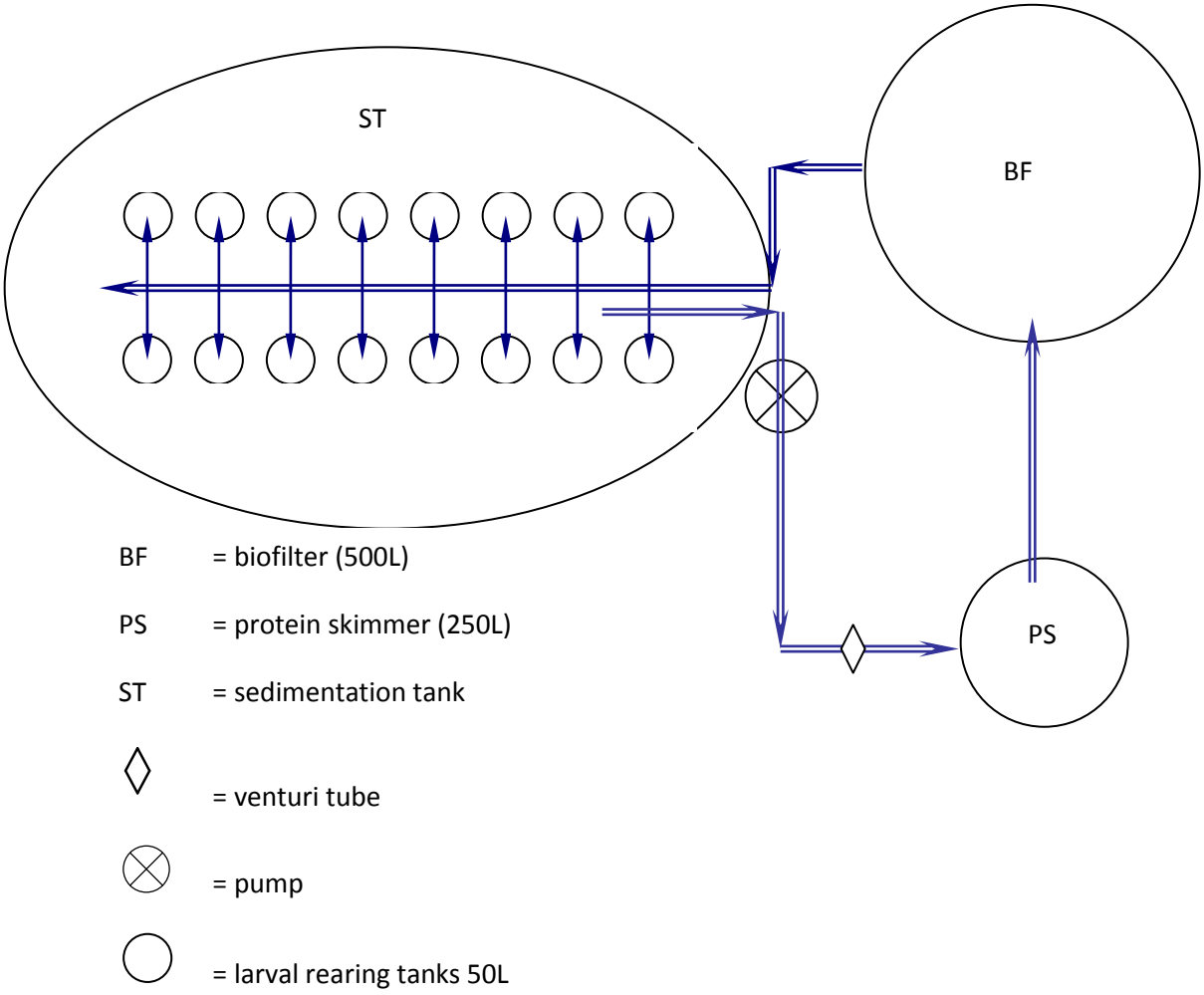


Figure 1. Schematic diagram of the experimental recirculating unit containing the culture tanks

Each system held 16 larval rearing tanks of 50 L containing a central tube covered with different mesh sizes (200 – 400 µm) according to larval stages. The tanks were placed in a 'water bath' containing 1500 L of sand-filtered and UV-disinfected seawater of 34 g L^{-1}. This tank functioned as a sedimentation tank, where effluents from the culture tanks charged with suspended solids (faeces, floccules and feed excess) could settle. Fine suspended solids, which were not removed in the sedimentation basin, were trapped in the foam produced by

the protein skimmer. To increase its efficiency a venturi tube was placed before the protein skimmer. The water was heated in the sedimentation tank by heat-exchanging tubes and a gas-boiler and controlled with a temperature sensor. The temperature in the rearing tanks was kept stable at 32 ± 1 °C.

Two weeks before the start of the experiments the two bio-filters were filled with purified and UV- disinfected seawater, filled with 100 L of artificial biofilter medium (Kinetco Macrolite®, Newbury, OH, USA, ceramic spheres of about 40µm), and inoculated with 10 mg L^{-1} Volatile Suspended Solids of the nitrifying bacteria suspension ABIL® (Avecom, Belgium). Every two days the nitrifying bacteria received a load of 5 mg L^{-1} of N in the form of ammonium chloride to support their growth. The pH of the bio-filter was controlled within the range of 7.0 - 8.0 by the addition of calcium carbonate. During the experiments the water recirculation rate through the protein skimmer and biofilter of the experimental recirculating units (containing the culture tanks) was set at 30 L min^{-1}, which provided an estimated hydraulic retention time of 17 minutes.

The experiments were terminated when the larvae reached PL 2 for experiments 1 and 2 and until PL 1 for experiment 3.

Experiment 1

Stocking density and water recirculation rate

Four increasing stocking densities were evaluated within the static culture system: 750, 1000, 1500 and 2000 larvae L^{-1}, coded D1, D2, D3 and D4 respectively, randomly assigned to the culture tanks with two replicates per treatment.

The recirculating units allowed a factorial design combining two factors, namely density and water recirculation. Larval densities were the same as those applied in the static culture phase. The applied water recirculation rates were 250, 500, 750 and 1000% day^{-1}, coded R1, R2, R3 and R4 respectively. The treatments were named henceforth D1R1, D1R2, D1R3, D1R4, D2R1, D2R2, D2R3, D2R4, D3R1, D3R2, D3R3, D3R4, D4R1, D4R2, D4R3 and D4R4. Two replicates per treatment were randomly assigned.

Feeding regime

During the stages N5 and Zoea 1 (Z1) the microalgae *Chaetoceros gracilis* was supplied. Starting from the transition of Z2/Z3 *Tetraselmis* sp. was introduced to gradually replace *Chaetoceros.* From Z2 until PL1, rotifers, *Brachionus* sp. Cayman (Dooms *et al*., 2007), frozen and live *Artemia* nauplii and artificial feed Epifeed® (Epicore, Eastampton, NJ, USA), Frippak® (Inve Aquaculture SA, Dendermonde, Belgium), Larva Z PLUS®(Zeigler, Gardners, PA, USA) were given following the standard feeding protocol of the ESPOL-CENAIM centre (Table 2). Microalgae were used as a concentrate obtained by means of a centrifuge. Due to the high larval stocking densities used in this study, the standard feeding levels for microalgae and rotifers were increased by a factor of 3, 5, 7 and 9 for densities D1, D2, D3 and D4, respectively. Live and artificial feeds were alternated with the daily ration equally divided over twelve feedings. To assure the concentration for microalgae, rotifers and *Artemia* nauplii during the RAS phase, counts were performed every two hours for microalgae and every three hours for rotifers and Artemia nauplii. During the experiments quantity of food available in RAS tank was measured. Every two hours microalgae counts were performed to determine its concentration. In case of not being as the assigned, the speed of entry of microalgae through the automatic feeder was increased or decreased depending on the case. Rotifers and Artemia nauplii were count every three hours by means of a 1 mL pipette. The same as with microalgae was applied and in some cases it was done manually. As a prophylactic treatment, the probiotic *Vibrio algynolyticus* (ILI strain) was added daily to the culture water at a concentration of 1.00E+10 colony forming units (CFU) mL^{-1} in order to get a final concentration of 1.00E+05 CFU mL^{-1} (Rodríguez *et al*., 2007).

Sampling Methods

Survival was estimated at every stage of development by taking three 125-mL samples from each culture tank and counting all live larvae. All sampled larvae were then returned to their respective culture tank.

For dry weight (DW, mg) determination, triplicate samples of 50 to 100 larvae were removed randomly from each replicate, washed briefly with distilled water and dried in an

oven at 60 °C for 24 h. Total biomass (B, g) was also determined at the end of the recirculating culture, by harvesting all the larvae over a 300 µm net, removing excess water and quantifying the total wet weight. The larval stage index (LSI) was determined by daily microscopic observations following the procedure described by Kanazawa *et al*. (1985).

Table 2. ESPOL-CENAIM's standard feeding protocol used for *Litopenaeus vannamei* larviculture (100 larvae L^{-1})

	Microalgae				Artificial Diets	
Larval stage	*Chaetoceros gracilis* (10^3 cells mL^{-1})	*Tetraselmis* sp. (10^3 cells mL^{-1})	Rotifers (rot mL^{-1})	*Artemia* (nauplii $larvae^{-1}$)	Liquid diet (g million $larvae^{-1}$)	Dry diet (g million $larvae^{-1}$)
N5	100					
Z1	120				10	
Z2	160		10		17	10
Z3	80	80	15	10	20	11
M1		60	25	14	28	14
M2		60	27	19	35	20
M3		60	30	24	45	23
PL1		60	33	30		34

Liquid diet: Epifeed® (Epicore, Eastampton, NJ, USA)
Dry diets: Frippak® (Inve Aquaculture SA, Dendermonde, Belgium), Larva Z PLUS®(Zeigler, Gardners, PA, USA)

Water Quality

Daily measurements of pH, Total Ammonia Nitrogen (TAN), nitrite NO_2-N and nitrate NO_3-N (mg L^{-1}) were done on water samples taken from all culture tanks during the static culture phase. TAN and nitrite analyses were performed following the protocol described by Solórzano (1983), and nitrate concentrations by a modified protocol from Solórzano (1983). In contrast to the static culture phase, water quality measurements in the recirculating culture phase were not measured in the separate culture tanks, instead TAN, nitrite and nitrate levels were measured from both the influent and effluent of the biofilters to verify the performance of the experimental recirculating units. TAN levels were recorded in order to calculate the Volumetric Nitrification Rates (VNR) expressed in g TAN removed (m^{-3} day^{-1}) following the procedure described by Carroll *et al.* (2003). The foam produced by the protein skimmer was removed daily and the dry matter content was measured to determine the total amount of removed organic matter at the end of the experiment using the protocol described by Boyd (1992).

Experiment 2

The same set-up, culture conditions, stocking densities and water recirculation rates as in experiment 1 were used. A similar feeding strategy as in experiment 1 was adopted, but microalgae were administered continuously by means of a peristaltic pump® (Ecoline multi-channel, Harvard Apparatus Inc., Holliston, MA, USA) and maintained at a level of 100 000 cells mL^{-1}, irrespective of stocking density.

During the recirculating culture phase in experiment 1, it was observed that the digestive tract of the larvae was not completely full, therefore feeding quantities of formulated diet were also increased, *i.e.* multiplied (compared to the standard feeding schedule) by a factor 2, 4, 6 and 8 for the water recirculation rates R1, R2, R3 and R4 respectively. *Artemia* nauplii feeding rates (nauplii $larvae^{-1}$) were adjusted daily in each treatment after observations of the digestive tract.

Experiment 3

The same set-up and conditions as in experiment 1 were used. Only two stocking densities, namely D2 and D4 (1000 and 2000 larvae L^{-1}) were tested however. The feeding strategy during the static culture was similar to experiment 1, but larvae were fed different microalgae concentrations, resulting in four treatments: D2A1, D2A2, D4A3 and D4A4. For the combinations D2A1 and D2A2, 100 000 cells mL^{-1} and 200 000 cells mL^{-1} were given, respectively. A microalgae concentration of 400 000 cells ml^{-1} was used for the combination D4A3 and 700 000 cells mL^{-1} for the combination D4A4. Each treatment had three replicates.

Larvae from treatments which had high survival (D2A2 and D4A4) at the end of the static culture were transferred to the recirculating system. These two densities (D2 and D4) were then combined with two flow rates R2 (500 % day^{-1}) and R4 (1000 % day^{-1}), resulting in the treatments named henceforth D2R2, D2R4, D4R2 and D4R4. Each combination had again three replicates. The same feeding strategy as in experiment 2 was used.

Statistical analyses

All values shown are expressed as means ± standard error of mean (SEM). Normal probability plots and the Bartlett test for homogeneity of variances were used to verify the assumptions for further analysis. When necessary, data were log transformed to obtain normal distribution, although unadjusted means are presented. Data were analyzed with analysis of variance: a one-way ANOVA for the results of the static culture system and a two-way ANOVA for the results of the recirculating culture system, with stocking density and water flow rate as main factors. For experiment 3, data from the results of the recirculating culture system was analyzed by a one-way ANOVA. Tukey´s HSD multiple comparison test was used to identify differences among treatments. All references to statistical significance were at the 5% level or lower. The statistical analyses were performed using STATISTICA 4.1 (Statsoft®, Tulsa, Oklahoma, USA). Data of density D4 (2000 larvae L^{-1}) at the end of the recirculating system in experiments 1 and 2 were not submitted to statistical analysis due to total mortality registered in some of the combinations. Data of the recirculation rate R4 (1000% day^{-1}) in experiment 3 was not submitted to statistical analysis due to total mortality registered with these combinations.

Results

Experiment 1

Larval performance during the static culture phase

Table 3 presents survival, dry weight, length and larval stage index at the end of the static culture phase. During the first phase of the experiment, larval survival was generally low (51-67%). Significant higher survival ($p<0.05$) where recorded for the stocking density 1000 larvae L^{-1} (D2) compared to that of 2000 larvae L^{-1} (D4). A negative trend was observed for individual dry weight and length with increasing larval density, although no significant differences were detected ($p > 0.05$).

Table 3. Survival, dry weight, length and larval stage index (LSI) of Zoea 3 *Litopenaeus vannamei* larvae reared under different stocking densities at the end of the static culture phase in experiment 1.

	Parameters			
Stocking density (larvae L^{-1})	Survival (%)	Dry weight (mg larvae L^{-1})	Length (mm)	Larval Stage Index
D1 (750)	61 ± 2^{ab}	0.034 ± 0.004^{a}	2.27 ± 0.07^{a}	3.09 ± 0.00^{a}
D2 (1000)	67 ± 7^{a}	0.028 ± 0.001^{a}	2.25 ± 0.08^{a}	3.17 ± 0.03^{a}
D3 (1500)	53 ± 8^{ab}	0.027 ± 0.002^{a}	2.18 ± 0.02^{a}	3.03 ± 0.05^{a}
D4 (2000)	51 ± 3^{b}	0.025 ± 0.006^{a}	2.14 ± 0.05^{a}	3.09 ± 0.04^{a}

Within columns, superscript letters indicate significant differences ($p < 0.05$).

Larval performance in the recirculating culture phase

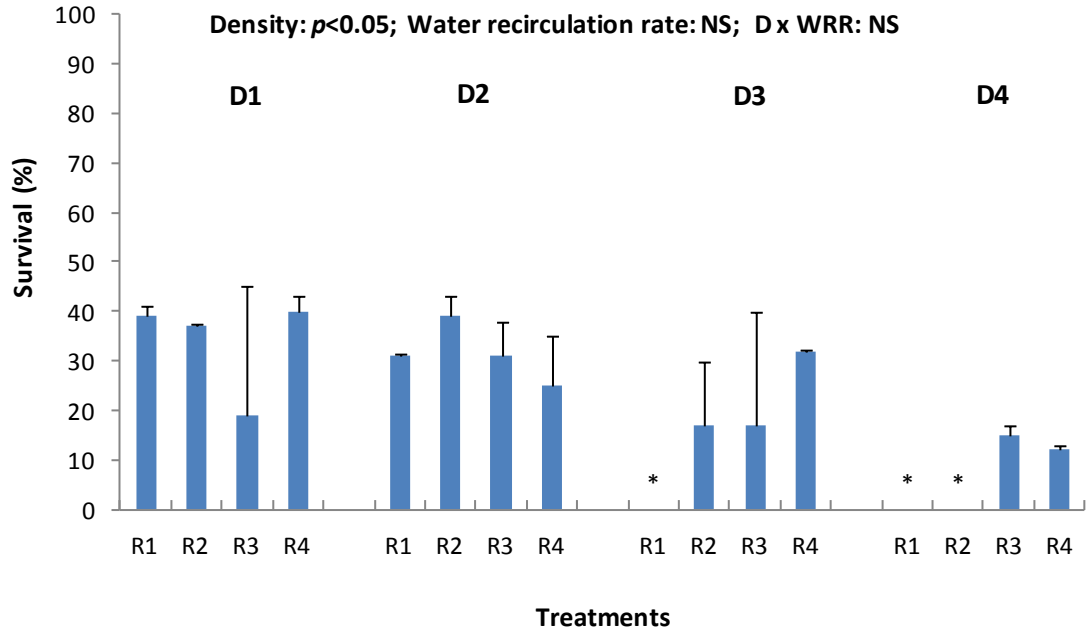
Figure 2 illustrates larval survival in the treatments with different combinations of stocking density and water recirculation rates during the recirculating culture phase. Survival of larvae was affected by density ($p<0.05$). The highest survival were obtained with the combinations D1R1 (39 ± 2%), D1R4 (40 ± 3%) and D2R2 (39 ± 4%). Total mortality was observed for the combinations D3R1, D4R1 and D4R2.

Dry weight and length of the postlarvae 2 and total biomass per tank were not affected by stocking densities nor by water recirculation rates ($p>0.05$) (Table 4). The highest dry weight and total biomass were recorded in D3R4 (0.120 mg $larvae^{-1}$ and 18.45 g), D2R2 (0.088 mg $larvae^{-1}$ and 15.91 g) and D1R1 (0.081 mg $larvae^{-1}$ and 12.72 g).

Water quality

Mean concentration of TAN (mg L^{-1}) of the influent of the biofilter at the beginning of the recirculating culture phase was 1.19 ± 0.30, while a mean concentration of 0.22 ± 0.1 was registered at the end of the culture period. The mean Volumetric Nitrification Rate (VNR) was 62 g TAN m^{-3} day^{-1} at a mean influent TAN of 1.10 mg L^{-1}. With a maximum influent TAN level of 1.40**,** the maximum VNR recorded was 81 g TAN m^{-3} day^{-1}. Mean NO_2-N concentrations of 0.360 and 1.130 mg L^{-1} were registered in the effluent of the biofilters at the beginning and at the end of the recirculating culture period, respectively. While mean concentrations of nitrate NO_3-N were 0.500 mg L^{-1} in the effluent of the

biofilters at the beginning and 2.500 mg L^{-1} at the end of the recirculating culture period. Mean pH was 7.8 (range 7.5 - 8.1). The amount of organic matter removed from the protein skimmer at the end of the experiment was 29.6 ± 3.8 g.



*Total mortality in tanks for this combination of density-water recirculation rate.

Figure 2. Mean survival (%) of *Litopenaeus vannamei* postlarvae 2 reared under different stocking densities and water recirculation rates in a recirculating system. Standard deviation bars are shown. The results of the two-way ANOVA are shown at the top (DxWRR stands for density –water recirculation rate interaction).

Table 4. Effect of different stocking densities and water recirculation rates on dry weight, length and total biomass of PL2 *Litopenaeus vannamei* larvae cultured in an experimental recirculating system in experiment 1. Results from two-way ANOVA analyses are presented. NS means not significant

Treatments		Dry weight (mg larvae^{-1})	Length (mm)	Biomass (g)
D1	R1	0.081 ± 0.006	3.43 ± 0.04	12.72 ± 0.05
	R2	0.072 ± 0.001	3.39 ± 0.04	11.36 ± 0.43
	R3	0.046 ± 0.032	1.69 ± 2.39	5.96 ± 8.43
	R4	0.076 ± 0.001	3.41 ± 0.05	12.45 ± 0.33
D2	R1	0.085 ± 0.002	3.37 ± 0.03	12.31 ± 0.21
	R2	0.088 ± 0.002	3.34 ± 0.04	15.91 ± 0.76
	R3	0.083 ± 0.001	3.39 ± 0.03	11.82 ± 2.39
	R4	0.046 ± 0.030	1.65 ± 1.17	5.36 ± 7.59
D3	R1	*	*	*
	R2	0.076 ± 0.001	3.30 ± 0.03	9.19 ± 7.19
	R3	0.048 ± 0.030	1.65 ± 1.17	10.14 ± 14.35
	R4	0.102 ± 0.006	3.26 ± 0.04	18.45 ± 1.33
D4	R1	*	*	*
	R2	*	*	*
	R3	0.029 ± 0.020	1.62 ± 1.14	14.00 ± 19.81
	R4	0.053 ± 0.030	1.57 ± 1.11	9.13 ± 12.91
Statistical significance				
Density		NS	NS	NS
Water recirculation rate		NS	NS	NS
Interaction		NS	NS	NS

Experiment 2

Larval performance during the static culture phase

On average, the survival recorded in this experiment were higher than those recorded in experiment 1. The most striking differences were obtained in treatments with densities of 1000 and 2000 larvae L^{-1}(D2 and D4), with a survival of 89 and 78% in experiment 2 versus only 67 and 51% in experiment 1. Survival was significantly higher (p<0.05) for D2 and D4

compared to the other densities tested. Length and LSI of Z3 for D2 were generally higher than in the other treatments (Table 5), but only a significant difference (p<0.05) was detected for total length between D2 and D4.

No significant differences were detected for individual dry weight, although larvae of treatment D1 had an average dry weight of 0.060 mg larvae^{-1} compared to 0.032 to 0.042 mg larvae^{-1} in the other treatments.

Table 5. Survival, dry weight, length and larval stage index (LSI) of Zoea 3 *Litopenaeus vannamei* larvae reared at different stocking densities at the end of the static culture of the experiment 2

	Parameters			
Stocking density (larvae L^{-1})	Survival (%)	Dry weight (mg larvae L^{-1})	Length (mm)	Larval Stage Index
D1 (750)	50 ± 6^{b}	0.060 ± 0.010^{a}	2.13 ± 0.020^{ab}	2.71 ± 0.05^{a}
D2 (1000)	89 ± 10^{a}	0.041 ± 0.004^{a}	2.21 ± 0.020^{a}	2.90 ± 0.02^{a}
D3 (1500)	52 ± 6^{b}	0.041 ± 0.003^{a}	2.15 ± 0.003^{ab}	2.70 ± 0.06^{a}
D4 (2000)	78 ± 5^{a}	0.032 ± 0.002^{a}	2.09 ± 0.009^{b}	2.80 ± 0.05^{a}

Within columns, superscript letters indicate significant differences (p<0.05).

Results of water quality analysis at the Z3 stage are summarized in Table 6. During the static culture phase the pH remained within the range of 7.43 to 8.12. Concentrations of TAN (mg L^{-1}) increased with increasing larval stocking densities but were not significantly different between treatments (p>0.05). Nitrite and nitrate did not present significant differences (p>0.05) among density treatments.

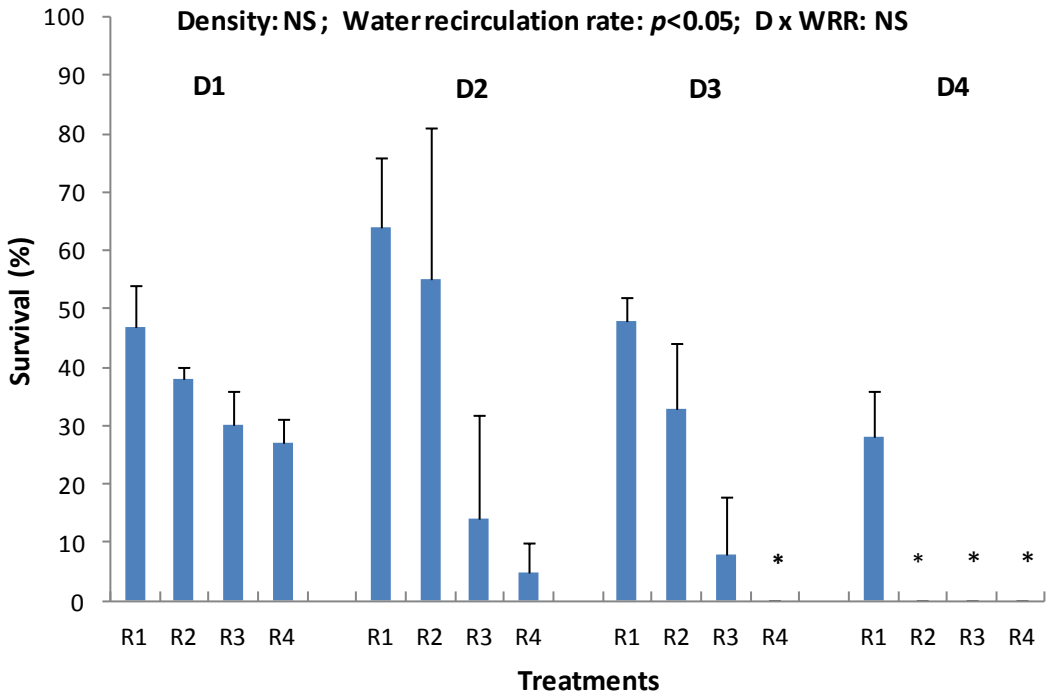
Table 6. Means ± SE of total ammonia nitrogen (TAN), nitrite (NO_2-N) and nitrate (NO_3-N) at the end of static culture period of experiment 2

	Parameters		
Stocking density (larvae L^{-1})	TAN (mg L^{-1})	NO_2-N (mg L^{-1})	NO_3-N (mg L^{-1})
D1 (750)	1.88 ± 0.29^{a}	0.015 ± 0.007^{a}	0.088 ± 0.010^{a}
D2 (1000)	2.27 ± 0.20^{a}	0.009 ± 0.001^{a}	0.078 ± 0.007^{a}
D3 (1500)	3.25 ± 0.31^{a}	0.017 ± 0.007^{a}	0.059 ± 0.003^{a}
D4 (2000)	5.45 ± 1.93^{a}	0.020 ± 0.004^{a}	0.045 ± 0.030^{a}

Within columns, superscript letters indicate significant differences (p<0.05).

Larval Performance during the recirculating culture phase

Higher survival percentages were obtained in experiment 2 compared to those obtained in experiment 1 during the recirculating culture (Figure 3). A significant (p <0.05) effect of water recirculation rate (R1 and R2) on survival was observed. The greater the water exchange rates the lower the survival for the different combinations. Total mortality was registered for D3R4, D4R2, D4R3 and D4R4.



*Total mortality in tanks for this combination of density-water exchange rate

Figure 3. Mean survival (%) of *Litopenaeus vannamei* larvae reared under different stocking densities and water flow rate combinations in a recirculating system in experiment 2. Standard deviation bars are shown. The results of the two-way ANOVA are shown at the top (DxWRR stands for density –water recirculation interaction)

Also most other larval performance parameters were affected by the water exchange rate (table 7). Dry weight of PL2 in the recirculating culture system was significantly affected by both density ($p<0.05$) and by water exchange rate ($p<0.01$). The highest dry weights were observed in treatments with low exchange rates (R1 and R2). The same trend was observed for biomass ($p<0.001$) and length ($p<0.05$), which were affected by water exchange rates.

Table 7. Effect of different stocking densities and water recirculation rates on dry weight, length and biomass of *Litopenaeus vannamei* postlarvae 2 cultured in an experimental recirculating system in experiment 2. Means ± SE are given. *p* - values are presented for two-way ANOVA analyses. NS means not significant

Treatments		Dry weight (mg larvae^{-1})	Length (mm)	Biomass (g)
D1	R1	0.097 ± 0.001	3.54 ± 0.01	8.40 ± 0.80
	R2	0.101 ± 0.009	3.47 ± 0.05	8.35 ± 1.10
	R3	0.084 ± 0.003	3.34 ± 0.02	5.15 ± 0.15
	R4	0.086 ± 0.001	3.37 ± 0.12	4.85 ± 0.65
D2	R1	0.103 ± 0.005	3.57 ± 0.03	18.85 ± 1.15
	R2	0.099 ± 0.008	3.47 ± 0.06	15.30 ± 6.3
	R3	0.046 ± 0.045	1.71 ±1.71	4.0 ± 4.0
	R4	0.043 ± 0.043	1.68 ± 1.68	1.30 ± 1.30
D3	R1	0.073 ± 0.024	3.58 ± 0.03	18.65 ± 2.15
	R2	0.089 ± 0.001	3.43 ± 0.01	13.40 ± 2.10
	R3	0.044 ± 0.043	1.65 ± 1.65	2.75 ± 2.75
	R4	*	*	*
D4	R1	0.094 ± 0.003	3.42 ± 0.01	11.95 ± 2.15
	R2	*	*	*
	R3	*	*	*
	R4	*	*	*
Statistical significance				
Density		*p < 0.05*	NS	NS
Water recirculation rat		*p < 0.01*	*p < 0.05*	*p < 0.001*
Interaction		NS	NS	NS

*Total mortality in tanks for this combination of density-water recirculation rate.

Water quality

During the culture in the recirculating system, the pH remained within the range of 7.5 - 7.8. Mean concentration of TAN (mg L^{-1}) at the beginning of the recirculating culture phase in the influent of the biofilters was 1.10 ± 0.13, while a mean concentration of 0.50 ±0.10 was registered at the end of the culture period. Mean Volumetric Nitrification Rate (VNR) was 124 g TAN m^{-3} day^{-1} at a mean influent TAN of 0.93 mg L^{-1}. With a maximum influent TAN level of 1.30, the maximum VNR recorded was 175 g TAN m^{-3} day^{-1}. Mean NO_2-N concentrations of 0.300 and 0.270 mg L^{-1} were registered in the effluent of the biofilters at the beginning and at the end of the recirculating culture period, respectively. While mean concentrations of NO_3-N were 0.700 mg L^{-1} in the effluent of the biofilters at the beginning and 4.500 mg L^{-1} at the end of the recirculating culture period. The total average amount of organic matter removed from the protein skimmer at the end of the experiment was 77.8 ± 7 g.

Experiment 3

Larval Performance during the static culture phase

Table 8 presents survival, dry weight, length and larval stage index results at the end of the static culture phase. Significant lower survival, length and LSI ($p<0.05$) where recorded for the stocking density 2 000 larvae L^{-1} (D4) fed with a low microalgae concentration (A3, 400 000 cells mL^{-1}) as compared to the same density fed with a high microalgae concentration (A4, 700 000 cells mL^{-1}). For most parameters, performance in this treatment (D4A3) was also worse than in both treatments with lower larval density (D2, 1 000 larvae L^{-1}), independent of the microalgae concentration used. Dry weight was significantly higher ($p<0.05$) for the stocking density 1 000 larvae L^{-1} fed high microalgae concentration (D2A2) compared to the other treatments.

Table 8. Larval performance of *Litopenaeus vannamei* larvae fed different microalgae concentrations (A1, 100 000; A2, 200 000; A3, 400 000 and A4, 700 000 cells mL^{-1}) and reared at two larval densities (D2, 1000 and D4, 2000 larvae L^{-1}) at the end of the static culture phase in experiment 3. Means ± SD are given.

Treatments	Survival (%)	Dry weight (mg)	Length (mm)	Larval Stage Index
D2A1	88 ± 3^{a}	0.031 ± 0.002^{b}	2.14 ± 0.02^{a}	3.42 ± 0.15^{ab}
D2A2	84 ± 8^{a}	0.045 ± 0.001^{a}	2.18 ± 0.02^{a}	3.84 ± 0.06^{a}
D4A3	61 ± 8^{b}	0.031 ± 0.001^{b}	1.99 ± 0.05^{b}	2.99 ± 0.01^{b}
D4A4	82 ± 10^{a}	0.037 ± 0.001^{b}	2.13 ± 0.03^{a}	3.58 ± 0.17^{a}

Within columns, superscript letters indicate significant differences ($p<0.05$).

Water quality

In this experiment TAN concentrations did not increase with increasing stocking densities as in experiment 2. TAN values remained below 2.50 mg L^{-1} and presented no significant differences ($p>0.05$) between treatments. Nitrite concentrations did not differ ($p>0.05$) among the different stocking densities. Nitrate concentrations were significantly higher in D2A1compared to the other treatments (table 9).

Table 9. Means ± SE of total ammonia nitrogen (TAN), nitrite (NO_2-N) and nitrate (NO_3-N) at the end of the static culture phase in experiment 3

Stocking density (larvae L^{-1})	Parameters TAN (mg L^{-1})	NO_2-N (mg L^{-1})	NO_3-N (mg L^{-1})
D2A1	1.51 ± 0.16^{a}	0.030 ± 0.005^{a}	0.065 ± 0.020^{a}
D2A2	1.82 ± 0.45^{a}	0.090 ± 0.030^{a}	0.003 ± 0.003^{b}
D4A3	2.09 ± 0.17^{a}	0.012 ± 0.020^{a}	0.003 ± 0.003^{b}
D4A4	1.94 ± 0.32^{a}	0.090 ± 0.030^{a}	0.006 ± 0.006^{b}

Within columns, superscript letters indicate significant differences ($p < 0.05$).

Larval Performance during the recirculating culture phase

A similar survival (60 ± 2%) was obtained for treatment D2R2 as in experiment 2 (55 ± 18%) (Table 10). In this experiment, D4R2 presented a mean survival of 39 ± 25%, while total mortality was registered for this combination in experiments 1 and 2. Treatments with high recirculation rates (D2R4 and D4R4) resulted in total mortality (Table 10). D2R2 yielded higher values for survival, dry weight, length and LSI compared to D4R2, although not significantly different (p>0.05). Although in this experiment the animals were already harvested at PL1, higher values of biomass were obtained for D2R2 and D4R2 compared to biomass values in experiments 1 and 2, where the animals were harvested at the PL2 stage (Table 10).

Table 10. Larval performance of *Litopenaeus vannamei* postlarvae 1 reared at two larval densities (D2, 1000 and D4, 2000 larvae L^{-1}) combined with two water recirculation rates (R2, 500 and R4, 1000% day^{-1}) in an experimental recirculating system in experiment 3. Means ± SE are given

	Parameters				
Treatments	Survival (%)	Dry weight (mg larvae^{-1})	Length (mm)	Larval Stage Index	Biomass (g)
D2 R2	60 ± 2^{a}	0.141 ± 0.030^{a}	2.93 ± 0.06^{a}	5.99 ± 0.03^{a}	20.81 ± 4.40^{a}
D2 R4	*	*	*	*	*
D4 R2	39 ± 25^{a}	0.073 ± 0.063^{a}	2.24 ± 1.50^{a}	3.97 ± 3.08^{a}	24.73 ± 8.22^{a}
D4 R4	*	*	*	*	*

Within columns, superscript letters indicate significant differences (p < 0.05).
*Total mortality in tanks for this combination of density-water recirculation rate.

Water quality

Fluctuations of the pH were in the range of 7.4 - 8.15. Mean concentration of TAN (mg L^{-1}) at the beginning of the recirculating culture phase in the influent of the biofilter was 0.39 ± 0.002 and a mean concentration of 0.28 ± 0.01 was registered at the end of the culture period in the effluent of the biofilter of the recirculating units. The mean volumetric nitrification rate (VNR) was 25 g TAN m^{-3} day^{-1} at a mean TAN influent of 0.38 mg L^{-1}. With a maximum influent TAN level of 0.60 mg L^{-1}, the maximum VNR recorded was 52 g TAN m^{-3} day^{-1}. Mean NO_2-N concentrations of 2.170 and 0.940 mg L^{-1} were registered in

the effluent of the biofilters at the beginning and at the end of the recirculating culture period, respectively. While mean concentrations of NO_3-N were 0.010 mg L^{-1} in the effluent of the biofilters at the beginning and 2.300 mg L^{-1} at the end of the recirculating culture period. The total average amount of organic matter removed from the protein skimmer at the end of the experiment was 30.0 ± 17.0 g.

Discussion

Recirculating Aquaculture Systems (RAS) offer several advantages in the management of waste materials from aquaculture activity, in increasing the environmental sustainability and biosecurity, therefore allowing intensification of the rearing process. Intensive aquaculture systems have been used to efficiently produce fish (Poxton *et al.*, 1982; Kikuch *et al.*, 2006), rotifers (Suantika *et al.*, 2001), *Artemia* biomass (Zmora and Shpigel, 2006), and shrimp (Sturmer *et al*., 1992; Davis and Arnold, 1998; Samocha *et al*., 1993; Atwood *et al*., 2005; Mishra *et al*., 2008). As far as we know this is the first study on shrimp larvae performance at super high stocking densities reared in a RAS. Applying a RAS to intensify culture conditions might offer a production strategy that could ultimately improve productivity and profitability of shrimp larviculture.

Effects of stocking density

In the present study, increasing stocking densities affected survival and growth of the larvae in the static culture system, although the observed differences were not statistically significant. In the recirculating system, a similar tendency was observed for survival, mostly with statistical significant effects (*e.g.* experiment 1). Total mortalities were observed for some combinations with a density of 2000 larvae L^{-1}. Williams *et al.* (1996) observed a reduction in survival and growth of *L. setiferus* and *L. vannamei* juveniles when using higher stocking densities and reported that this may be due to inadequacies of the feed and partially also to the social function of crowding. Among the studies related to stocking densities of penaeid shrimp larvae are those reported by Cook and Murphy (1969) who obtained an average survival of 50% for three species of marine shrimp at densities of up to

266 larvae L^{-1} ; Jones *et al*. (1979) observed survival for *M. japonicus* larvae of 53 - 86% at densities of 240-260 larvae L^{-1}; Beard *et al*. (1977) found survival of 42 - 83% at densities of 71 - 400 larvae L^{-1} of *F. merguiensis.* Vinatea and Andreatta, 1997 observed that treatments with an initial stocking density of 200 larvae L^{-1} had significantly higher survival and dry weight than those with stocking densities of 350 larvae L^{-1}. Emmerson and Andrews (1981) observed that the growth of *F. indicus* larvae decreases linearly as stocking density increases, and established maximum limits for the Z1 - M2 M3 and PL1 stages of 100 larvae L^{-1} and 50 larvae L^{-1} respectively. In our study, survival of 32 - 40% were obtained for the stocking densities of 750, 1000 and 1500 larvae L^{-1} resulting in a final density of 300, 390 and 480 larvae L^{-1} respectively. The survival obtained in experiment 1 was low, but still represents densities that are 5 to 8 times higher than those obtained in traditional larviculture systems.

Although difficult to compare since different stocking densities were used, survival and dry weight recorded in the present study were similar to those obtained in the study of Martins *et al*. (2006), Al Azad *et al*. (2002) and D´Abramo *et al*. (2006) in traditional culture systems for *F. paulensis* (60 larvae L^{-1}) *P. monodon* (100 - 200 larvae L^{-1}) and *L. vannamei* (200 - 300 larvae L^{-1}), respectively. On the other hand, total length values of PL1 - PL2 in our study were lower than those in the cited studies. This can be explained since different methodologies were used between studies making comparison of results difficult. Gandy (2004) demonstrated that a closed recirculating larval rearing system successfully produced PL5 from Z1 with similar dry weights, lengths and stress resistance compared to PL produced under standard water exchange practices at a stocking density of 100 larvae L^{-1}. A recirculating system used in Vietnam for *P. monodon* larvae production at commercial scale with stocking density of 200 -250 larvae L^{-1} obtained survival at harvest of PL12 ranging from 20 to 80%, with half of the batches giving ≥ 60 percent (Thach *et al.,* 2005). Truong *et al.* (2009) evaluated six stocking densities from 150 up to 900 N L^{-1} for *P. monodon* in an improved RAS composed of a biofilter, protein skimmer and ozone generator at laboratory scale. The best performance was at a density of 450 N L^{-1} with a survival of 56% and a yield of 252 larvae L^{-1}. Apparently, there is no other comparable study that has reported such super-high stocking densities (1 000 and 2 000 N L^{-1}) as the ones applied in this study.

Effects of water recirculation

The present results suggest that increasing water recirculation rates during the recirculating culture phase does not improve larvae survival or growth. Although in this study water quality parameters were not evaluated for each treatment, values of TAN, NO_2-N and NO_3-N in the recirculating system were within recommended levels; therefore these water parameters probably did not limit or affect growth or survival of the larvae. Higher water recirculation rates may have resulted in increased food loss to the system and as a consequence could have reduced its availability for the larvae affecting growth and survival. This was also observed by Vinatea and Andreatta (1997) who obtained a significantly higher survival at a renewal rate of 100% day^{-1} (58.6%), as compared to water renewal rates of 200 and 300% day^{-1} for *F. paulensis* larvae when comparing a static and continuous renewal strategy. Mishra *et al*. (2008) evaluated the effect of two water exchange regimes and the use of foam fractionators (FF) for the culture of *L. vannamei* in raceways, obtaining greater mean final weight (1.91 and 2.0 g vs. 1.73 and 1.43 g), survival (100 and 92.4% vs. 55.9 and 81.8%), and yield (7.64 and 6.89 kg m^{-3} vs. 3.92 and 4.74 kg m^{-3}) with the use of FF and lower water exchange than those operated without FF and with higher water exchange rate.

Water quality measurements were not recorded for every tank; nevertheless, values of the biofilter effluents of the recirculation units generally did not exceeded the recommended level of 1.9 mg L^{-1} TAN reported by Cobo *et al* (*in press*) to reduce ammonia toxicity during the rearing *L. vannamei* larvae. Also, it was observed that at the maximum TAN influent concentration, VNR was still rising, showing that the media of the bio-filter still had the capacity to remove more TAN. Similar VNR values were obtained by Carroll *et al.* (2003) in marine recirculating systems with microbead media.

Effects of feeding strategy

Feeding strategy, frequency and feed amount per feeding are integral parts of feed management. It is generally believed that distributing a daily ration in multiple feedings or even feeding continuously improves shrimp growth and helps to maintain water quality (Velasco *et al*., 1999). Our results indicated that in static culture systems survival was improved by the constant supply of microalgae by means of an automatic feeder. The use of

an automatic feeder provided small and frequent additions of food, avoiding over or under feeding which may negatively affect the larval performance. One of the main problems in hatcheries is the infrequent application of food causing firstly an excess and then a shortage of feed. A sudden increase in food levels can cause a proliferation of bacteria and protozoans, while low food levels may compromise survival, growth and PL quality.

Both in experiment 2 and 3, the density that gave the highest survival and length results was that of 1000 larvae L^{-1}. A considerable increment in survival was obtained in experiment 3 for the density of 2000 larvae L^{-1} when microalgae were supplied at high concentrations at a constant rate. Also individual dry weight, length and LSI were significantly improved by increasing the microalgae concentration. Kurmaly *et al*. (1989) stated that due to the short gastroevacuation time of larvae during herbivoral stages there is a need of an increase in food amount and constant supply of food for larvae to increase feed consumption, contributing to better survival and growth. Also, Kumlu (1997), observed that during planktonic stages, decapod crustacean larvae are chance encounter feeders and therefore need a high density of food particles in suspension at all times to assure good survival and growth. Better larval performance at high microalgae concentrations may also be due to improvements in water quality. It was observed that in the static culture phase in experiment 3, TAN and nitrate values were reduced by 27% and 63% for combinations of 1000 larvae L^{-1} and 2000 larvae L^{-1}, respectively. And although the reduced values of TAN exceeded the recommended level of 0.42 mg L^{-1} TAN reported by Cobo *et al.* (*in press*) to reduce ammonia toxicity during the rearing *L. vannamei* larvae, survival were higher during this experiment. This reduction could be explained by the capacity of the microalgae to absorb several nitrogenous compounds (Conway and Harrison, 1977; Paslow *et al*., 1985; Suttle and Harrison, 1988).

Interaction between feeding strategy and water recirculation

An improvement in survival and water quality during the static culture due to the improved feeding practices, contributed to higher survival in the RAS in experiment 2 and 3. In experiment 2, a pattern in which survival was significantly affected by water recirculation rate was observed: the lower the water recirculation rate the higher the survival. This pattern

was directly related with the increment in the amount of food. With the lower recirculation rate (250% day^{-1}), the average larval densities obtained at the end of the culture were 705 larvae L^{-1} for a stocking density of 1500 larvae L^{-1}, 650 larvae L^{-1} for a stocking density of 1000 larvae L^{-1}, 345 larvae L^{-1} for a stocking density of 750 larvae L^{-1} , while it was only 550 larvae L^{-1} for a stocking density of 1000 larvae L^{-1} with a water recirculation rate of 500% day^{-1}. As a result, 7 to 10 times more larvae were produced in the RAS than in TAS. Total mortality was obtained in experiment 2 at the density of 2000 larvae L^{-1}. However, in experiment 3 a survival of 39% was obtained for the same density. Only the lowest water recirculation rate gave survival for all densities tested. This is in accordance with Timmons and Losordo (1994) who stated that turnover time in culture chambers may exceed 24 h for larval stages, whereas for juveniles it may be several times per hour.

Based on the survival and growth results, the combination of a stocking density of 1000 larvae L^{-1} and a water exchange rate of 500% day^{-1} consistently presented higher mean survival (39, 55 and 60%) and biomass output (0.32, 0.31 and 0.42 kg m^{-3}) compared to the other treatments. Biomass yields of 0.53 to 1.66 kg m^{-3} for *L. vannamei* were obtained by Samocha *et al*. (1993) in an intensive raceway production system which was similar to the production results summarized by Samocha and Lawrence (1992) for intensive nursery systems under a variety of management conditions. Implementing a simple recirculating nursery system for *L. vannamei* and *L. setiferus*, Davis and Arnold (1998) obtained final biomass yields of 2.80 kg m^{-3} and 0.60 kg m^{-3} with stocking densities of 582 to 8300 PLs m^{-3}, respectively.

The present study demonstrates the feasibility to intensively produce *L. vannamei* larvae in an experimental recirculating system. These laboratory results show that when applying proper feeding regimes, feeding frequencies and daily rations larvae can be raised at densities up to 2000 L^{-1} using a recirculating aquaculture system. However, these results need to be verified under pilot and large-scale commercial production once biological parameters at this high stocking density are defined more precisely.

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CHAPTER V

HIGH DENSITY REARING OF *Litopenaeus vannamei* (Boone) LARVAE IN A RECIRCULATING SYSTEM

María de Lourdes Cobo[1], José Luis Vélez[2], Mathieu Wille[2], Rubén Román[1], Jorge Calderón[1], Patrick Sorgeloos[2]

[1] Escuela Superior Politécnica del Litoral (ESPOL), Campus Gustavo Galindo Velasco, Km 30.5 vía Perimetral, P.O. Box 09-01-5863, Guayaquil, Ecuador. Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM).

[2] Pontificia Universidad Católica del Ecuador. Sede Regional Manabí, Campus Bahía de Caráquez, Ecuador.

[3] Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University, Rozier 44, B-9000 Ghent, Belgium

HIGH DENSITY REARING OF *Litopenaeus vannamei* (Boone) LARVAE IN A RECIRCULATING SYSTE

Abstract

Rearing experiments of *Litopenaeus vannamei* larvae were carried out under high density conditions using a 1 m^3 seawater recirculating aquaculture system enclosed in a greenhouse and equipped with cartridge, biological and UV filters. Survival, growth, microbial aspects and physiological condition of the larvae reared in the recirculating aquaculture system were compared with those reared in a traditional culture system at conventional larval density. Water quality parameters were measured in the two culture systems. N5 were stocked at a density of 100 nauplii L^{-1} for the traditional culture system and 1000 nauplii L^{-1} for the recirculating aquaculture system. Recirculation started at Z3 with a daily water recirculation rate of 500%. Survival averaged 62 ± 8% and 50 ± 7% for the traditional culture system and recirculating aquaculture system, respectively. Length (mm) was similar in the two culture systems (3.76 ± 0.58 for the traditional culture system and 3.12 ± 0.61 for recirculating aquaculture system). Total biomass (g) was over 5 times greater in the recirculating aquaculture system than in traditional culture system (55 ± 4 *vs.* 304 ± 5). The recirculating aquaculture system operated satisfactorily maintaining ammonia and nitrite concentrations of the rearing water at less than 1 mg L^{-1} through the whole culture period. In general, water quality parameters were lower in the recirculating aquaculture system than those measured in the traditional culture system. No differences in bacterial numbers were detected between both culture systems. Mortality was not significantly different ($p<0.05$) between larvae reared in traditional culture system and recirculating aquaculture system when challenged with the pathogenic bacteria *Vibrio harveyi* and *V. splendidus* at concentration of $1x10^8$ colony forming units. These results suggest the feasibility to produce healthy *L. vannamei* postlarvae at 1000 larvae L^{-1} in a recirculating aquaculture system.

Introduction

Industrial shrimp farming started around 1970, exhibiting a fast growth due to the development of seed production in hatcheries, improved feeds and the high profit of the activity (Leung *et al*., 2000). Due to the expanding and competitive nature of shrimp production, its present and future depends and will depend largely on the stable supply of high quality larvae produced in hatcheries. Disease outbreaks are considered as the main constraint in the shrimp culture industry (Subasinghe, 2005), urging to carry out changes in the culture systems currently used. Environmental friendly treatments or strategies such as recycling the cultured water appear as a promising approach to overcome the problems found in traditional systems. During the last decade, recirculating aquaculture systems (RAS) have gained interest among aquaculturists. Many advantages can be obtained from these systems, the most important ones being the improvement of animal productivity at high densities and the reduction of wastewater discharged from aquaculture facilities (Kim *et al*., 2000; Timmons *et al.*, 2002). Recirculating systems may not only improve water quality in terms of suspended solids and nitrogen removal, there is also evidence that they may reduce the bacterial proliferation and/or that microbial communities are more stable during the culture period because water is continuously recycled and newly introduced water can be treated with cartridge filters or UV sterilizers (Suantika, 2001; Rombaut *et al*., 2001; Kikuchi *et al.*, 2006).

Most of the studies investigating recirculating systems deal with fish species *e.g.* turbot (Poxton *et al.*, 1982; Kikuchi *et al.*, 2006), walleye (Aneshansley *et al*., 2001), rainbow trout (Krumis *et al.*, 2001) and seabass (Franco-Nava *et al*., 2004). Nevertheless, the use of recirculating systems in intensive and superintensive aquaculture is a relatively new field, especially for shrimp culture (Horowitz *et al*., 2001). Conventional RAS have been tested for different species and different life stages of marine shrimp, *e.g.* for broodstock maturation, larval rearing, and for nursery and grow-out production of *P. monodon* (Menasveta *et al.*, 1991; Tach *et al*., 2005; Tseng *et al*., 1998, respectively), and for *L. setiferus* grow-out production (Williams *et al*., 1996; Davis and Arnold, 1998; Horowitz *et al*., 2001). Also information regarding efficient RAS application for nursery and grow-out culture of *L.vannamei* is available (Williams *et al*., 1996; Davis and Arnold, 1998;

Velasco *et al*., 2001; Samocha *et al*., 2002), but its application for the larval stages is less documented.

The effects of high-density rearing conditions on *L. vananmei* larval performance, quality and on the microbial community in a RAS need to be elucidated. Therefore, the development of a hatchery technology for intensive shrimp larviculture, based on a RAS, constitutes a cornerstone and is the challenge of the present study. A pilot-scale production system was used to evaluate the benefits of a RAS compared to the traditional aquaculture system (TAS).

Materials and Methods

The experiments were conducted at the Escuela Politécnica del Litoral (ESPOL) – Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM) facilities in San Pedro de Manglaralto (Santa Elena Province, Ecuador).

Experimental animals, culture systems and experimental design

Due to limitations in the number of experimental units, the rearing trial was repeated in time (three times). For each trial N5 of *L. vannamei* were obtained from EGIDIOSA hatchery (San Pablo, Santa Elena Province) and transported to the research facilities. All nauplii were disinfected with 100 mg L^{-1} of Argentyne® (Argent, Redmond, WA, USA) for 1 min and acclimatized to experimental conditions. The experiments were terminated when the larvae in the TAS reached the PL1 stage.

The RAS used was the Bioreactor EU-B 1024- H1F7 Pilot which was enclosed in a greenhouse. Figure 1 provides a schematic outline of the RAS. The unit consisted of a 1000 L cylinder-conical culture tank (CT) containing a central tube which is covered with different mesh sizes (200 - 400 µm) for the different larval stages, in order to retain larvae within the CT and to permit the discharge of organic matter and material in suspension. Sand-filtered and UV-treated seawater with a salinity of 34 g L^{-1} was used. Aeration via a pvc-pipe with holes placed at the bottom of the CT maintained dissolved oxygen

concentration above 4 mg L^{-1}. The temperature in the CT was kept stable at 32 ± 1 °C and controlled with a temperature probe.

Water was pumped from the CT to a mechanical filter composed of 7 cartridge filters to retain particles larger than 3µm. The effluent water flowed in two directions; one part went to a submerged biofilter with a capacity of 100 L filled with 70 L of artificial carrier material Kinetco Macrolite® (Newbury, OH, USA, ceramic spheres of about 40µm); the other part of the effluent passed through a UV sterilizer. After the biological filtration and UV sterilization the treated water was re-injected into the CT. The biofilter was inoculated two weeks prior to the experiment with 10 mg L^{-1} Volatile Suspended Solids of the nitrifying suspension Abil® (Avecom, Belgium). During this start-up period, every two days the nitrifying bacteria received a load of 5 mg L^{-1} of Nitrogen by adding Ammonium chloride to support its growth. The pH of the biofilter was maintained above 7.0 by the addition of $CaCO_3$. Recirculation was started at Z3 applying a daily water recirculation rate of 500%. An initial stocking density of 1000 N5 L^{-1} was used in the three trials.

Performance of the RAS was compared with a TAS. N5 were stocked at 100 N5 L^{-1} in 500 L round-shaped (115-cm diameter by 75 cm height) transparent polyethylene tanks. Water pre-treatment, temperature and dissolved oxygen concentration were equal to those in the RAS. From Z3 onwards, 30% water renewal was applied daily.

The standard feeding regime protocol of ESPOL - CENAIM was used for TAS (Table 1). For the RAS several modifications were made as described in Table 2. Concentrated microalgae (by centrifugation) were supplied to the RAS. Live and artificial foods were administered alternatively and provided manually in twelve daily rations for TAS and by means of an automatic feeder for the RAS. The quantity of live food available in RAS tank was continuously measured. Every two hours microalgae counts were performed to determine its concentration. In case of not being as assigned, the speed of entry of micro-algae through the automatic feeders was increased or decreased depending on these measurements. Rotifers and *Artemia* nauplii concentrations were counted every three hours by means of a 1 mL pipette. Similarly as for microalgae, levels were adjusted if necessary.

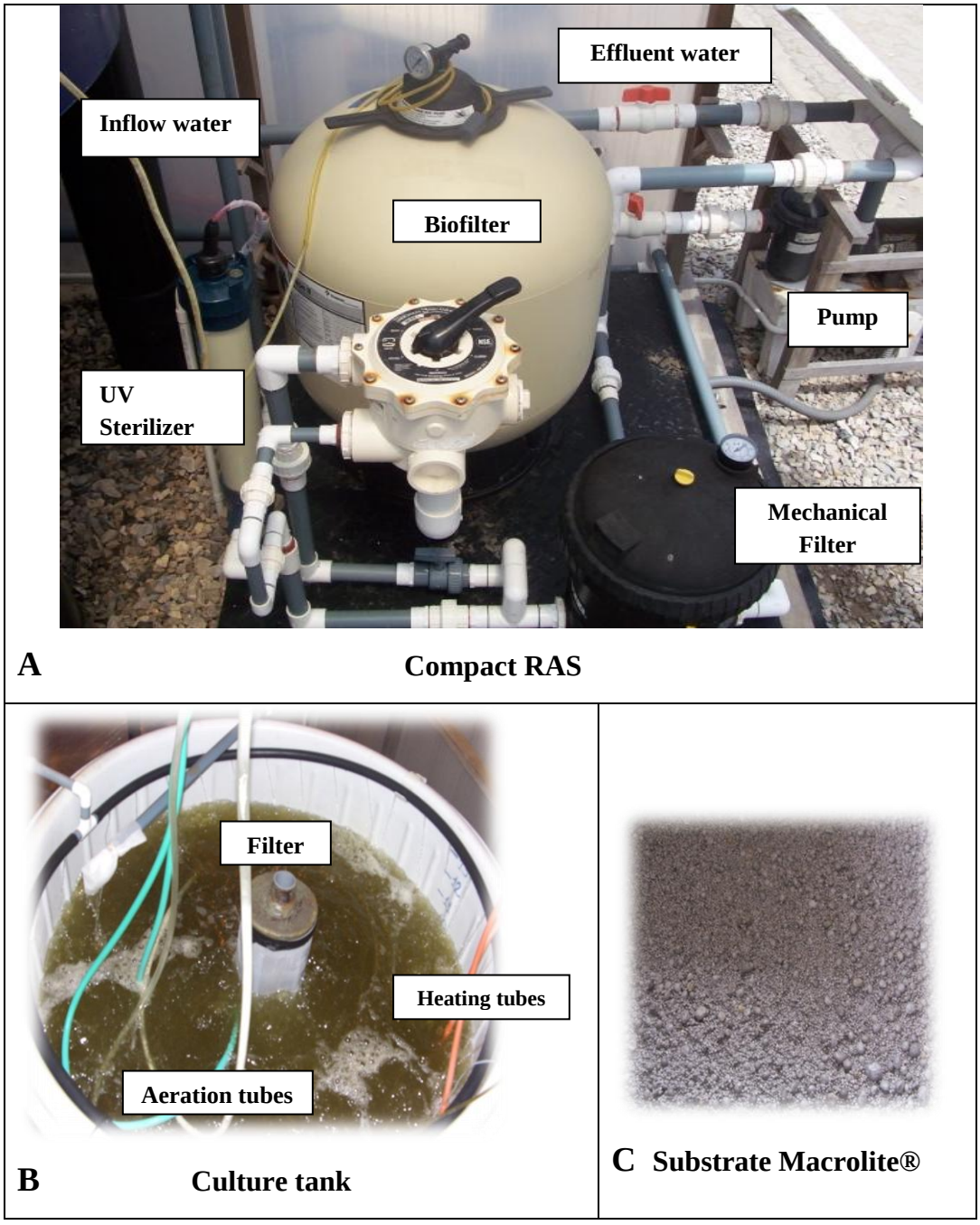

Figure 1. **A**. Photographs of the compact RAS (Bioreactor EU-B 1024- H1F7 Pilot), **B**. culture tank (1 m^3) and **C**. macrolite (MA) used as substrate in the biofilter

Artificial feeds, consisting of the liquid feed Epifeed-LHF® (Epicore, Eastampton, NJ, USA), and dry feeds Frippak® (INVE Aquaculture NV, Dendermonde, Belgium) and Larval Z Plus ® (ZEIGLER, Gardners, PA, USA), were given from Z1 until PL1 in the TAS while for the RAS only the dry feed Frippak® was administered. As a prophylactic treatment, the probiotic *Vibrio algynolyticus* (ILI strain) was added daily to the culture water at a concentration of 1.00E+10 colony forming units (CFU) mL^{-1} in order to get a final concentration of 1.00E+05 CFU mL^{-1} (Rodríguez *et al*., 2007) for the TAS and five times more the volume used for TAS was used for the RAS.

Larval performance

Larval survival was estimated at every stage of development. Survival was determined by taking five 125-mL samples from each culture tank and counting all live larvae. All sampled larvae were then returned to their respective culture tank.

Total length of the larvae (TL, mm) was measured at PL1 from the eyestalk base until the end of the last abdominal segment by means of a profile projector. This measurement was done on samples of 30 larvae per replicate. For the determination of dry weight (DW, mg), triplicate samples of 50 PL1 per replicate were taken, washed briefly with distilled water and placed into pre-weighed aluminium foil cups. The samples were dried in an oven at 60 °C for 24 h and then weighed. Total biomass (B, g) was determined at the end of the culture period by harvesting all larvae and quantifying the total weight. The larval stage index (LSI) was determined by daily microscopic observations according to the procedure described by Kanazawa *et al.* (1985).

Table 1. ESPOL-CENAIM's standard feeding protocol used for *Litopenaeus vannamei* in the traditional aquaculture system (TAS) (100 N5 L^{-1}).

	Microalgae				Artificial Diets	
Larval stage	*Chaetoceros gracilis* (10^3 cells mL^{-1})	*Tetraselmis* sp. (10^3 cells mL^{-1})	Rotifers (rot mL^{-1})	*Artemia* (nauplii $larvae^{-1}$)	Liquid diet (g million $larvae^{-1}$)	Dry diet (g million $larvae^{-1}$)
N5	100					
Z1	120				10	
Z2	160		10		17	10
Z3	80	80	15	10	20	11
M1		60	25	14	28	14
M2		60	27	19	35	20
M3		60	30	24	45	23
PL1		60	33	30		34

Table 2. Feeding protocol used for *Litopenaeus vannamei* larviculture in the recirculating aquaculture system (RAS) (1000 N5 L^{-1})

Larval stage	Microalgae *C. gracilis* (10^3cells mL^{-1})	Rotifers (rot mL^{-1})	*Artemia* (nauplii $larvae^{-1}$)	Dry diet (g million $larvae^{-1}$)
N5	150			
Z1	175			
Z2	200	100		10
Z3	250	120	15	11
M1	200	192	20	14
M2	150	360	30	20
M3	100	192	40	23
PL1	100	192	50	34

Challenge test

Physiological condition of the larvae was tested through a challenge test. Postlarvae from TAS and RAS were collected and placed in 2 L containers. Infections were performed via immersion with two pathogenic bacteria: 246 (*Vibrio splendidus* I) and 2590 (*Vibrio harveyi*) CENAIM strains at a concentration of 1.00E+108 CFU. Mortality was recorded after 24 h of exposure. Each test was run in five replicates with 100 postlarvae per container.

Statistical analyses

Results for larval performance and the challenge test were subjected to a one-way analysis of variance (ANOVA). Normal probability plots and the Bartlett test for homogeneity of variances were used to verify the ANOVA assumptions. When necessary, percentage data were √arcsine transformed before being submitted to ANOVA. Scheffé multiple comparison test was used to identify differences among treatments. All references to statistical significance were at the 5% level or lower. The statistical analysis was performed using Data Desk® 6.0 PPC for Mac.

Water Quality

Daily measurements of pH, Total Ammonia Nitrogen (TAN), nitrite NO_2-N and nitrate NO_3-N (mg L^1) were carried out on the culture water. TAN and nitrite analyses were performed following the protocol described by Solórzano (1983), and nitrate concentrations by a modified protocol from Solórzano (1983). Total suspended solids (TSS) were determined following the method described in APHA, AWWA and WPCF (1992).

Microbiological analyses

Larvae were collected at N5, Z2, M2 and PL1 from the TAS and RAS to perform microbiological analysis. They were rinsed with distilled water on a 60 µm sieve to remove all flocks and bacteria loosely attached to the body surface. Samples were macerated with a stomacher blender and serial dilutions were prepared in sterilized seawater with 1 g of the macerated samples. Two sub-samples of 100 µL from the suspension were spread plated on

MA (marine agar 2216) and (Thiosulphate Citrate Bile Sucrose) (DIFCO Co. Ltd., USA) for total bacteria and TCBS counts, respectively. The plates were incubated at 30 °C and bacterial counts performed after 24 h.

Microbial community structure

The profiles of the microbial community of the RAS and TAS were examined by means of denaturing gradient gel electrophoresis (DGGE) of amplified 16S rRNA gene fragments.

DNA extraction and polymerase chain reaction (PCR) amplification

Water samples of 25 L were collected daily from the RAS and TAS to be centrifuged at 3000 rpm until a volume of 500 mL was obtained. Total DNA from the water samples was obtained following the method described by Smalla *et al.* (1993). To extract bacterial DNA, the 500 mL water sample was centrifuged for 10 minutes at 3500 x *g*. The total amount of nucleic acids extracted from the 500 mL water samples was finally re-suspended in 50 μL DNase free water.

Amplification of 16S rRNA genes

The 16S rRNA genes from the microbial community in the water samples were amplified following the procedure described by Schaefer and Muyzer (2001). The extracted prokaryotic DNA was amplified by PCR in two subsequent cycles using a PTC 200 MS Research. PCR amplification was carried out in 25 μL reactions and 2 μL of extracted total DNA was added. Using the primers PRBA-338-F-GC (5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG-3') and 518R - (5'ATTACCGCGGCTGCTGG-3) DNA fragments of 450 bp were amplified. The PCR master mix contained 0.5 μL of each primer, 200 μM of each dNTPs, 1.5 nM $MgCl_2$, 10 μL of thermophilic DNA Polymerase 10 x reaction buffer ($MgCl_2$ - free). 0.75 U of Taq DNA polymerase (Promega ®) and sterile water, to final volume of 25 μL. PCR was performed in a PTC-200MJ Research ® cycler as follows: 94

°C for 5 min, followed by 30 cycles of 92 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. All PCR products (10 μL volumes) were analyzed by electrophoresis in 2% (w/v) agarose gels before DGGE analysis was performed.

Analysis of PCR products by DGGE

DGGE was performed as described by Muyzer *et al.* (1993), using the CBS Scientific Co. DGGE-2001-Rev.B System with 6% (w/v) polyacrylamide gels in 1 x TAE (20 nM Tris, 10 nM acetate, 0.5 mM EDTA, pH 7.4), containing a linear chemical gradient ranging from 40 to 60% denaturant (where 100% denaturant contains 7 M urea and 40% formamide). PCR products (5 μL) obtained from genomic DNA of water samples were used for separation in denaturing gradient gels. The electrophoresis ran for 5 h at 60 °C and at 150 V.

After completion of electrophoresis, the gels were Silver stained ($AgNO_3$) following the protocol described by Dinesh *et al*. (1995). The stained gels were immediately photographed on a white light box with a digital camera Olympus C-5000 (5 megapixels: optical zoom 3x of 38mm - 14mm).

An individual binary matrix (bands present or absent) was generated in all lines that were transformed to a distance matrix using the genetic distance (Link *et al.*, 1995) in the Treecon software (version 1.3b). The clustering analysis of multivariate UPGMA (Unweighted Pair Group Method with Arithmetic Averages) was used to calculate dendrograms.

Results

Larval Performance

Survival, dry weights, lengths, larval stage indices and total biomass production per tank of PL1 *L. vannamei* reared under TAS and RAS conditions are presented in table 3. Survival and length of PL1 were similar between the two culture systems, however recurrent mortalities were observed from M2 onwards in the RAS due to fouling. PL1 from TAS exhibited a significantly higher dry weight compared to those reared intensively in the RAS ($p<0.05$). A significantly higher LSI ($p<0.05$) was observed for larvae reared in TAS reaching the post-larval stage one day earlier than those reared in RAS. The biomass obtained from the RAS was significantly higher ($p<0.05$), being almost six-fold higher than in the TAS.

Table 3. Survival, dry weight, length, larval stage index (LSI) and biomass of PL1 *Litopenaeus vannamei* larvae reared in a traditional culture system (TAS) and recirculating aquaculture system (RAS). Means ± SD are presented

	Parameters				
Culture system	Survival (%)	Dry weight (mg larvae $^{-1}$)	Lenght (mm)	Larval Stage index	Biomass (g)
TAS	62 ± 8^{a}	0.170 ± 0.020^{a}	3.76 ± 0.58^{a}	7.50 ± 0.20^{a}	55 ± 4.0^{b}
RAS	50 ± 7^{a}	0.090 ± 0.010^{b}	3.12 ± 0.61^{a}	6.70 ± 0.20^{b}	304 ± 5.0^{a}

Means within the same column and followed by the same letter are not significantly different ($p>0.05$).

Water quality

Results of water quality in the TAS and in the RAS are summarized in Table 4. During the whole culture period the pH remained within the range of 7.27 to 8.17 and 7.56 to 8.12 for the TAS and RAS, respectively. Concentrations of TAN (mg L^{-1}) and nitrate were significantly higher ($p<0.05$) in the TAS than in the RAS, while nitrite (mg L^{-1}) was significantly higher ($p<0.05$) in the RAS. Similar results for TSS were registered for both culture systems.

Table 4. Initial and final values of TAN (total ammonia nitrogen, mg L^{-1}), nitrite N-NO_2 (mg L^{-1}), nitrate N-NO_3 (mg L^{-1}) and total suspended solids (TSS, mg L^{-1}) of culture water in the traditional culture system (TAS) and recirculating aquaculture system (RAS). Means ± SD are presented.

		Parameters			
Culture system		TAN (mg L^{-1})	NO_2 - N (mg L^{-1})	NO_3 - N (mg L^{-1})	TSS (mg L^{-1})
TAS	Initial	0.03 ± 0.02	0.001 ± 0.001	0.540 ± 0.030	63 ± 15
	Final	1.30 ± 0.04[a]	0.070 ± 0.010[b]	3.250 ± 0.200[a]	162 ± 108[a]
RAS	Initial	0.07 ± 0.02	0.001 ± 0.001	0.140 ± 0.020	62 ± 12
	Final	0.34 ± 0.21[b]	0.420 ± 0.220[a]	1.900 ± 0.700[b]	131 ± 62[a]

Means within the same column and followed by the same letter are not significantly different ($p>0.05$).

Challenge test

No significant difference was observed between larvae reared in the TAS and the RAS when exposed to bacterial strains 264 and 2590. Mortality of larvae from the TAS was 13 ± 12% and 12 ± 7% for the bacteria 264 and 2590, respectively. Mortality of larvae from the RAS was 12 ± 11% for the bacteria 264 and 12 ± 9% for the bacteria 2590.

Microbiological analyses

Bacterial counts performed on larvae during N5, Z2, M2 and PL1 stages are provided in Table 5. In Z2 the amount of total bacteria on MA in the RAS system was significantly higher ($p<0.05$) than in the TAS, while on TCBS Agar the bacterial load was similar for both systems. Total bacteria and TCBS counts reached the highest counts during M2 in the RAS, which were significantly different from the TAS ($p<0.05$). Subsequently counts decreased during the PL1 stage.

Table 5. Bacterial counts obtained from larvae sampled at different larval stages during the culture in the traditional system (TAS) and recirculating system (RAS)

Larval stages	Culture System	Marine Agar CFU g^{-1}	TCBS CFU g^{-1}
N5	TAS	2.40E+06	1.90E+04
	RAS	2.40E+06	1.90E+04
Z2	TAS	3.10E+07 ± 1.20E+07[b]	8.10E+05 ± 1.80E+05[a]
	RAS	1.70E+08 ± 1.10E+08[a]	5.30E+05 ± 3.50E+05[a]
M2	TAS	7.30E+06 ± 1.30E+06[b]	7.00E+05 ± 1.50E+05[b]
	RAS	1.40E+08 ± 1.10E+08[a]	2.00E+06 ± 1.70E+06[a]
PL1	TAS	5.60E+06 ± 1.80E+06[a]	2.20E+04 ± 1.85E+04[b]
	RAS	2.30E+06 ± 1.80E+06[a]	1.20E+05 ± 0.90E+05[a]

Means within the same column referring for each larval stage followed by the same letter are not significantly different ($p>0.05$).

Microbial Community Structure

The cluster analysis of the microbial community structure of the TAS and RAS during the 9 days culture period is visualized in a dendrogram shown in Figure 2. In the TAS three main clusters could be observed, one cluster grouped the first 6 days of the culture, except for day 4 (start of water renewal) which clustered differently. During days 7 and 8 a new cluster was expressed which related more to the clusters of the RAS. A new shift was observed at the end of the culture during day 9 (not grouping with any other day). For the RAS also three main clusters could be appreciated, observing differences in clustering of the first three days of culture, while from day 4 (start of recirculation) onwards all days clustered together except for days 5 and 9. The variability of the banding patterns within the clusters indicated that the structure of the microbial communities of the culture systems was not static but rather dynamic. These observations suggested that the microbial community associated with the larval cultures could evolve in relation to the culture systems used in addition to the contribution of the food supply and the zoo-technical parameters.

A high range of dissimilarity (50 - 60%) was observed for the microbial groups in the TAS and the RAS suggesting the presence of microbiota with different genetic information

during the culture period (Figure 2). Nevertheless, there were groups in common between the two culture systems.

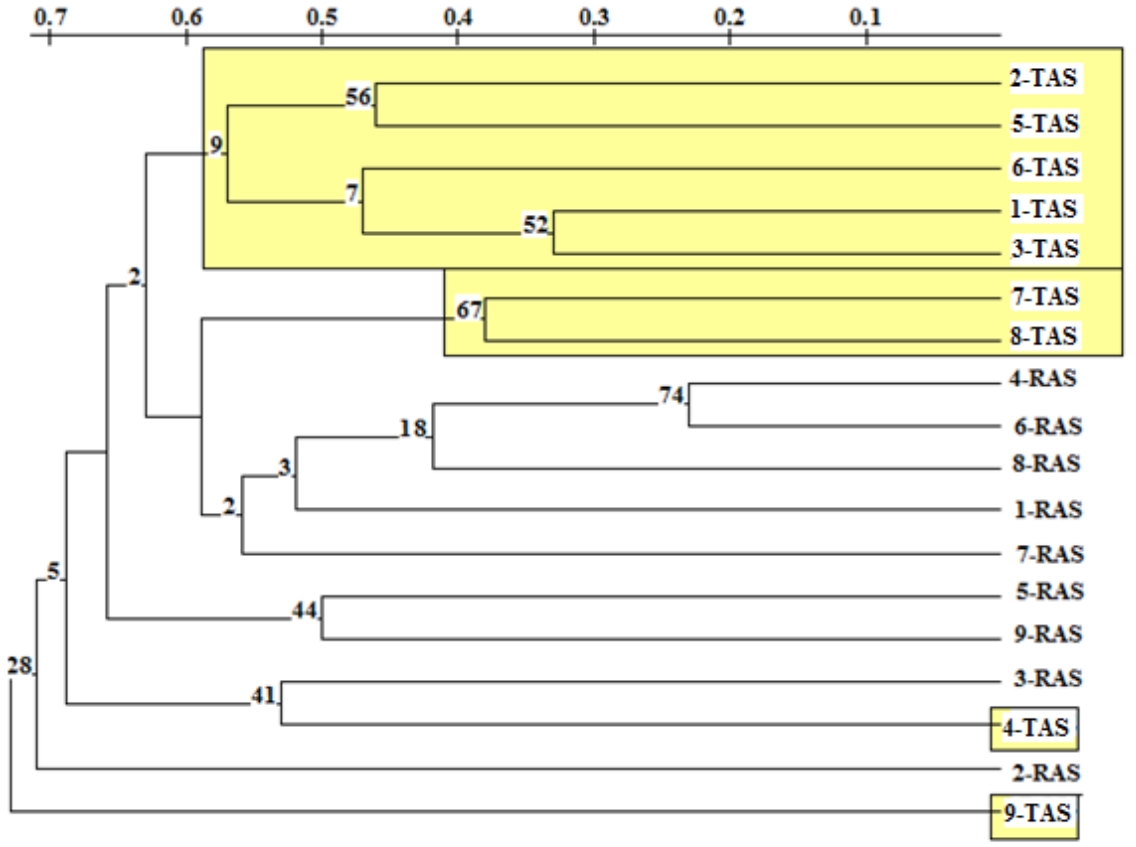

Figure 2. Cluster analysis of microbial community structure analysed using denaturing gradient gel electrophoresis (DGGE) of amplified 16S rRNA gene fragments. Number of days since the start of the culture is indicated before the culture system: traditional system (TAS) and recirculating system (RAS). Scale (0.1 - 0.7) indicates the non-similarity level. Numbers on the branches refer to bootstrap values for 100 times.

Discussion

Implementation of RAS in shrimp culture at high stocking density has mainly been documented for post-larval and juvenile stages, while studies referring to the use of RAS in shrimp larviculture using high stocking densities are less documented. In our study, when implementing a pilot 1 m^3 RAs to produce *L. vannamei* larvae using a high stocking denisty (1000 N5 L^{-1}), survival and length results were similar to TAS at conventional density (100 N5 L^{-1}). Overall results were also similar to those reported in literature by Martins *et al*. (2006), Al Azad *et al*. (2002), D´Abramo *et al*. (2006) in TAS for *F. paulensis* (60 NL^{-1}), *P. monodon* (100 - 200 N L^{-1}) and *L. vannamei* (200 - 300 N L^{-1}), respectively. Our results were also similar to those obtained by Beard *et al*. (1977) when using laboratory recirculating systems to culture *F. merguiensis* at a stocking density up to 400 N L^{-1} yielding survival percentages of 42 to 83%. Gandy (2004) evaluated RAS for the production of *F. aztecus* PL. The PL survival in RAS was similar or higher than that in control tanks when using initial stocking densities of 100 N L^{-1}, obtaining survival ranges of 27 to 65%. Thach *et al.* (2005) used a recirculating system to produce *P. monodon* larvae at commercial scale, obtaining survival at harvest of PL12 ranging from 20 to 80%, with half of the batches ≥ 60% at stocking densities of 200 - 250 N L^{-1}. Truong *et al.* (2009) evaluated stocking densities from 150 up to 900 nauplii L^{-1} for *P. monodon* in an improved RAS. The best performance was at a density of 450 N L^{-1} with a survival of 56% and a yield of 252 larvae L^{-1}. In terms of percentage survival our results are comparable with the results of the cited studies, although the stocking density used in our study was 2 to 16 times higher, yielding an average output of 500 PL1 L^{-1}.

Recirculating systems are considered to be as highly productive systems, which maximize biomass output per unit of area, volume or investment (Rosenthal and Black, 1993). Davis and Arnold (1998) demonstrated that with different stocking strategies and system configurations, ranges of biomass production between 1.22 to 11.23 kg m^{-3} for *L. vannamei* and 0.36 to 5.70 kg m^{-3} for *L. setiferus* could be obtained. These production results are also similar to those summarized by Samocha and Lawrence (1992) and Mishra *et al*. (2008) for intensive nursery systems and raceways under different management conditions.

Our results indicate that *L. vannamei* larvae can be raised at high density using a RAS with a 6 times higher production yield than in a TAS.

In the present study, larvae reared intensively in the RAS showed a lower dry weight compared to those reared in the TAS. Higher stocking densities could increase competition for food and space consequently resulting in lower weight. Secondly, loss of feed was observed when the recirculation was started as feed particles were retained in the cartridge filters. This may have lead to underfeeding, thus resulting in a reduced growth rate. This could also explain the lower LSI of larvae reared in RAS, which was reflected in a one day delay of metamorphosis to the PL stage.

In general, water quality parameters values were within recommended levels for different penaeid species. Chin and Chen (1987), Lin *et al*. (1993) reported 24 h LC_{50} values of 4.7 mg L^{-1} NH_3-N for *P. monodon* (PL6) and 2.3 mg L^{-1} NH_3-N for *M. japonicus* (PL1). For both systems, values for nitrite in our study for both systems were far below those reported as toxic. Alcaraz *et al.* (1999) determined that the 24 h LC_{50} for *L. setiferus* was 268.1 mg L^{-1} NO_2-N. Significantly lower final values for TAN, but significantly higher values for nitrite were registered in the RAS compared to the TAS. These results indicate that the biofilter within the RAS was activated, however that the conversion rate from ammonia to nitrite was higher than the one from nitrite into nitrate, resulting in increased nitrite levels. Surprisingly, significantly higher values of nitrate were measured in the TAS at the end of the experiment. This may be explained by the concentration of nitrate salts contained in the algae culture media from the algae supplied during larval feeding. High values of TSS were observed for both culture systems in this study which were above the recommended values for aquaculture activities given by Timmons *et al*. (2002).

Rombaut *et al*. (2001) demonstrated the positive effect of RAS for rotifer production on the bacterial numbers within the system, being 1 to 2 log units lower than in a batch rotifer culture system. In our study we could not find evidence of this advantage. On the contrary, the larvae reared in the RAS presented significantly higher bacterial counts of 1 to 2 log than in the TAS. Moreover, both systems registered higher values compared to the ones obtained in commercial hatcheries (Vandenberghe *et al*., 1998). These high values for both systems could be attributed to: 1) the use of the probiotic strain *Vibrio alginolyticus* as a prophylactic treatment during the culture, and 2) the microbial load coming with the live

food (microalgae, rotifers, and *Artemia* nauplii) used as food source for the larvae, especially in the RAS where a high amount of live feed was given. Lavens and Sorgeloos (2000) indicated that live feed is an important source of microbial contamination *e.g. Artemia* nauplii have a bacterial load up to 10^{10} CFU g^{-1} being mostly Vibrios. Suantika (2001) found levels of 10^7 and 10^6 CFU mL^{-1} on marine agar in the rearing water of rotifers cultured in batch and recirculating systems, respectively.

Several studies have attempted to characterize the microbial communities within different aquaculture rearing systems by means of several techniques, *e.g.* in fishes (Brunvold *et al*., 2007; Jensen *et al*., 2004; McIntosh *et al*., 2008), mollusks (Sandaa *et al*., 2003) and in shrimp, especially for on-growing cultures (Sung *et al.,* 2001; Li *et al*., 2005). Nevertheless, there is scarce knowledge on the microbial ecology and its evolution during the culture period for shrimp larviculture systems, and especially in RAS. The current study expands the knowledge base when comparing the microbial community of two different culture systems: TAS and RAS. Clusters were delineated at 55% similarity showing a high variability among bacterial communities between systems and within systems. This is in disagreement with Suantika (2001) who observed a high similarity (98%) on the DGGE profiles of the water samples taken during day 8 and 21 of rotifers cultured in a recirculating system having a microbiota with similar genetic information. Vandenberghe *et al.* (1999) observed also a high degree of heterogeneity (45% similarity) even among *Vibrio alginolyticus* strains when studying Vibrios associated with *L. vannamei* larvae and postlarvae at different hatcheries in Ecuador. In the bacteriological survey performed in different operational hatcheries of *F. chinensis* and *P. monodon,* Vandenberghe *et al.* (1998) and Hisbi *et al*. (2000), observed that the flora associated with larvae was not very stable and that it was influenced by the bacterial flora of the administered food and by the environment. Similar observations were made in our study when different culture systems were applied. More research however, needs to be carried out to characterize the microbial community structure of *L. vannamei* larviculture and its evolution during the culture period with different culture systems and practices for a better understanding of its role, in order to improve protocols and the welfare of the reared larvae.

The usefulness of *in vivo* infection or "challenge tests" has been demonstrated in the evaluation of virulence of *Vibrio* isolates associated with mortalities, to test prophylactic and

curative treatments and in the study of host-factors influencing the expression of bacterial virulence (Saulnier *et al.*, 2000; Alabi *et al.*, 1999; Roque *et al.*, 1998). The purpose of using a bath challenge test in our study was to evaluate differences in larval fitness of larvae reared in different systems. Results of our study showed that there were no significant ($p>0.05$) differences between the larvae reared in the TAS and the RAS, demonstrating that the type of culture system and the difference in stocking densities did not influence the physiological condition of the larvae in terms of tolerance to a pathogen. Our results are similar to those reported by Pico (2004), who obtained a mortality of 20.4% of PL3 *L. vannamei* when bath challenged with 246 CENAIM strain using a bacterial concentration of 1.00E+07 CFU, but lower when challenged with the higher bacterial concentration 1.00E+08 CFU. Similar and higher values in survival were obtained in our work compared to Alabi *et al.* (1999) who reported values within ranges of 6.5 to 79% survival when challenging Z3 of *Penaeus indicus* with different strains of *V. harveyi*.

In conclusion, it can be stated that the recirculating system evaluated in this study allows the intensive production of *L. vannamei* larvae, maximizing biomass production and controlling water quality, although a reduction in other production parameters (growth, larval development) was observed. Further improvements (feeding regime and feed ration) are needed to tackle these shortcomings and to ensure consistent output at commercial level. Studies need to be carried out to investigate subsequent performance (during nursery and grow-out) of larvae reared under intensive condition in a RAS.

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CHAPTER VI

EVALUATION OF UMBRELLA-STAGE *Artemia* AS FIRST ANIMAL LIVE FOOD FOR *Litopenaeus vannamei* (Boone) LARVAE

María de Lourdes Cobo[1], Roeland Wouters[2], Mathieu Wille[3], Stanislaus Sonnenholzner[1] and Patrick Sorgeloos[2]

[1] Escuela Superior Politécnica del Litoral (ESPOL), Campus Gustavo Galindo Velasco, Km 30.5 vía Perimetral, P.O. Box 09-01-5863, Guayaquil, Ecuador. Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM).

[2] INVE Technologies N.V., Dendermonde, Belgium

[3] Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University, Rozier 44, B-9000 Ghent, Belgium

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EVALUATION OF UMBRELLA-STAGE *Artemia* AS FIRST ANIMAL LIVE FOOD FOR *Litopenaeus vannamei* (Boone) LARVAE

Abstract

An alternative larval shrimp feeding regime - in which umbrella-stage *Artemia* did constitute the first zooplancton source - was evaluated in the culture of *Litopenaeus vannamei*. In a preliminary experiment, umbrella-stage *Artemia* were fed to larvae from zoea 2 (Z2) to mysis 2 (M2) sub-stages to identify the larval stage at which raptorial feeding starts and to determine daily feeding rates. The following experiment evaluated the performance of three feeding regimes that differed during the late zoea / early mysis stages: 1) a control treatment with frozen *Artemia* nauplii (FAN), 2) a treatment with frozen umbrella-stage *Artemia* (FUA), and 3) ESPOL-CENAIM's internal standard treatment combining enriched rotifers and frozen *Artemia* nauplii from Z2 until PL1 and from Z3 till M2, respectively (ER+FAN).

The ingestion rate of umbrella-stage *Artemia* increased from 9 umbrella larvae^{-1} day^{-1} at Z2 stage to 21 umbrella larvae^{-1} day^{-1} at M2 at a feeding density of 1 and 5 umbrella ml^{-1} respectively. Individual dry weight of the larvae increased from 0.019 mg at Z2 to 0.085 mg at M2. The steep increase in ingestion and dry weight from Z3 to M2 suggests a transition in feeding mode starts from mysis stage Treatments FUA and ER+FAN exhibited a significantly higher larval stage index ($p<0.05$) during the period that zoea metamorphosed to the mysis stage, and a higher final biomass, compared to treatment FAN. Based on these results and on practical considerations, a feeding regime starting with umbrella-stage *Artemia* from Z2 sub-stage can be recommended for *L. vannamei* larvae rearing.

Introduction

Feeding regimes and feeding practices are major factors affecting the success of a commercial shrimp hatchery (D'Abramo *et al*., 2006; Yúfera *et al*., 1984). The development of shrimp larvae is characterized by a number of stages and sub-stages that differ in behaviour, morphology and nutritional requirements (Lavens and Sorgeloos, 1996; Samocha *et al.,* 1989). In nature, the larval shrimp diet generally consists of diverse phytoplankton, zooplankton and bioflocs of various sizes and with different biochemical composition, therefore more likely to meet the overall nutritional requirements of the larvae. In contrast, feeding regimes used in commercial shrimp hatcheries rely on a limited number of live food items or on a combination of live and artificial food. The larval rearing diet used in commercial hatcheries consists of a few species of microalgae (Gallardo *et al.,* 1995; Loya-Javellana, 1989) and newly hatched *Artemia* nauplii (Kumlu and Jones, 1995; New, 1976; Sorgeloos, 1980). *Artemia* nauplii constitute the principal animal protein source used in commercial shrimp larviculture, because of their nutritional value and size, easy acceptance by the larvae and convenient storage as cysts.

Determining the suitable particle size, feed quantity and feeding schedule is of great importance and greatly affects larval development (Jones *et al*., 1997a; Kurmaly *et al*., 1989; Samocha *et al.,* 1989). Zoeal stages feed mainly on phytoplankton (3 - 30 µm diameter) by filter-feeding, although from stage Z2, depending on the penaeid species, they can capture animal prey of up to 100 µm raptorially (Jones *et al*., 1997b). Feeding on animal organism as early as Z2 has been documented by several authors, using rotifers (Emmerson, 1984; Naessens *et al*., 1995; Samocha *et al.,* 1989), nematodes (Biedenbach *et al*., 1989; Focken *et al*., 2006; Wilkenfeld, 1984), copepods (D'Abramo *et al*., 2006) and *Artemia* nauplii in different forms such as live freshly hatched nauplii (Kuban *et al*., 1985; Lavens and Sorgeloos, 1996; Samocha *et al.,* 1989) and frozen nauplii (Soares *et al*., 2006; Wilkenfeld *et al*., 1981). Some commercial hatcheries start feeding Instar 1 *Artemia* nauplii as early as possible, because a number of studies have documented the benefit of the introduction of an animal protein source in the zoea stages (Naessens *et al.,* 1995; Samocha *et al.,* 1989; Wilkenfeld, 1984). However, shrimp larvae at zoea stages are poor hunters and the use of live *Artemia* nauplii results in an inefficient food uptake. Therefore, heat-killed, blended or

frozen *Artemia* Instar 1 nauplii can be fed to late zoea stages and mysis to ease capture. Also, the use of inactive *Artemia* displays the advantage to overcome problems associated with ongrowing of uneaten *Artemia* in the larval rearing tanks (Sorgeloos *et al*., 1998). On the other hand, the used of heat-killed and blended *Artemia* nauplii may affect the nutritional quality and may deteriorate rearing water quality. García-Ortega *et al*. (1995) studied the effect of heat treatment on the nutritional quality of decapsulated *Artemia* cysts, observing that already at 60 °C around 30% of the protein was denaturalised and the enzymatic activity decreased by more than 50%. The continued use of frozen *Artemia* may deteriorate water quality as well. Soares *et al.* (2006) furthermore observed that feeding frozen *Artemia* nauplii resulted in significantly lower weight in *Farfantepenaeus paulensis* postlarvae.

Umbrella-stage *Artemia* can be harvested during the hatching process of *Artemia* cysts after only 12 h. When the cyst shell or chorion breaks, a pre-nauplius larva, which is still surrounded by its hatching membrane, protrudes from the cyst shell hanging underneath the empty shell, thus the name umbrella-stage. The "breaking" process of the cyst ends when the hatching membrane breaks and the newly hatched Instar 1 nauplius emerges (Figure 1). Umbrella stage *Artemia* display some advantages over *Artemia* nauplii such as higher energy content, smaller size and are easy to be captured (Lavens and Sorgeloos, 1996). Umbrella-stage *Artemia* is sometimes used to feed shrimp and fish larvae (Nhu *et al*., 2009; Wouters *et al*., 2009).

Rotifers (*Brachionus* spp.) are considered to be an excellent food source for fish and crustacean larvae (Léger and Sorgeloos, 1992; Lubzens *et al*., 1997; Naessens *et al.*, 1995; Sorgeloos *et al*., 1991). Rotifers are widely used in aquaculture because they present several characteristics that make them a suitable prey for fish and shrimp larvae; such as slow swimming behavior and small size. Rotifers are hardy organisms which have the potential to be mass produced at high densities due to a rapid reproduction and present the possibility to be used as carriers for dietary components (Léger and Sorgeloos, 1992; Lubzens *et al*., 2001; Roiha *et al*., 2011; Wouters *et al*., 1997).

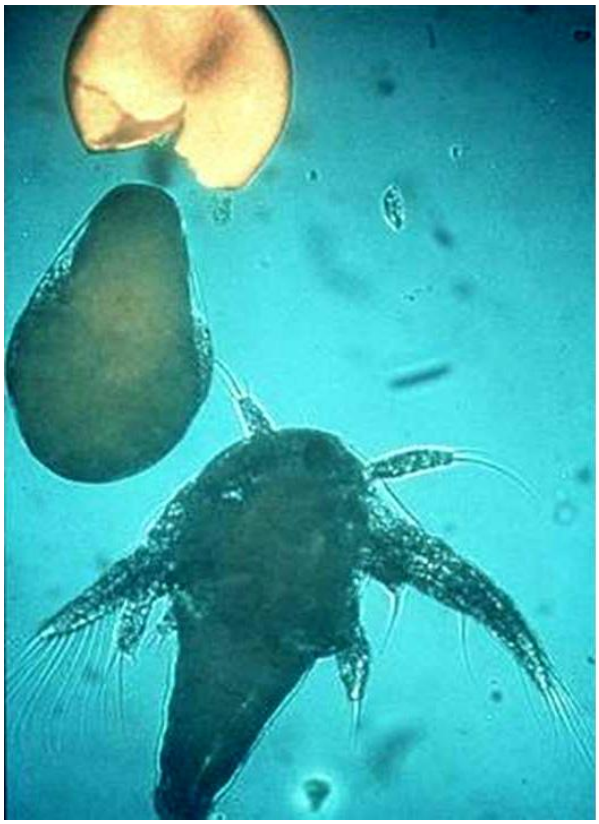

Figure 1. Umbrella-stage hanging underneath the empty cyst shell and instar I nauplius stage of *Artemia* (Lavens and Sorgeloos, 1996)

Feeding practices for shrimp larvae are commonly based on two prey sizes, algae less than 20 µm, followed by *Artemia* nauplii of about 450 µm. From the energetic point of view, a prey should be as large as possible to have the most efficient energy intake by the larvae per hunting effort. Léger and Sorgeloos (1992) and Naessens *et al*. (1995) have recommended rotifers to fill the size gap between algae and *Artemia* nauplii. Jones *et al*. (1991) indicated that rotifers also facilitate the early introduction of high quality protein of animal origin in the larval diet and the predator may take the advantage of the prey's set of digestive enzymes to better utilize simultaneously administered feeds. For the abovementioned reasons, the ESPOL-CENAIM Research Center has been using rotifers within the feeding regime of *L. vannamei* larvae as a standard practice for many years.

In the present work, we studied the use of a feeding regime for *L. vannamei* larvae, introducing umbrella stage *Artemia*. To set-up a feeding regime with umbrella-stage

Artemia, it was necessary to determine at what stage they could be offered to the shrimp larvae and document ingestion rates. Subsequently, the effect of their use on the larval performance was evaluated by comparison with a feeding regime that is typically used in commercial hatcheries. As the use of rotifers is being applied routinely at the ESPOL-CENAIM research center for over a decade, as the center's standard feeding regime, this regime was also compared to the commercial standard feeding regime.

Materials and methods

Experimental Animals

N5 of *L. vannamei* were obtained from EGIDIOSA hatchery (San Pablo, Santa Elena Province) and transported to the research facilities of the Escuela Politécnica del Litoral (ESPOL) – Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM) Nauplii were disinfected with 100 mg L^{-1} Argentyne® (Argent, Redmond, WA, USA) for 1 min and acclimatized to the experimental conditions.

Two experiments were performed: firstly, an ingestion experiment to determine the consumption rates of *L. vannamei* larvae feeding on umbrella-stage *Artemia*. Secondly, an experiment to evaluate the larval performance with two alternative feeding schedules.

Ingestion experiment

Ingestion of umbrella-stage *Artemia* was assessed at different larval stages from Z2 until M2. Z2 larvae were stocked in 1-L plastic Imhoff settling cones at a density of 100 larvae L^{-1}. The settling cones were held in a water bath to maintain temperature at 32 ± 1 ºC. Salinity was 35 ± 1 g L^{-1} and pH averaged 8.22 ± 0.21. Gentle aeration was provided through a 1 mL glass pipette from the bottom of each cone to assure homogeneous distribution of the prey and larvae and to maintain dissolved oxygen near saturation levels.

During the experiment, larvae were fed the microalga *Chaetoceros gracilis* at 150 000 cells mL^{-1}. Great Salt Lake (UT-USA) *Artemia* cysts (INVE Aquaculture NV, Dendermonde, Belgium) were incubated at 28 °C in 35 g L^{-1} seawater under continuous light supplied by two fluorescent white lamps of 40 W (Sylvania ®), which provided a

luminance of 2000 lux (27 µmol m^{-2} s^{-1}) and strong aeration (Lavens and Sorgeloos, 1996). After 12 h incubation, umbrella-stage *Artemia* were collected on a 125 µm sieve and washed several times with tap-water in order to remove empty shells and membranes. Umbrella-stage *Artemia* were fed at 1 umbrella mL^{-1} day^{-1} for the zoea sub-stages and 5 umbrella mL^{-1} day^{-1} for the mysis sub-stages**.** Four replicates per larval stage were used.

Daily ingestion was monitored every three hours. The umbrella count was computed by 3 replicate counts of 5 mL samples fixed with lugol. The ingestion rate, *I*, was calculated using the equation described by Paffenhoffer (1971):

$$I= V (C_t-C_o)/nt$$

Where (C_t-C_o) is the decrease in concentration of umbrella within the experimental period, *t* (24 h). V is the water volume and n is the number of shrimp larvae (the mean of the initial and final number of larvae in the 24 h period).

Larval counts were recorded every 24 h to calculate the survival at each sub-stage. Total length of the larvae (TL, mm) was measured from the eyestalk base until the end of the last abdominal segment by means of a profile projector. This measurement was done on samples of 30 larvae per replicate. For determination of dry weight (DW, mg), triplicate samples of 50 larvae per replicate were taken randomly, washed briefly with distilled water and placed into pre-weighed aluminium foil cups. The samples were dried in an oven at 60 °C for 24 h and then weighed. The larval stage index (LSI) was determined by daily microscopic observations following the procedure described by Kanazawa *et al*. (1985).

Larval rearing experiment

Experimental design

This experiment evaluated the performance of three feeding regimes (Table 1) that differed during the late zoea/early mysis stages: 1) a control treatment in which frozen *Artemia* nauplii were fed (FAN), 2) a treatment in which frozen umbrella-stage *Artemia* were fed (FUA), and 3) CENAIM's internal standard treatment combining enriched rotifers

and frozen *Artemia* nauplii from Z2 until PL1 and from Z3 till M2, respectively (ER+FAN). Each dietary treatment was randomly assigned to ten replicate tanks.

Rearing conditions, live food and feeding regime

N5 were stocked at 100 N5 L^{-1} and maintained in 50-L cylindro-conical fibreglass tanks filled with sand-filtered and UV-treated seawater (salinity 34 g L^{-1}). Temperature was maintained at 32 ± 1 °C. Dissolved oxygen concentration was kept above 4 mg L^{-1} in each tank.

During the substages N5 and Z1, the microalgae *C. gracilis* was supplied. Starting from the transition of Z2 - Z3, *Tetraselmis* sp. gradually replaced *C. gracilis* (Table 1). The feeding regimes of the three treatments began to differ when 90% of the larvae had moulted to Z2. Rotifers and *Artemia* were incubated, harvested and enriched according to standard procedures as described in Lavens and Sorgeloos (1996). Rotifers, *Brachionus* sp. Cayman (Dooms *et al*., 2007) used in the experiment were obtained from a batch culture fed live *Tetraselmis* sp. in 5 m^3 rectangular fiber-glass tanks. Rotifers were harvested and placed in a 50-L cylindro-conical polycarbonate tank and enriched with EPICORE® emulsion (Epicore, Eastampton, NJ, USA) for 6 h. *Artemia* cysts (INVE Aquaculture NV, Dendermonde, Belgium) were incubated at 28 °C in 35 g L^{-1} seawater with continuous light provided by two fluorescent white lamps of 40 W (Sylvania ®), which provided a luminance of 2000 lux (27 µmol m^{-2} s^{-1}) and strong aeration. After 12 h incubation, umbrella-stage *Artemia* were collected as described earlier. They were kept in a freezer at - 20 °C. For Instar I production, *Artemia* cysts (INVE Aquaculture NV, Dendermonde Belgium) were decapsulated, incubated as described above and harvested after 24 h. Instar I *Artemia* nauplii were used as live newly-hatched nauplii, frozen nauplii (frozen at -20 °C) and enriched metanauplii. The same emulsion as for rotifers was used for the *Artemia* enrichment. Enriched rotifers, *Artemia* nauplii, *Artemia* umbrella and enriched metanauplii *Artemia* were disinfected with 50 mg formaldehyde L^{-1} for 30 min before being given to the larvae. Artificial feeds Epifeed-LHF® (Epicore, Eastampton, NJ, USA), Frippak® (INVE Aquaculture NV, Dendermonde, Belgium), Larval Z Plus® (ZEIGLER, Gardners, PA, USA) were given from Z2 until PL12 (Table 1). Live and artificial feeds were administered alternately and provided

manually in twelve daily rations. Survival of *L. vannamei* larvae was counted at every stage of development in order to adjust the feeding regime. As a prophylactic treatment, the probiotic *Vibrio algynolyticus* (ILI strain) was added daily to the culture water at a concentration of 1.00E+10 colony forming units (CFU) mL^{-1} in order to get a final concentration of 1.00E+05 CFU mL^{-1} (Rodríguez *et al*., 2007).

Evaluation criteria

Survival was determined by taking three 125-mL samples from each culture tank and counting all live larvae. All sampled larvae were then returned to their respective culture tank. Survival was determined at PL1 and at the termination of the experiment when the larvae reached PL12. Total length, dry weight and larval stage index were determined as described above.

A salinity stress test was applied at PL12 on three replicate groups of 100 postlarvae from each dietary treatment. For the salinity stress test, PL12 were transferred abruptly from 35 g L^{-1} seawater to 1-L plastic beakers containing tap water (< 3 g L^{-1}) for a period of 30 min. Thereafter, postlarvae were transferred to their original beaker for another 30 min. Percentage survival was assessed considering postlarvae that did not present movement of pleopods and did not react when prodding with a pipette (Martins *et al*., 2006; Palacios *et al*., 1999).

Table 1. Feeding regimes applied in the *Litopenaeus vannamei* larval rearing experiment

	Microalgae		Artificial Diets		*Artemia*		Dietary Treatments			
Larval stage	*Chaetoceros gracilis* (10^3cells mL^{-1})	*Tetraselmis* sp. (10^3cells mL^{-1})	Liquid diet (g million $larvae^{-1}$)	Dry diet (g million $larvae^{-1}$)	Live nauplii $larvae^{-1}$	Enriched metanauplii $larvae^{-1}$	Frozen *Artemia* nauplii (FAN)	Frozen umbrella-*Artemia* (FUA)	Enriched rotifers mL^{-1} + frozen *Artemia* nauplii (ER +FAN)	
N5	100									
Z1	120		10							
Z2	160		17	10			8	10	10	
Z3	80	80	20	11			10	15	15	10
M1		60	28	14			12	20	25	12
M2		60	35	20			14	25	27	14
M3		60	45	23	19				30	
PL1		60		34	24				33	
PL2		60		44		17				
PL3		60		64		20				
PL4		60		76		25				
PL5		60		88		30				
PL6		60		100		35				
PL7		60		112		40				
PL8		60		124		45				
PL9		60		136		50				
PL10		60		150		55				
PL11		60		162		60				
PL12		60		175		65				

Statistical analyses

Results are expressed as means ± standard error of mean (SEM). Normal probability plots and the Bartlett test for homogeneity of variances were used to verify the assumptions for further analysis. A one way ANOVA was used to detect differences between the treatments. An √arcsine transformation of percentage data was applied before the data were analyzed. Tukey´s HSD multiple comparison test was used to identify differences between treatments. All references to statistical significance were at the 5% level or lower. The statistical analyses were performed using STATISTICA 4.1 (Statsoft®, Tulsa, Oklahoma, USA).

Results

Ingestion experiment

Survival, dry weight, length and larval stage index are presented in Table 2. Survival was higher for zoeal stages than for mysis stages. For length, dry weight and LSI increasing values were observed demonstrating a normal larval stage development. Figure 2 shows the consumption rates of umbrella by *L. vannamei* larvae from Z2 to M2. Larvae consumed a significant amount of umbrella (a mean of 6.33 umbrella larvae^{-1} day^{-1}.) from the initial concentration (1 umbrella mL^{-1}) in the early stage Z2. Consumption did not increase at Z3. However, umbrella consumption doubled at M1 and further increased at M2 at a concentration of 5 umbrella mL^{-1} day^{-1}.

Table 2. Survival, dry weight, length and larval stage index of *Litopenaeus vannamei* larval stages during the ingestion experiment. Means ± standard error are presented

	Parameteres			
Larval stages	Survival (%)	Dry weight (mg larvae^{-1})	Length (mm)	Larval stage Index
Z2	94 ± 2	0.019 ± 0.001	1.45 ± 0.01	2.970± 0.001
Z3	96 ± 3	0.029 ± 0.002	2.29 ± 0.01	4.010 ± 0.005
M1	89 ± 1	0.065 ± 0.003	2.84 ± 0.07	4.940 ± 0.001
M2	87 ± 3	0.085 ± 0.003	3.47 ± 0.11	6.000 ± 0.001

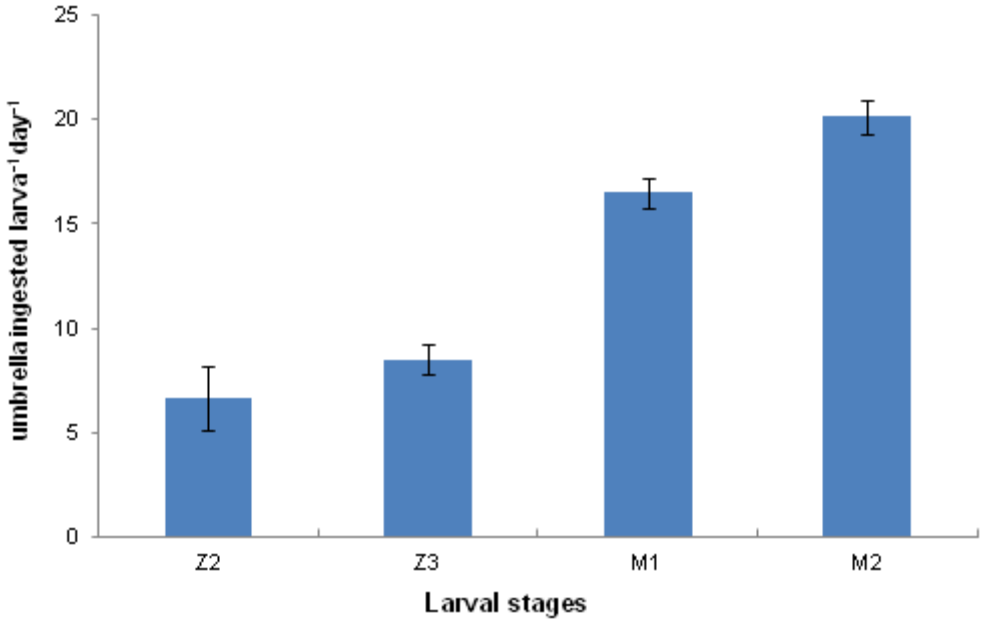

Figure 2. Ingestion rates of umbrella-stage *Artemia* by *Litopenaeus vannamei* larvae from Z2 to M2

Larval rearing experiment

Table 3 presents survival, dry weight and length results at PL1 stage of larvae reared under the different dietary treatments. Survival of larvae were generally high (88-91%) and did not differ between treatments ($p>0.05$). Dry weight and length seemed to be higher in treatments FUA and ER+FAN as compared to control FAN, but the results were not statistically different ($p<0.05$).

At culture day 3 and 4, a significant ($p<0.05$) higher LSI was observed for larvae of treatments FUA and ER+FAN as compared to control treatment FAN (Figure 3). From culture day 5 onward, the LSI was equal in all treatments. The share of larvae that metamorphosed to PL1 ranged from 90 to 100% in all treatments.

Survival at PL12 stage ranged from 52 to 64%. No significant differences were found between dietary treatments for survival and length ($p> 0.05$, Table 4). Dry weight exhibited the same trend as in PL1 with higher values for larvae fed FUA and ER+FAN, although not

significantly different than FAN ($p>0.05$). On the other hand, significantly higher biomass ($p<0.05$) was obtained in treatments FUA and ER+FAN compared to FAN. PL12 exposed to a salinity stress presented the same percentage survival for all the dietary treatments ($p>0.05$).

Table 3. Survival, dry weight and length at PL1 of *Litopenaeus vannamei* larvae receiving different dietary treatments: FAN (frozen *Artemia* nauplii), FUA (frozen umbrella-stage *Artemia*) and ER+FAN (enriched rotifers + frozen *Artemia* nauplii). Means ± standard error are presented

	Parameters		
Dietary treatments	Survival (%)	Dry weight (mg larvae^{-1})	Length (mm)
FAN	91 ± 2^{a}	0.180 ± 0.013^{a}	3.70 ± 0.03^{a}
FUA	89 ± 4^{a}	0.197 ± 0.012^{a}	3.80 ± 0.08^{a}
ER + FAN	88 ± 3^{a}	0.207 ± 0.011^{a}	3.85 ± 0.08^{a}

Within columns, superscript letters indicate significant diffferences ($p<0.05$)

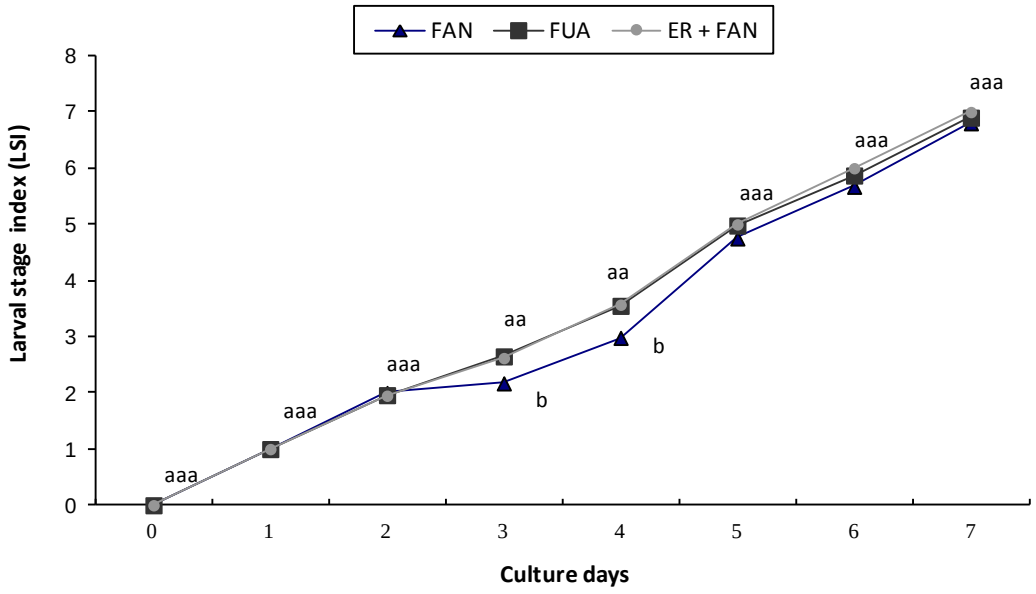

Figure 3. Larval stage index (LSI) of *Litopenaeus vannamei* larvae receiving different dietary treatments: FAN (frozen *Artemia* nauplii), FUA (frozen umbrella-stage *Artemia*) and ER+FAN (enriched rotifers +frozen *Artemia* nauplii)

Table 4. Survival, dry weight, length, biomass and osmotic stress survival at PL12 of *Litopenaeus vannamei* larvae receiving different dietary treatments: FAN (frozen *Artemia* nauplii), FUA (frozen umbrella-stage *Artemia*), and ER+FAN (enriched rotifers +frozen *Artemia* nauplii). Means ± standard error are presented

	Parameters				
Dietary treatments	Survival (%)	Dry weight (mg larvae^{-1})	Length (mm)	Biomass (g)	Osmotic stress survival (%)
FAN	56 ± 2^{a}	0.440 ± 0.021^{a}	6.00 ± 0.12^{a}	8.46 ± 0.42^{a}	61 ± 3.7^{a}
FUA	58 ± 3^{a}	0.471 ± 0.022^{a}	6.05 ± 0.09^{a}	9.70 ± 0.50^{b}	59 ± 3.5^{a}
ER + FAN	63 ± 4^{a}	0.488 ± 0.018^{a}	6.14 ± 0.10^{a}	11.56 ± 0.72^{b}	61 ± 3.6^{a}

Within columns, superscript letters indicate significant diffferences ($p<0.05$)

Discussion

Live freshly-hatched *Artemia* nauplii are the most commonly used animal food source in penaeid larviculture. It has been demonstrated by Emmerson (1984), Hirata *et al*. (1985) and Wilkenfeld *et al.* (1981) that *Artemia* nauplii consumption starts from Z3 in *F. indicus M. japonicus* and *L. setiferus*, respectively. Samocha *et al.* (1989) discerned no significant difference in survival when *Artemia* nauplii were offered to *L. setiferus* larvae from Z2, Z3 or M1, although trends towards increasing larval dry weights were evident as the stage of *Artemia* nauplii introduction was advanced from M1 to Z2. These findings are in accordance with Kuban *et al.* (1985) who supplemented larval diets for four penaeid species (*F. aztecus, L. setiferus, Litopenaeus. stylirostris* and *L. vannamei*) with *Artemia* nauplii beginning at Z2 versus M1 improved their growth rates in terms of dry weights but did not improve their survival or metamorphosis rate. Introducing *Artemia* nauplii already from Z2 as compared to the classical feeding regime with *Artemia* supplementation as of M1 stage, better growth rates in terms of dry weights were obtained but survival nor metamorphosis rate had changed. Introducing *Artemia* nauplii already from Z2 is a common practice in commercial hatcheries using different processing methods: frozen, blended or heat-killed. However, in order to avoid the use of processing methods that could degrade the quality of the *Artemia* offered to the larvae, we introduced the possibility to use umbrella-stage *Artemia* in the feeding regime of *L. vannamei* larvae.

Umbrella were readily ingested at stage Z2 since the prey size of umbrella-stage *Artemia* (348.9 ± 23.2 µm) is considerably smaller than that of *Artemia* nauplii (455.0 ± 25.4 µm). Nevertheless, a higher experimental variability of ingestion rate at Z2, suggests that not all Z2 larvae were equally efficient at catching and ingesting umbrella-stage *Artemia*. The highest consumption rate of umbrella-stage *Artemia* by *L. vannamei* larvae determined in this study (20 umbrella larvae-^{1} day^{-1} at M2) was considerably lower than ingestion rates of newly-hatched *Artemia* nauplii ranging from 30 to over 100 nauplii larvae^{-1} day^{-1} as reported by Cook and Murphy (1969), Gopalakrishnan (1976), Hirata *et al.* (1985) and Yúfera *et al*. (1984), for *L. setiferus, Penaeus marginatus, M. japonicus* and *M. kerathurus,* respectively. On the other hand, Chun and Shing (1986) reported an ingestion rate of 10 nauplii larvae^{-1} day^{-1} for M3 of *Metapenaeus ensis*. The results in our study showed a rather sharp increase in ingestion of umbrella-stage *Artemia* at metamorphosis from zoea to mysis stages. This can be explained by the feeding mode employed by penaeid larvae, shifting from filter to raptorial feeding, at the same time changing from herbivorous feeding at zoea stages to carnivorous during mysis stages (Jones, 1997 a; Kurmaly *et al.*, 1989; Wouters and Van Horeenbeck, 2003). Emmerson (1984) indicated that the changeover point occurs around M3 for *F. indicus,* while Chun and Shing (1986) reported that such transition in feeding mode takes place at a later stage for *M. ensis*. Our observations suggest that for *L. vannamei* transition in feeding mode starts from M1 stage.

In our study, a significantly ($p<0.05$) higher biomass was obtained for larvae fed umbrella (FUA) compared to those fed frozen *Artemia* nauplii (FAN). Moreover, larvae fed umbrella-stage *Artemia* exhibited a significantly ($p>0.05$) higher larval stage index at culture day 3 and 4 compared to those fed *Artemia* nauplii. It is observed that feeding umbrellas early at Z2 markedly accelerates the larval development to the mysis stage.

Emmerson (1984) demonstrated that *F. indicus* larvae consume rotifers as early as Z1, while Yúfera *et al.* (1984) and Hirata *et al.* (1985) showed rotifer consumption from Z2 - Z3 for *M. kerathurus* and *M. japonicus,* respectively. Naessens *et al.* (1995) performed tests at the CENAIM research center and observed that rotifers were ingested by *L. vannamei* from Z2 and that this resulted in an acceleration of the larval development. As a result, the metamorphosis to the postlarval stage was taking place almost one day earlier than in the

control group. Léger *et al.* (1986) suggested that a transitional prey to fill up the size gap between algae (8 - 20µm) and freshly hatched *Artemia* nauplii (430 - 455 µm) could enhance larval development and growth. Rotifers (50 - 300 µm) have been considered for this purpose, thus solving the problem of abrupt change in food particle size (Léger and Sorgeloos, 1992; Naessens *et al.*, 1995). In the present study, feeding enriched rotifers to *L. vannamei* larvae significantly ($p<0.05$) accelerated larval development in Z3 and M1 compared to those fed frozen *Artemia* nauplii (FAN); however, this effect was not apparent at the postlarval stage. Our results also indicated that an early introduction of rotifers into the feeding regime significantly improved biomass compared to the traditional feeding regime using *Artemia* nauplii as the first animal live food.

Hence, in the present study, both rotifers and umbrella have demonstrated to be valuable life food items, potentially improving culture performance as compared to *Artemia* nauplii. Some practical considerations can be taken into account regarding the culture and use of rotifers and umbrella. Rotifer cultures are often seen as a major expense in marine hatchery management due to the requirement for substantial floor space, microalgae, artificial feeds and man hours. Umbrella-stage *Artemia*, on the other hand, can be used as an intermediate prey replacing either the use of rotifers or *Artemia* nauplii. Results from our study demonstrate that umbrella-stage *Artemia* could replace live, frozen, heat-killed or blended *Artemia* nauplii typically used in many commercial shrimp hatcheries. Compared to rotifers, umbrella-stage *Artemia* are easy to produce (12 h hatching process), allowing the installation of more practical hatching routines. Moreover, umbrella have higher energy content and dry weight than either rotifers and newly-hatched *Artemia* nauplii.

In conclusion, taking into account the obtained culture results as well as the abovementioned considerations, umbrella-stage *Artemia* are being proposed for application in commercial feeding regimes of *L. vannamei* larvae, starting from the early Z2 sub-stage and continued until sub-stage M2. Although further studies are needed to establish the optimal concentration of FUA as an initial live food for *L. vannamei* larvae, based on the present results, it is recommended to start feeding 10 umbrella larvae^{-1} day^{-1} at Z2 and further increase to 25 umbrella larvae^{-1}day^{-1} at stage M2.

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CHAPTER VII

INTENSIVE CULTURE OF *Litopenaeus vannamei* (Boone) LARVAE IN A RECIRCULATING SYSTEM WITH THE USE OF UMBRELLA-STAGE *Artemia* AS A FOOD SOURCE FOR EARLY STAGES

María de Lourdes Cobo[1], Nestor Montaña[2], Mathieu Wille[3], Roeland Wouters[4], Rubén Román[1] and Patrick Sorgeloos[3]

[1] Escuela Superior Politécnica del Litoral (ESPOL), Campus Gustavo Galindo Velasco, Km 30.5 vía Perimetral, P.O. Box 09-01-5863, Guayaquil, Ecuador. Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM).

[2]Universidad de Ciencias Aplicadas y Ambientales. Facultad de Ciencias Agropecuarias, Bogotá, Colombia.

[3] Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University, Rozier 44, B-9000 Ghent, Belgium

[4] INVE Technologies N.V., Dendermonde, Belgium

INTENSIVE CULTURE OF *Litopenaeus vannamei* (Boone) LARVAE IN A RECIRCULATING SYSTEM WITH THE USE OF UMBRELLA-STAGE *Artemia* AS A FOOD SOURCE FOR EARLY STAGES

Abstract

A study was performed on the intensive rearing of *L. vannamei* larvae in a recirculating system using umbrella-stage *Artemia* as a feed for early larval stages. Umbrella-stage *Artemia* was fed from Z2 until M2 sub-stages in order to replace rotifers. Survival, growth and microbial aspects of the larvae reared in the recirculating culture system (RAS) were compared with those reared in a traditional culture system (TAS). N5 were stocked at a density of 100 N5 L^{-1} for the TAS and 1000 N5 L^{-1} for the RAS until they reached PL1. Water quality parameters were measured in the two culture systems. The physiological condition / quality of the larvae was assessed through an *in vivo* challenge test with a pathogenic bacteria strain (*Vibrio splendidus* I) at a concentration of 1.00E+08 CFU mL^{-1} and through evaluating their performance during a subsequent nursery culture (laboratory conditions). A financial analysis was performed to compare the economical profitability of the high density RAS with the TAS.

Survival for both culture systems at the end of the culture period were 71 ± 5% and 74 ± 6% for the RAS and the TAS, respectively. However, in terms of larval output, 710 PL1 L^{-1} were produced in the RAS compared to 74 PL1 L^{-1} produced in the TAS. Similar dry weights, length, and larval stage index results were recorded for the larvae reared in the two culture systems. No significant differences in survival were obtained after exposure to the *V. splendidus* I pathogenic bacteria, and in the nursery culture between larvae reared in the two culture systems. Compared to the TAS, the use of a RAS can contribute to a 42% saving on the total investment costs and also a saving up to 30% in the running costs, mainly due to reduced labour and depreciation costs per larviculture production cycle. This study demonstrated the feasibility of producing healthy *L. vannamei* larvae at high densities, in a reliable and cost-effective way, by using a simple recirculating system.

Introduction

Recirculating aquaculture systems (RAS) are increasingly being used in fish hatcheries (van Rijn, 1996; Blancheton, 2000; Faulk *et al*., 2007; Sammouth *et al*., 2009). They save on heating costs and space, increase bio-security and improve the stability of water quality. In hatcheries, stable water quality conditions are known to decrease stress and improve larviculture performance. For shrimp larval rearing however, the use of RAS is relatively new. It´s development may be very useful for the intensification of larviculture and could therefore be a powerful tool to improve larval output. RAS could improve the profitability of hatchery production, while being environmentally sound as water input and discharge are minimized.

Studies on the use of RAS for larval rearing of marine shrimp are scarce and less documented. Gandy (2004) evaluated RAS for production of *F. aztecus* postlarvae with multiple water re-use in a closed recirculating system. He obtained similar or higher survival in RAS than in control tanks. Menasveta *et al*. (1989) and Millamena *et al*. (1991) described the use of RAS for larval rearing of *P. monodon*. Ghanekar (2005) reported preliminary results with RAS for *P. monodon* larviculture with survival up to 66% at PL4. However, the larval development was reduced. Thach *et al*. (2005) applied a RAS for commercial-scale *P. monodon* larviculture, with stocking densities of 250 larvae L^{-1}. Survival at PL12 ranged from 20% to 80%, with half of the batches yielding 60% or more. Nonetheless, the stocking densities used in these studies were considerably lower than the 750 to 2000 larvae L^{-1} we have tested for *L. vannamei* in an experimental recirculating system in earlier trials (Wouters *et al*., 2009). There we reported a survival of 60% at a density of 1000 larvae L^{-1}, which is comparable to the survival obtained in traditional aquaculture systems (TAS). Repeated trials with stocking densities of 1000 larvae L^{-1} showed survival averaging 50% although larval dry weight was reduced. The total biomass was over 5 times higher than in TAS.

The success of commercial larviculture of penaeid shrimp is highly dependent on the feeding practices used. Important factors for the optimisation of feeding efficiency are the physical characteristics of the food and the specific nutritional requirements at each larval stage. Umbrella-stage *Artemia franciscana* has been suggested by Nhu *et al*. (2009) as a possible substitute for enriched rotifers in cobia larviculture, simplifying the rearing protocol and reducing production costs. Umbrella-stage *Artemia* is sometimes used to feed *P. monodon* from M1 to PL2 (Wouters *et al*., 2009). Replacing the fresh-frozen or heated

Artemia nauplii traditionally used by commercial shrimp hatcheries with umbrella-stage *Artemia*, would also reduce processing that might have an adverse effect on the nutritional value of the food (Wouters and Van Horenbeeck, 2003). Umbrella-stage *Artemia* could thus be an alternative food source for early shrimp larval stages.

The increased demand for shrimp postlarvae worldwide can either result in the increase in the hatchery space and personnel or in the intensification of the larval production. As economic profitability of shrimp larvae production depends to a large extent on the rearing technique, the development of a rearing system that increases productivity and production stability is highly recommended.

The objectives of this study were to: 1) compare the production output and economical profitability of a high density RAS with a TAS, and 2) test the quality of larvae reared in a RAS compared to a TAS.

Materials and methods

All trials were carried out at the Escuela Politécnica del Litoral (ESPOL) – Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM) in San Pedro de Manglaralto (Santa Elena Province, Ecuador).

Larviculture experiment

Experimental animals

N5 of *L. vannamei* were obtained from EGIDIOSA hatchery (San Pablo, Santa Elena Province, Ecuador) and transported to the facilities of the research center ESPOL-CENAIM. All nauplii were disinfected with 100 mg L^{-1} of Argentyne® (Argent, Redmond, WA, USA) for 1 min and acclimatized to the experimental conditions.

Culture conditions

Three RAS systems were fabricated in-house, based on the design of the Bioreactor EU-B 1024- H1F7 Pilot® (Chapter V) with some modifications. The recirculating units were enclosed in a greenhouse. Each unit consisted of a 1200-L cylindro-conical culture tank (CT) containing a central tube covered with a mesh. The size of the mesh differed (200 - 400 µm) according to the larval stage, in order to retain larvae within the CT while allowing organic matter and material in suspension to pass. Sand-filtered and UV-treated seawater of 34 g L^{-1} was used. Aeration through a pvc-pipe with holes placed at the bottom of the CT maintained dissolved oxygen concentration above 4 mg L^{-1}. The temperature in the CT was kept at 32 ± 1 °C and controlled with a temperature probe.

Water from the CT was pumped through a mechanical cartridge filter to retain particles larger than 3 µm and then flowed to a submerged biofilter (Atlantic Filter®, West Palm Beach, FL, USA). The biofilter, filled with 70 L of artificial carrier material Kinetco Macrolite® (Newbury, OH, USA, ceramic spheres of about 40 µm), had a capacity of 100 L. Thereafter, the effluent passed through a UV sterilizer before being re-injected into the CT at a daily water renewal rate of 500% day^{-1}. Two weeks prior to the experiment, the biofilter was inoculated with 10 mg L^{-1} Volatile Suspended Solids of the nitrifying suspension Abil® (Avecom, Belgium). To support their growth, every other day the nitrifying bacteria received a load of 5 mg N L^{-1} by adding ammonium chloride. The pH of the biofilter was maintained above 7.0 by the addition of Calcium Carbonate ($CaCO_3$). Larvae were stocked in the RAS at a density of 1000 N5 L^{-1}. Recirculation was activated when the larvae reached the Z3 stage. Between 5 to 10% of new water was added daily to offset water losses due to the cleaning of the bio-filter.

The three TAS were composed by 500-L round-shaped (115-cm diameter by 75 cm height) transparent polyethylene tanks in which sand-filtered and UV-treated seawater of 34 g L^{-1} was used. Temperature was maintained at 32 ± 1 °C. Dissolved oxygen concentration was kept above 4 mg L^{-1} in each tank. From Z3 stage onwards, approximately 30% daily water renewal was applied. The N5 were stocked at a density of 100 N5 L^{-1}. The experiment ended after 9 days, when larvae metamorphosed to PL1.

Feeding Protocols

For the TAS, the feeding protocol of CENAIM-ESPOL was used (table 1). The microalga *Chaetoceros gracilis* was supplied from N5 until Z3 and from this stage on replaced by *Tetraselmis* sp. From Z2 until PL1, the larvae were fed enriched rotifers *Brachionus* sp. Cayman (Dooms *et al*., 2007) and a combination of artificial feeds, namely the liquid diet EPIFEED-LHF® (Epicore, Eastampton, NJ, USA) and the dry diets Frippak® (INVE Aquaculture NV, Dendermonde, Belgium) and Larval Z Plus® (Zeigler, Gardners, PA, USA). *Artemia* cysts (Great Salt Lake USA, INVE Aquaculture NV, Dendermonde, Belgium) were decapsulated and incubated at 28 °C in 35 g L^{-1} seawater, with continuos light supplied by two fluorescent white lamps of 40 W (Sylvania ®), which provided a luminance of 2000 lux (27 µmol m^{-2} s^{-1}) and strong aeration (Lavens and Sorgeloos, 1996). *Artemia* nauplii Instar I were used as live freshly hatched nauplii and frozen nauplii (frozen at - 20 °C).

Table 1. Feeding protocol for *Litopenaeus vannamei* larviculture in the traditional aquaculture system (TAS) (100 N5 L^{-1})

	Microalgae				Artificial Diets	
Larval stage	*Chaetoceros gracilis* (10^3 cells mL^{-1})	*Tetraselmis* sp. (10^3 cells mL^{-1})	Rotifers (rot mL^{-1})	*Artemia* (nauplii $larvae^{-1}$)	Liquid diet (g million $larvae^{-1}$)	Dry diet (g million $larvae^{-1}$)
N5	100					
Z1	120				10	
Z2	160		10		17	10
Z3	80	80	15	10	20	11
M1		60	25	14	28	14
M2		60	27	19	35	20
M3		60	30	24	45	23
PL1		60	33	30		34

A simplified feeding regime was used for the RAS. For micro-algae, only concentrated *C. gracilis* was supplied. Additionally the dry diet Frippak® (INVE Aquaculture NV, Dendermonde, Belgium) was given from Z2 until PL1 (Table 2). Great Salt Lake (UT-USA) *Artemia* cysts (INVE Aquaculture NV, Dendermonde, Belgium) were incubated as described above (Lavens and Sorgeloos, 1996). After 12 h incubation, the

Artemia embryos, which were then in the umbrella-stage were collected on a 125 µm sieve and washed several times with tap-water in order to remove empty shells and membranes. Umbrella *Artemia* were supplied from Z2 to M2. During the experiment quantity of live food available in RAS tank was continuously measured. Every two hours micro-algae counts were performed to determine its concentration. In case of not being as assigned, the speed of entry of microalgae through the automatic feeders was increased or decreased depending on these measurements. *Artemia* umbrellas and nauplii concentrations were counted every three hours by means of a 1 mL pipette. Similarly as for microalgae, levels were adjusted if necessary.

Table 2. Feeding protocol for *Litopenaeus vannamei* larviculture in the recirculating aquaculture system (RAS) (1000 N5 L^{-1})

	Microalgae	*Artemia*		Artificial diet
Larval stage	*Chaetoceros* gracilis (10^3cells mL^{-1})	umbrella ($larvae^{-1)}$)	nauplii ($larvae^{-1}$)	Dry diet (g million $larvae^{-1}$)
N5	150			
Z1	175			
Z2	200	8		10
Z3	250	10	15	11
M1	150	18	20	14
M2		22	30	20
M3			40	23
PL1			50	34

Enriched rotifers, *Artemia* nauplii and *Artemia* umbrella were disinfected with 50 mg L^{-1} formaldehyde for 30 min before being fed to the larvae. Live and artificial food were alternated and administered manually in twelve daily feedings for the TAS and by an automatic feeder for the RAS. As a prophylactic treatment, the probiotic *Vibrio algynolyticus* (ILI strain) was added daily to the culture water at a concentration of 1.00E+10 colony forming units (CFU) mL^{-1} in order to get a final concentration of 1.00E+05 CFU mL^{-1} (Rodríguez *et al*., 2007) for the TAS and five times more the volume used for TAS was used for the RAS.

Larval performance

Survival of the larvae was estimated at every stage of development by taking five 125 mL samples from each culture tank and counting all live larvae. All sampled larvae were then returned to their respective culture tank. Survival (%) was calculated based on the number of surviving larvae at a particular stage compared to the total number of nauplii that was initially stocked.

Total length of the larvae (TL, mm) was measured from the eyestalk base until the end of the last abdominal segment by means of a profile projector. This measurement was done on samples of 30 larvae per replicate. Dry weight (DW, mg) was measured on five samples of 50 to 100 larvae at the N5, Z2, M2 and PL1 stage. The larvae were randomly removed from each system, washed briefly with distilled water, placed onto pre-weighed aluminium foil cups, dried in an oven at 60 °C for 24 h and then weighed. Total biomass (B, g) was determined at the end of the culture period, by harvesting all larvae and quantifying their weight.

The larval stage index (LSI), which is a measure for the rate of larval development was determined by daily microscopic observations according to the procedure described by Kanazawa *et al*. (1985).

Physico-chemical parameters

Daily measurements of pH, Total Ammonia Nitrogen (TAN), nitrite NO_2-N and nitrate NO_3-N (mg L^{-1}) were carried out on the culture water during the entire culture period. TAN and nitrite analyses were performed following the protocol described by Solórzano (1983), and nitrate concentrations by a modified protocol from Solórzano (1983). Total suspended solids (TSS) were determined following the method described by APHA, AWWA and WPCF (1992).

Microbiological analyses

Samples of N5, Z2, M2 and PL1 were collected from the TAS and RAS units for microbiological analysis. Larvae were rinsed with distilled water on a 60 µm sieve to remove all flocks and bacteria loosely attached to the external surface of the larvae. Samples were macerated with a stomacher blender and serial dilutions were prepared in sterilized seawater with 1 g of the macerated samples. Two sub-samples of 100 µL from the suspension were spread plated on marine agar 2216 (MA) (DIFCO ^{TM}, Sparks, MD, USA) and Thiosulphate Citrate Bile Sucrose Agar (TCBS) (DIFCO ^{TM}) for determination of total bacteria and TCBS counts, respectively. The plates were incubated at 30 °C and bacterial counts were performed after 24h. Enumeration of the *Vibrio alginolyticus* colonies was performed by colony blot using a monoclonal antibody against this bacterium (Donoso, 1996).

Larval condition evaluation

Challenge test

The physiological condition of the resulting postlarvae was tested through a challenge test. The PL were collected and placed in 2-L containers and acclimatized for 24 h. From both the TAS and RAS system, five replicates of 100 PL each were used. Infections were performed via immersion with the pathogenic bacteria 246 (*V. splendidus* I) CENAIM strain at a concentration of 1.00E+08 CFU mL^{-1}. Mortality was recorded after 24 h of exposure.

Performance during subsequent nursery culture under laboratory conditions

PL1 resulting from both systems were reared until PL25 following the standard feeding and management protocol of ESPOL - CENAIM. The PL were stocked at a density of 160 PL m^{-2} in 20 1000-L rectangular black fibreglass tanks (10 tanks for both the TAS and RAS system) filled with sand filtered and UV-treated seawater with a salinity of 35 g L^{-1}. All experimental units were continuously aerated maintaining dissolved oxygen levels above 5 mg L^{-1}. Water pH remained within the range 8.0 - 8.4. The experimental units were placed outdoors exposed to ambient temperature (~23°C). Water exchange was done daily at a rate of 30% day^{-1}. The PL were fed a commercial shrimp nursery diet containing 55% protein

(Molino 2®, Alimentsa, GYE, Ecuador). Feed was offered 3 times per day with feeding rates adjusted based on growth and on uneaten feed on the bottom of the culture tanks.

Economic and financial analysis

The capital and operational costs of the RAS were compared with those of the TAS and expressed per million larvae produced.

Statistical Analysis

Results from biological, physico-chemical and microbiological parameters, challenge test and performance in nursery culture were subjected to a one-way analysis of variance (ANOVA) followed by Tukey´s HSD multiple comparison test to identify differences among treatments. Normal probability plots and the Bartlett test for homogeneity of variances were used to verify the ANOVA assumptions. When necessary, data expressed in percentage were √arcsine transformed before submitting to ANOVA. All references to statistical significance were at the 5% level or lower. The statistical analysis was performed using STATISTICA 4.1 (Statsoft®, Tulsa, OK, USA).

Results

Larviculture experiment

Biological parameters

Larval survival were significantly higher ($p<0.05$) in the RAS than in the TAS in the first two zoeal stages, Z1 and Z2, and in M3. During the transition from mysis to postlarval stage, larvae reared in the RAS experienced a pronounced mortality (20%), resulting in similar ($p>0.05$) survival percentage at the end of the culture period: 71% and 74% for RAS and TAS, respectively (Figure 1).

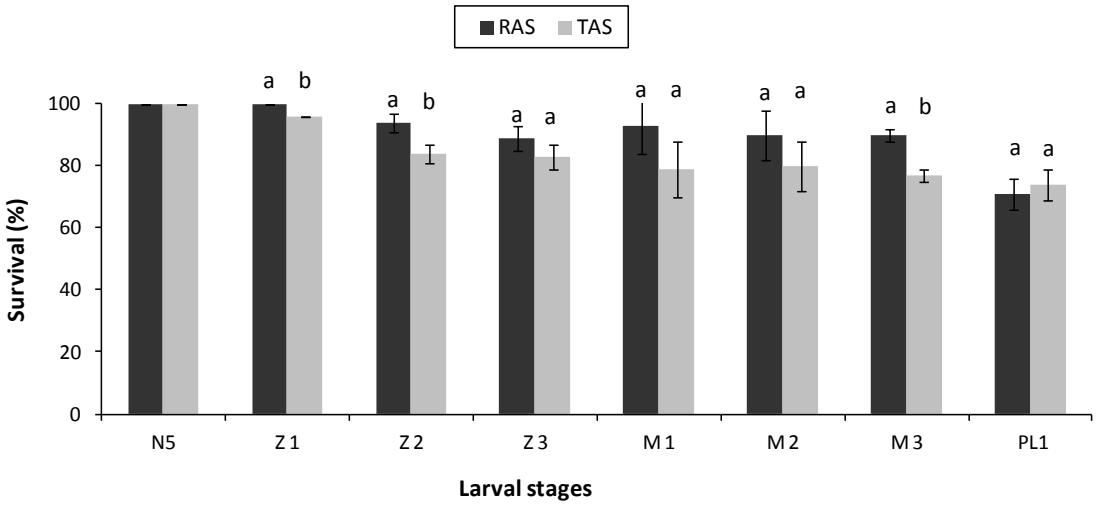

Figure 1. Mean survival (%) of *Litopenaeus vannamei* larvae reared in a traditional aquaculture system (TAS) at 100 N5 L^{-1} and a recirculating aquaculture system (RAS) at 1000 N5 L^{-1}. Standard deviation bars are shown. Means within a given larval stage sharing a common letter are not significantly different ($p>0.05$). Nauplii 5 (N5), zoea 1 (Z1), zoea 2 (Z2), zoea 3 (Z3), mysis 1 (M1), mysis 2 (M2), mysis 3 (M3) and postlarvae 1 (PL1).

Average length, dry weight, larval stage index (LSI) and biomass at the different larval stages are shown in Table 3. The values for length and dry weight were similar ($p>0.05$) in both culture systems during the whole culture period. In both systems the larvae reached the postlarval stage after 9 days of rearing. In the TAS, 93% of the larvae completed their metamorphosis to PL1, in the RAS 87%. LSI values were significantly higher ($p<0.05$) for larvae reared in RAS compared to TAS at Z2 stage; while the inverse was observed at the M2 and PL1 stages where LSI values were significantly higher for TAS compared to RAS (Table 3).

In the RAS the mean biomass was significantly higher ($p<0.05$) being 7.5- fold greater than in the TAS.

Table 3. Length, dry weight, larval stage index (LSI) and biomass of *Litopenaeus vannamei* larvae reared in a traditional aquaculture system (TAS) at 100 N5 $L^{-1}$and recirculating aquaculture system (RAS) at 1000 N5 L^{-1}. Means ± SD are presented

		Parameters			
Larval stages	Culture System	Length (mm)	Dry weight (mg larvae^{-1})	Larval Stage Index	Biomass (g)
N5	TAS	0.482 ± 0.01	0.016 ± 0.001		
	RAS	0.482 ± 0.01	0.016 ± 0.001		
Z2	TAS	1.33 ± 0.14^{a}	0.024 ± 0.013^{a}	1.83 ± 0.02^{b}	
	RAS	1.35 ± 0.08^{a}	0.023 ± 0.005^{a}	1.93 ± 0.01^{a}	
M2	TAS	2.71 ± 0.34^{a}	0.07 ± 0.02^{a}	5.26 ± 0.05^{a}	
	RAS	2.65 ± 0.28^{a}	0.09 ± 0.03^{a}	4.76 ± 0.02^{b}	
PL1	TAS	3.57 ± 0.42^{a}	0.13 ± 0.02^{a}	6.92 ± 0.03^{a}	55.67 ± 12.14^{b}
	RAS	3.23 ± 0.34^{a}	0.11 ± 0.01^{a}	6.82 ± 0.02^{a}	411.60 ± 12.00^{a}

Means within the same column within a given larval stage sharing a common superscript letter are not significantly different ($p>0.05$)

Physico-chemical parameters

Table 4 shows the range in physico-chemical parameters recorded in the RAS and the TAS at different larval stages. Dissolved oxygen decreased during the culture period but levels remained above 4 mg L^{-1}. In the RAS water temperatures increased significantly ($p>0.05$), reaching 34 °C, due to the effect of the greenhouse and the high ambient temperatures during the experiment (warm-rainy season). Therefore, heaters in the RAS were turned off from Z3 onwards. The pH ranged from 7.92 - 8.18 and 7.53 -7.78 for the TAS and the RAS, respectively. The pH was more stable and significantly lower (p>0.05) form Z3 onwards in the RAS than in the TAS, and was lower after recirculation was started and after each water renewal. TAN levels in the RAS were significantly lower ($p<0.05$) than in the TAS at Z3, while at M1 values of TAN were similar (p>0.05) for both culture systems. An increase in TAN concentrations was found in both systems at PL1, at which the level became significantly higher ($p<0.05$) in the RAS (Table 4). Significantly ($p<0.05$) lower values of NO_2-N were registered for the RAS at Z3 and M1 compared to TAS. However, the level of

nitrite increased at PL1 in the RAS, and became significantly higher ($p<0.05$) compared to the TAS, in which levels remained stable from M1 until the end of the experiment (Table 4). During the whole culture period the levels of nitrate NO_3-N were significantly higher ($p<0.05$) in the TAS than in the RAS, except at PL1 at which levels were similar ($p>0.05$) for both culture systems. Total Suspended Solids (TSS) increased during larval development in the TAS. In contrast, in the RAS a peak of 156.4 mg L^{-1}, which was significantly higher ($p>0.05$) than in the TAS, was reached at Z3, but then the level decreased again after recirculation was activated, achieving similar values ($p>0.05$) compared to the TAS at PL1 stage (Table 4).

Microbiological analyses

Bacterial counts performed on larvae at N5, Z2, M2 and PL1 stages are presented in Table 5. Similar TCBS and total bacteria counts were obtained in both culture systems at all larval stages except for M2, where TCBS counts were significantly lower in larvae reared in RAS.

Table 5. Bacterial counts (colonies forming units per g larvae, CFU g^{-1}) obtained from larvae sampled at different larval stages during the culture in a traditional aquaculture system (TAS) at 100 N5 L^{-1} and a recirculating aquaculture system (RAS) at 1000 N5 L^{-1}. Mean ± SD are presented.

Larval stages	Culture Systems	Marine Agar (CFU g^{-1})	TCBS (CFU g^{-1})
N5	TAS	5.10E+05	4.30E+04
	RAS	5.10E+05	4.30E+04
Z2	TAS	3.27E+06 ± 1.30E+06^{a}	2.27E+04 ± 2.25E+04^{a}
	RAS	2.80E+07 ± 1.93E+07^{a}	2.03E+05 ± 4.04E+04^{a}
M2	TAS	5.63E+06 ± 3.06E+06^{a}	2.33E+04 ± 2.52E+04^{a}
	RAS	1.05E+07 ± 3.44E+06^{a}	8.33E+03 ± 9.71E+03^{b}
PL1	TAS	2.97E+07 ± 4.16E+06^{a}	4.13E+05 ± 1.53E+05^{a}
	RAS	4.40E+07 ± 1.47E+07^{a}	7.07E+05 ± 2.25E+05^{a}

Means within the same column and within the same larval stage sharing a common superscript letter are not significantly different (p>0.05)

Table 4. The physico-chemical parameters measured in the recirculating aquaculture system (RAS) at 1000 N5 L^{-1} and the traditional aquaculture system (TAS) at 100 N5 L^{-1} at different larval stages. Means ± SD are presented

		Physico-chemical parameters						
Larval stages	Culture Systems	pH	Temperature (° C)	DO (mg L^{-1})	TAN (mg L^{-1})	NO_2-N (mg L^{-1})	NO_3-N (mg L^{-1})	TSS (mg L^{-1})
Before water renewal and water recirculation								
Z1	TAS	7.92 ± 0.03^{a}	31.7 ± 0.2	5.03 ± 0.54^{a}	0.02 ± 0.02	0.009 ± 0.002	0.651 ± 0.020^{a}	40.7 ± 4.1^{a}
	RAS	7.78 ± 0.34^{a}	32.2 ± 0.1	5.47 ± 0.04^{a}	0.0	0.0	0.112 ± 0.010^{b}	66.3 ± 26.1^{a}
Z3	TAS	8.18 ± 0.04^{a}	32.0 ± 0.6	4.57 ± 0.31^{a}	0.36 ± 0.03^{a}	0.007 ± 0.001^{a}	2.462 ± 0.171^{a}	76.0 ± 10.0^{b}
	RAS	7.75 ± 0.19^{b}	32.6 ± 0.1	4.53 ± 0.06^{a}	0.15 ± 0.03^{b}	0.001 ± 0.002^{b}	0.150 ± 0.045^{b}	156.4 ± 47.0^{a}
During water renewal and water recirculation								
M1	TAS	8.08 ± 0.01^{a}	32.1 ± 0.2	4.44 ± 0.02^{a}	0.89 ± 0.04^{a}	0.013 ± 0.001^{a}	2.393 ± 0.061^{a}	nd
	RAS	7.62 ± 0.10^{b}	34.2 ± 0.1	4.07 ± 0.12^{b}	0.62 ± 0.33^{a}	0.009 ± 0.001^{b}	1.364 ± 0.024^{b}	nd
PL1	TAS	8.09 ± 0.03^{a}	32.0 ± 0.1	4.78 ± 0.14^{a}	2.23 ± 0.22^{b}	0.010 ± 0.006^{b}	2.297 ± 0.028^{a}	131.6 ± 23.3^{a}
	RAS	7.53 ± 0.06^{b}	33.3 ± 0.2	4.45 ± 0.19^{a}	3.39 ± 0.39^{a}	0.191 ± 0.054^{a}	1.950 ± 0.628^{b}	137.5 ± 19.2^{a}

nd= not determined
DO = Dissolved oxygen
TAN = Total Ammonia Nitrogen
TSS = Total Suspended Solids

Means within the same column within a given larval stage sharing a common superscript letter are not significantly different ($p>0.05$)

In the RAS, the percentage of the probiotic bacteria *Vibrio algynolyticus* (ILI strain) in the vibrio count was highest at Z2 (85%), with a gradual reduction through the larval developmental stages (60% at M2 and 42% at PL1). An opposite trend was observed for the larvae reared in the TAS with 34%, 57% and 66% at Z2, M2 and PL1, respectively (Figure 2).

Larval condition evaluation

Challenge test

No significant difference in mortality was observed between larvae reared at low density in the TAS and at high density in the RAS when exposed to the bacterial strain 264. Mortality of larvae in the RAS was 18 ± 7% and in the TAS 6 ± 9%.

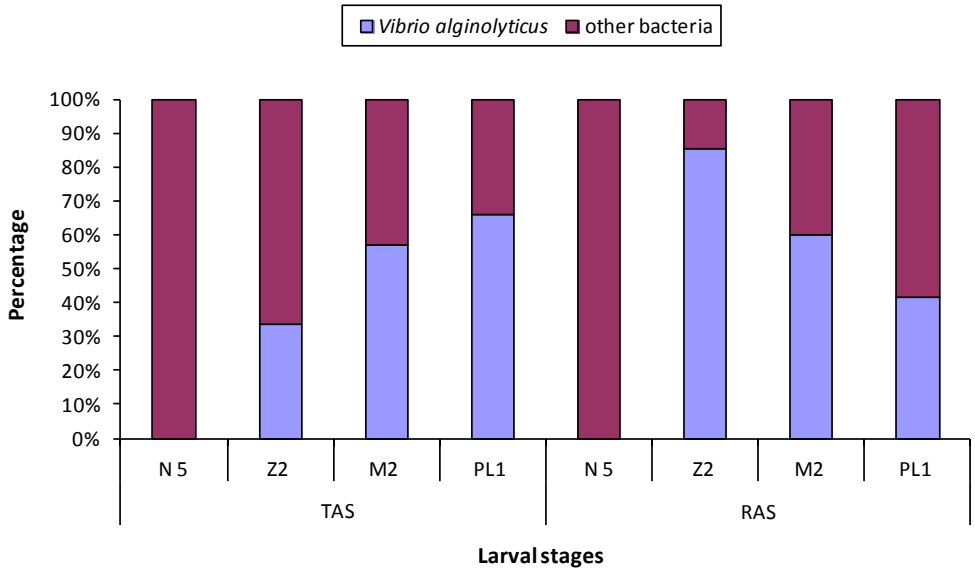

Figure 2. Ratio (%) of *Vibrio algynolyticus* (ILI strain) and other bacteria present in larvae reared at 100 N5 L^{-1} in the traditional culture system (TAS) and at 1000 N5 L^{-1} in the recirculating aquaculture system (RAS) at different larval stages (N5= Nauplii 5, Z2= Zoea 2 sub-stage, M2= Mysis 2 sub-stage and PL1= postlarvae 1).

Performance during subsequent nursery culture under laboratory conditions

Performance of larvae originating from the RAS en the TAS during the 28-days nursery culture under laboratory conditions are presented in table 6. The survival were

equally high (*p>0.05*) for both PL sources (89%). No significant differences were observed in dry weight, specific growth rate (SGR), length and biomass between PLs originating from the two larviculture systems.

Economical and financial analyses

Table 7 presents an overview of the total capital investment required to run a TAS and a RAS with a production of 1 million *L. vannamei* larvae under laboratory conditions. For the TAS the total capital cost is around US$ 23 000, while for the RAS a lower capital investment of US$ 13 000 is required. For both culture systems investment on fixed assets is the highest cost (77% of total capital cost for TAS and 61% for RAS), while 23% and 39% of total capital cost is spent on general and scientific equipment for the TAS and RAS respectively.

Table 6. Survival, dry weight, length and biomass of *Litopenaeus vannamei* after a 28-days nursery culture under laboratory conditions of postlarvae originating from a traditional aquaculture system (TAS) at at 100 N5 L^{-1} and a recirculating aquaculture system (RAS) are presented

	Parameters				
Culture Systems	Survival (%)	Dry weight (mg larvae^{-1})	Length (mm)	Specific growth rate (SGR)	Biomass (g)
TAS	89 ± 7^{a}	0.026 ± 0.001^{a}	18.46 ± 2.52^{a}	10.32 ± 0.34^{a}	18.81 ± 2.83^{a}
RAS	89 ± 3^{a}	0.024 ± 0.001^{a}	19.03 ± 2.11^{a}	10.03 ± 0.63^{a}	17.19 ± 3.24^{a}

*Specific growth rate (SGR) = $[(\ln W_1 - \ln W_0)/t] \times 100$, where W_0 and W_1 are the mean dry weight (mg) at the start and at the end of the experiment and t denotes total rearing period in days.
Means in the same columm sharing a common superscript are not significantly different (*p>0.05*)

The total running cost per production cycle of 1 million *L. vannamei* larvae in a TAS is around US$ 2000 with 31% of total cost for labour, 17% for depreciation of investment, 12% for food, 11% for energy and 8% for nauplii (Figure 3). The total running cost for RAS is 30% lower than for TAS. Labour still represents the main share in the running cost, but is lower than in TAS (Figure 3 and Table 8).

Table 7. Estimated capital investment for a traditional aquaculture system (TAS) at 100 N5 L^{-1} and a recirculating aquaculture system (RAS) at 1000 N5 L^{-1} with a production capacity of 1 million *Litopenaeus vannamei* larvae under laboratory conditions

Facility	Description	TAS				RAS			
		Quantity	Total cost (U.S. Dollars)	Depreciation (%)	Annual cost (U.S. Dollars)	Quantity	Total cost (U.S. Dollars)	Depreciation (%)	Annual cost (U.S. Dollars)
A. Buildings	Concrete base, lab. floor, channels, building	55 m^2	3.300,00	10	330,00	6 m^2	120,00	10	12,00
	Greenhouse building					m^2	650,00	33	214,50
Sub-total A			**3.300,00**		**330,00**		**770,00**		**226,50**
B.Systems equipments	Blower	1	793,00	20	158,60	1	793,00	20	158,60
	Gas Heater	1	574,90	20	114,98	1	574,90	20	114,98
	Pump for gas heater	1	229,30	20	45,86	1	229,30	20	45,86
	Pump for temperature maintainace	1	229,30	20	45,86				
	Pump					1	334,80	20	66,96
	UV Sterilizer					1	168,90	20	33,78
	Automatic feeder					1	2.274,00	20	454,80
Sub-total B			**1.826,50**		**365,30**		**4.374,90**		**874,98**
C. Tanks	500 l round-shaped transparent polyethylene tanks	20	10.000,00	20	2.000,00				
	1200 l cylinder-conical tank					1	450,00		90,00
	1l plastic cones					5	75,00		15,00
Sub-total C			**10.000,00**		**2.000,00**		**525,00**		**105,00**
D. Filtration	Mechanical filter					1	320,00	20	64,00
	Cartridge filter					1	51,00	50	25,50
	100 l biofilter					1	790,00	20	158,00
	Macrolite (substrate biofilter)					100 l	200,00	10	20,00
	Filter frame	20	20,00	20	4,00	1	20,00	20	4,00
Sub-total D			**20,00**		**4,00**		**1.381,00**		**271,50**
E. General and scientific equipment	Microscope	1	1.350,00	10	135,00	1	1.350,00	10	135,00
	Stereo-microscope	1	2.240,00	10	224,00	1	2.240,00	10	224,00
	Haemocytometer	1	54,00	50	27,00	1	54,00	50	27,00
	Portable pH meter	1	171,00	20	34,20	1	171,00	20	34,20
	Portable O_2 meter	1	655,00	20	131,00	1	655,00	20	131,00
	Refrigerator	1	600,00	20	120,00	1	600,00	20	120,00
	Laboratory balance	1	300,00	20	60,00	1	300,00	20	60,00
Sub-total E			**5.370,00**		**731,20**		**5.370,00**		**731,20**
F. Tubing	PVC tubing	150 m	800,00	20	160,00	1	235,00	20	47,00
	Flexible black tube	25 m	200,00	20	40,00	1.25 m	10,00	20	2,00
Sub-total F			**1.000,00**		**200,00**		**245,00**		**49,00**
G. Services installations	Freshwater	1	95,00	20	19,00	1	95,00	20	19,00
	Pipework seawater	1	325,00	20	65,00	1	150,00	20	30,00
	Pipework air	1	358,00	20	71,60	1	112,00	20	22,40
	Electrical	1	1.568,00	20	313,60	1	852,00	20	170,40
Sub-total G			**2.346,00**		**469,20**		**1.209,00**		**241,80**
Total capital and annual depreciation costs			**23.862,50**		**4.099,70**		**13.874,90**		**2.499,98**

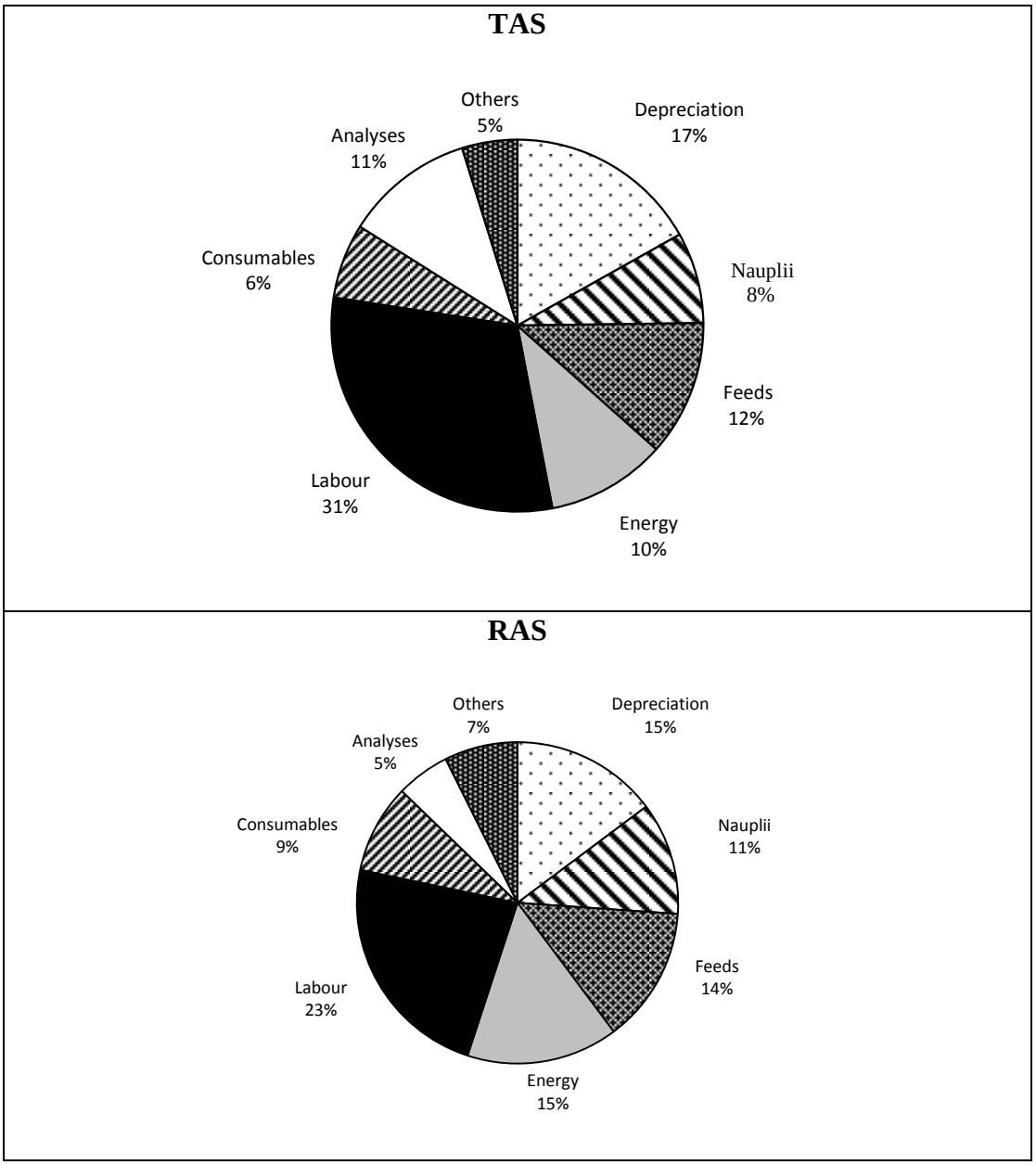

Figure 3. Break down of estimated production costs for the production of 1 million postlarvae in a traditional culture system (TAS) at 100 N5 L^{-1} and in a recirculating culture system (RAS) at 1000 N5 L^{-1}

Table 8. Estimated operation cost to produce 1 million *Litopenaeus vannamei* larvae under laboratory conditions in a traditional aquaculture system (TAS) at 100 N5 L^{-1} and a recirculating aquaculture system (RAS) at 1000 N5 L^{-1}

Item	Description	TAS		RAS	
		Quantity	Total cost (U.S. Dollars)	Quantity	Total cost (U.S.Dollars)
A. Energy	air supply/blower, water supply/pumps,				
Electricity	equipments: microscope, refrigerator,	1240 kw	194,68	1356 kw	212,90
Gas	water heating	8 gas charge	16,00	1 gas charge	2,00
Sub-total A			**210,68**		**214,90**
B. Consumables	Cleaning material	6	6,84	6	6,84
	Netting	3	9,00	1	3,00
	Miscellaneous material	5	50,00	5	50,00
	Water quality tests		66,00	3	66,00
Sub-total B			**131,84**		**125,84**
C. Chemicals	Argentine, Sodium Hipoclorite, Chlorhidric acid, Neutral soap, Alcohol		12,42		18,92
Sub-total C			**12,42**		**18,92**
D. Animals and probiotics	Nauplii (milliar)	1000	150,00	1000	150,00
	Probiotic (l)	1	10,50	0,900	9,45
Sub-total D			**160,50**		**159,45**
E. Feed	*Artificial feed*				
	Frippak (g)	57,68	6,68	86,30	9,69
	Lanzy (g)	47,18	2,35		
	Epifeed (ml)	143	10,01		
	Live food				
	Microalgae MT	7	42,00		
	Microalgae concentrate MT			4,8	72,00
	Rotifers (million)	973	58,38		
	Artemia cysts for nauplii	2.724,00	120,00	1.952,20	86,00
	Artemia cysts for umbrella			454	25,00
Sub-total E			**239,42**		**192,69**
F. Labour	Technician (hours per production cycle)	68	276,25	40	162,50
	Operators	248	341,00	120	165,00
Sub-total F			**617,25**		**327,50**
G.Analyses	Microbiological	12	120,00	4	40,00
	Water quality (Total suspended solids)	12	108,00	4	36,00
Sub-total G			**228,00**		**76,00**
H. Miscellaneous varia			85,00		85,00
Sub-total G			**85,00**		**85,00**
I. Depreciation per production cycle			341,64		208,33
Sub-total G			**341,64**		**208,33**
Total running costs			**2.026,75**		**1.408,63**

Discussion

Several studies have shown that in shrimp culture, increasing stocking density resulted in higher mortality and lower feeding efficiency (Martin *et al*., 1998; Ray and Chien, 1992; Tseng *et al*., 1998). An explanation by Ray and Chien (1992) is that excretory products, faeces and unconsumed food accumulate in the culture water causing toxic effects on the shrimp. In high density rearing of *P. monodon* in a RAS, the animals experienced stress leading to lower survival and growth (Tseng *et al*., 1998). These authors suggested that higher stocking densities could increase competition for food and space and might account for the observed effects. A mechanism that is suggested in several studies is that crowding may create a hostile environment through strong competition for resources, chemical interactions, mechanical / physical interferences and even cannibalism. On the other hand, Nga *et al*. (2005) concluded that physical interference, stress and cannibalism are not causal factors for the negative impact of crowding on *P. monodon* postlarvae survival and growth, but that ammonium toxicity is. They also suggested that other crowding chemicals such as alarm pheromones could be causal factors affecting survival and growth, as was detected for other crustaceans (Hazlett, 1994; Hazlett and McIay, 2000).

Effect of rearing system on water quality

In our study, ammonia toxicity at PL1 stage could not be excluded since ammonia levels in both culture systems were higher than the 1.32 mg L^{-1} TAN recommended for rearing *L. vannamei* PL1 (Cobo *et al., in press*). However, TAN levels in both culture systems were far below median lethal concentrations (24h-LC_{50}) reported for different penaeid species. The LC_{50} for TAN for *P. monodon* nauplii, zoea, mysis and postlarva (PL6) stages are 6.00, 8.48, 24.04 and 52.11 mg L^{-1}, respectively. Ostrensky and Wasielesky (1995) reported values of LC_{50} for *F. paulensis* nauplii, zoea, mysis and PL1 of 102.30, 22.93, 74.87 and 24.19, respectively. For *L. vannamei*, TAN LC_{50} of 10.03, 17.93 and 13.2 mg L^{-1} were recorded for zoea, mysis and PL1 stages (Cobo *et al*., *in press*).

Nitrite concentrations were within safe levels. The 'safe level' for rearing *L. vannamei* juveniles was estimated to be 25.7 mg L^{-1} for nitrite (NO_2-N) in 35 g L^{-1} salinity (Lin and Chen, 2003). For post-larval stages, Ostrensky and Poersch (1992) and Chen and Tu (1990) reported 24h- LC_{50} nitrite values of 277.8 and 83.4 mg L^{-1} NO_2-N for *F. paulensis* PL1 and *M. japonicus* PL2 respectively. Chen and Chin (1988) determined that the 24 h LC_{50} for PL20 *P. monodon* was 61.9 mg L^{-1} NO_2-N. At the end of our experiment, NO_2-N was much lower (0.191 ± 0.054, Table 4) than the values reported in these studies. During the entire culture period, NO_3-N concentrations were significantly higher in the TAS than in the RAS. The higher nitrate values in the TAS could be attributed to residual nitrate salts from the culture media of the algae, which were used to feed the larvae.

Logically, the TSS values were higher in the RAS until the recirculation was started, then reaching similar values as in the TAS (table 2). TSS values in both systems were however above the limit of 80 mg L^{-1} recommended for aquaculture activities by Timmons *et al*. (2002).

The pH was generally stable, but lower in the RAS than in the TAS. During nitrification, bacteria within the bio-filter of the RAS produce carbon dioxide (CO_2), which could explain the lower pH values measured in the RAS (Kim *et al*., 2000).

Until larval stage M3, survival was higher in the RAS. However, a rather sudden and abrupt mortality (20%) occurred during the transition from M3 to PL1. This mortality could partly be explained by the high temperatures (34 °C) experienced in the RAS, being above the optimum (28 - 32°C) suggested by Van Wyk *et al*. (1999). Temperature is one of the most important abiotic factors affecting the growth and survival of aquatic organisms (Van Wyk *et al*., 1999), particularly in penaeids (Parado-Estepa, 1998) that during larval development do not withstand major environmental changes. However, it is known that larvae of *P. monodon* are grown successfully at extremely high temperatures (32 - 34°C) in commercial hatcheries in Southeast Asia (Chen, 1990; Fegan, 1992). Most of the *L. vannamei* hatcheries in Ecuador use water temperatures up to 32 °C as they affirm that high temperatures increase moulting frequency.

Temperature has long been recognized as a factor affecting ammonia excretion of marine invertebrates. An increase in temperature results in progressive increases in ammonia-N excretion as was documented for *F. chinensis* between 15 and 25°C (Chen and Nan, 1993) and *M. japonicus* between 15 and 35°C (Chen and Lai, 1993). Alcaraz *et al.* (1997) observed that the increase of water temperature and the subsequent rise of the metabolic rate of *L. setiferus* post-larvae further increased ammonia and nitrite accumulation by the organisms. In our study, an increase in TAN values was registered with the increase of water temperature from 32°C to 34°C of 3.39 mg L^{-1}. This value exceeds the recommended level for TAN of 1.32 mg L^{-1} for rearing *L. vannamei* PL1 and could partly explain the mortality of larvae in the transition of M3 to PL1. Lin *et al.* (1993) observed that susceptibility to ammonia increased during and just after moult in larvae, postlarvae and juveniles of the shrimp *P. japonicus*. Ostrensky and Wasielesky (1995) considered postlarvae of *P. paulensis* as a transitional stage in terms of sensitivity to ammonia. It was also observed that the PL1 stage was more sensitive to ammonia than the mysis stage of *L. vannamei* (Cobo *et al.*, *in press*). This may also partly explain the sudden mortality of the larvae in our experiment when moulting from M3 to PL1.

Effect of stocking density, rearing system and feeding on larval performance and system productivity

Shrimp culture at high stocking density using RAS has mainly been documented for postlarval and juvenile stages, while studies referring to the use of RAS in shrimp larval rearing using high stocking densities are rather scarce. Recirculating systems are considered to be highly productive systems. This high productivity can be measured in terms of larval and/or biomass output per unit of area, volume or investment (Rosenthal and Black, 1993). In our study, implementing a RAS using umbrella-stage *Artemia* as a food source to produce *L. vannamei* resulted in comparable survival in the RAS (71%) and TAS (74%). However, the stocking density in the RAS was 10 times higher, yielding an average output of 710 larvae L^{-1} compared to only 74 larvae L^{-1} in the TAS. Gandy (2004) evaluated a RAS for the production of *F. aztecus* postlarvae using stocking densities of 100 N L^{-1} with survival of 64.8% giving a larval output of 64 larvae L^{-1}. Ghanekar (2005) reported preliminary results with a small-scale RAS for *P.*

monodon larviculture using a stocking rate of 300 N L^{-1}, with a survival to PL4 of 66% and a larval output of 198 larvae L^{-1}. Thach *et al*. (2005) also applied a RAS for commercial-scale *P. monodon* larviculture with stocking densities of 250 N L^{-1}, an average survival of 60% and a larval output of 150 larvae L^{-1}. Truong *et al.* (2009) evaluated six stocking densities from 150 up to 900 nauplii L^{-1} for *P. monodon* in an improved RAS composed of a biofilter, protein skimmer and ozone generator at laboratory scale. They found the best performance at a density of 450 N L^{-1} with a survival of 56% and a yield of 252 larvae L^{-1}.

In the present study, dry weight and length of larvae reared in the RAS using high stocking density (1000 N5 L^{-1}) were similar to those of larvae reared in TAS at normal density (100 N5 L^{-1}). These results were also similar to the ones reported in literature by Martins *et al*. (2006), Al Azad *et al*. (2002), D´Abramo *et al*. (2006) in TAS for *F. paulensis* (60 N L^{-1}), *P. monodon* (100-200 N L^{-1}) and *L. vannamei* (100-150 N L^{-1}), respectively. However, a total biomass of 0.410 kg m^{-3} was obtained in the RAS compared to 0.056 kg m^{-3} in the TAS.

The increment in biomass could also be attributed to the introduction of the umbrella-stage *Artemia* in the RAS larval feeding regime. Léger *et al*. (1986) and Naessens *et al*. (1995) suggested including an alternative prey to fill up the size gap between algae and freshly hatched *Artemia* nauplii in order to improve larval development and growth. Umbrella-stage *Artemia* are easy to produce. Also in a TAS, feeding frozen umbrella-stage *Artemia* proved to improved biomass production of *L. vannamei* larvae compared to frozen *Artemia* nauplii (Chapter 7). The results of this experiment indicate that when using umbrella-stage *Artemia*, *L. vannamei* larvae can also be raised at high density in a RAS with comparable performance as in TAS.

Effect of stocking density and rearing system on larval condition

In general, bacterial numbers in the RAS were higher than in the TAS. This difference was however not that dramatic considering the huge difference in stocking densities used. Suantika (2001) demonstrated that the use of a recirculating system in rotifer production resulted in a 1 to 2 log units lower bacterial number than in a batch

rotifer culture system. In our study this advantage could not be demonstrated. This could be due to the use of the probiotic strain *Vibrio alginolyticus* as a prophylactic procedure during the culture, contributing to an increase in bacteria load. Another reason could be the use of different food items: Suantika (2001) used artificial diets, while in our study a combination of live food and artificial diets were used for both culture systems. Vandenberghe *et al*. (1998) indicated that the flora associated with shrimp larvae is not very stable and it is influenced by the bacterial flora of the administered food and by the environment. Lavens and Sorgeloos (2000) also indicated that live food is an important source for microbial contamination *e.g. Artemia* nauplii may have a bacterial load up to 10^{10} CFU g^{-1}, mostly consisting of Vibrio's. The microbial load brought in through the large quantities of live food (microalgae, rotifers, *Artemia* nauplii and umbrella) used to feed the high density of larvae, may therefore explain the higher bacterial counts in the RAS.

Vibrio spp. are one of the most important causative agents of bacterial infections and diseases in the culture of penaeid shrimp (Lightner, 1993). On the other hand, some *Vibrio's* have been isolated from healthy penaeid shrimp. Vandenberghe et *al.* (1998) observed that the bacterial flora of successful *F. chinensis* larvae production was mainly dominated by *V. alginolyticus.* In Ecuador, a *V. alginolyticus* strain with antagonistic properties towards the pathogen *V. harveyi* has been used both at experimental scale (San Miguel, 1996; Zherdmant *et al*., 1997) and in commercial hatcheries (ESPOL-CENAIM's larval rearing protocol; Garriquez and Arevalo, 1995). In our study, we could demonstrate the establishment of *V. alginolyticus* in the larvae of the two culture systems. The percentage of *V. alginolyticus* in the RAS was high at Z2, then decreased when the recirculation started, indicating this strain had difficulties to establish itself under the RAS conditions, while in contrast its relative share increased in TAS during the water exchange phase (Figure 2).

The usefulness of *in vivo* infection or "challenge test" has been demonstrated in the evaluation of virulence of *Vibrio* isolates associated with mortalities (Saulnier *et al.*, 2000; Chythanya *et al.,* 2002), to test prophylactic and curative treatments (Alabi *et al.*, 1999) and in studies of host-factors influencing the expression of bacterial virulence (Roque *et al.*, 1998). In our study we used a challenge test to evaluate the physiological

condition (quality) of the *L. vannamei* larvae reared in the TAS and RAS. The results of our challenge test are similar to the ones of Pico (2004) who obtained mortality of 20.4% in *L. vannamei* PL3 when bath challenged with the same 246 strain. An important result of this study is that no significant ($p>0.05$) differences in larval quality were found between the larvae reared at high densities in the RAS and those cultured a low density in a traditional system.

During the subsequent nursery phase, the performance of the larvae from the RAS did not differ from those cultured in the TAS (Table 4). Higher survival (89%) was obtained in our experiment compared to the 73% and 63% that Rodríguez *et al.* (2011) reported in an experiment testing the application of β-1,3/1,6- glucans as a health management strategy during nursery rearing (PL2-PL25) under controlled conditions.

Cost Analysis

A culture system can only be successful if it is cost-effective. With the specific conditions used in this study, the use of the RAS clearly had advantages compared to the TAS from an economical point of view. For the same production output, total investment costs are considerably reduced in the RAS compared to the TAS (Table 7 and 8), the cost of the TAS being more than 70% higher than of the RAS. The major expenses in the TAS are attributed to buildings and tanks, while for RAS the major expense rely on system equipment (Table 7). As far as running costs are concerned, per production cycle the use of the RAS proved more than 40% cheaper than the TAS. Costs for feed, energy, nauplii, consumables and others were similar in both culture systems, but the RAS is more cost-effective as far as labour, depreciation of investment and analysis costs are concerned, owing to the high larval density that can be achieved in this system.

Wickins and Lee (2002) pointed out labour as the greatest component of production costs for a shrimp hatchery accounting to 33% of costs. In our study, a similar value was observed for labour in the TAS being 31%, while labour can be reduced to 23% by rearing the larvae at high density in the RAS (Figure 3). Also, Suantika *et al.* (2003) observed that when using a RAS for rotifer production, saving

was mainly made by a reduction on labour from 14% in the batch culture system to 7% in the RAS. In percentage, energy accounts for 15% in the RAS compared to 10% in the TAS, due to the daily functioning of the water recirculation equipment. Nevertheless, production costs of larvae under laboratory conditions (expressed in US$ per 1000) is considerably lower in the RAS compared to the TAS: $ 2.00 and $ 2.90, respectively.

In conclusion, it can be stated that the intensive production of *L. vannamei* larvae in a RAS has shown its effectiveness in controlling water quality and maximizing biomass production. The results from the challenge test and nursery culture give strong evidence that larval quality is not affected, being similar to that in traditional systems. Moreover, the RAS is more cost-effective. Therefore, the present study indicates that the culture system and feeding practices applied here hold promise in increasing production of good quality larvae, reducing operating costs, and increasing biosecurity.

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CHAPTER VIII

GENERAL DISCUSSION, CONCLUSIONS AND FURTHER RESEARCH

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General Discussion

Aquaculture contribution to world supplies of crustaceans has grown rapidly in the last decade. For 2010, marine crustacean production accounted for as much as 70.6% of the world supply. The production of marine species is dominated by the white leg shrimp *Litopenaeus vannamei* (FAO, 2012). As the demand for shrimp is expected to increase, efforts to increase the production of the shrimp industry are also driven towards more intensive practices. Shrimp farming will depend largely on the production of more and good quality postlarvae, at a more cost-effective level. It has been identified that in traditional larviculture systems, losses are mainly due to the occurrence of diseases, water quality aspects and environmental issues. Therefore, the long-term viability of shrimp aquaculture depends on appropriate technologies that are designed to maintain good water quality, reduce the introduction and spread of pathogens and mitigate negative environmental impacts. Recirculating aquaculture systems (RAS) appear as a promising approach to overcome constraints found in traditional aquaculture systems. Several advantages have been attributed to recirculating technology, such as, efficient removal of suspended solids and ammonia controlling water quality, offering a stable environment for the reared organisms which leads to a higher productivity and good animal quality. Also, due to limiting water exchange, it is possible to avoid the entrance of disease causing pathogens, while being environmental friendly with little water discharge.

Different from conventional nursery and grow-out procedures, larviculture is generally carried out under controlled conditions and requires specific culture techniques especially with respect to husbandry techniques, feeding strategies, and microbial control. The main reasons for this are that larvae are usually very small, extremely fragile and generally not fully developed physiologically (Lavens and Sorgeloos, 1996). In shrimp, these are not the only limitations as the developing larvae also have to pass

through different larval stages, changing feeding behavior from a herbivorous filter-feeding to a carnivorous hunting behavior and finally becoming omnivorous. The aim of this study was to evaluate the feasibility to develop culture techniques for high density rearing of *L. vannamei* larvae using a RAS.

Intensive Shrimp Larviculture

Intensive and super-intensive aquaculture is a relatively new field, especially for shrimp culture. There are different approaches to achieve intensification. One method involves the use of high or super high density recirculating type culture systems. High density RAS have been tested since years especially for fishes such as red drum *Sciaenops ocellatus* (Arnold *et al*., 1990), catfish *Clarius gariepinus* (Bovendeur *et al*., 1987), tilapia *Oreochromis mossambicus* (Provenzano and Winfield, 1987) and salmon *Salmo salar* (Williams *et al.*, 1982; Sutterlin *et al*., 1984; Poston and Williams, 1988). But, also for prawns and shrimps, for example freshwater prawns *Macrobrachium rosenbergii* (Sandifer and Smith, 1975), penaeid shrimp *F. aztecus* (Mock *et al.*, 1973, 1977; Neal and Mock, 1976) and *Penaeus californiensis* (Salser *et al*., 1978), being predominantly at juvenile and adult stages. Relatively few studies have been performed on shrimp larvae rearing in RAS, *e.g*. *F. aztecus* (Gandy, 2004) and *P. monodon* (Ghanekar, 2005; Thach *et al*., 2005; Truong *et al.*, 2009). And, most of these studies did not use high density cultures. To our knowledge, our study is the first one to explore high or super high stocking densities up to 2000 larvae L^{-1} of *L. vannamei* using a RAS.

The different steps to achieve the objective of this study and their results were presented in the previous chapters and are discussed successively.

Husbandry Techniques

Water quality parameters: Ammonia tolerance of *L. vannamei* larvae

One of the most important limiting factors in intensive culture systems is the build-up of nitrogenous waste, such as ammonia and nitrite, resulting from shrimp excretory products, faeces and unconsumed food items which accumulate in culture

water causing toxic effects on shrimp. The knowledge of tolerance limits of a species regarding water quality and the maintenance of water quality are indispensable requirements in any rearing system. It is even more so for intensive systems and more specifically also for the efficiency of the biofilter within RAS. In an intensive culture system, ammonia and nitrite increase exponentially over time. High concentrations of ammonia may result in retardation of shrimp growth, and in extreme cases can cause mortality (Zin and Chu, 1991). *L. vannamei* is currently the main penaeid shrimp species produced through aquaculture. Although several studies have been carried out to determine toxicity levels of ammonia in different life stages of *L.vannamei*, no studies for larval stages have been reported. Prior to evaluating the feasibility to produce *L. vannamei* larvae intensively in a RAS, we determined the ammonia tolerance of *L. vannamei* larvae at the different developmental stages and sub-stages (**Chapter III**). Results of short-term experiments conducted by several authors revealed that there is a progressive increase in ammonia tolerance during the larval stages of several species of penaeids (Jayasankar and Muthu, 1938; Chin and Chen, 1987; Chen and Lin, 1991). However a comparison of "safe levels" between penaeid species is difficult as different methodologies, test conditions, time exposures and larval stages have been used among related studies. Our results from 24 h short-term toxicity tests of *L.vannamei* larvae exposed to increasing concentrations of Total Ammonia Nitrogen (TAN) at the different larval stages showed a progressive tolerance to ammonia with larval development until mysis stage. The mysis stages were considerably more resistant to ammonia than zoea and PL1 stages. A similar trend in tolerance to ammonia was observed for *Farfantepenaeus paulensis* by Ostrensky and Wasielsky (1995). A pronounced decrease in ammonia tolerance was observed at PL1 stage. This was also observed by Ostrensky and Wasielsky (1995) and could be attributed to the final and most important metamorphosis that occurs in this stage before becoming a juvenile shrimp. The 24h LC_{50} (Median Lethal Concentration) values were high, suggesting a relatively high resistance of *L. vannamei* larvae to ammonia. The data generated in our study are thought to contribute to the knowledge of tolerance limits of *L. vannamei* larvae and are

valuable for water quality management in traditional as well as in intensive culture systems.

Water quality parameters: effect of culture sytems

In our high density rearing experiments (**Chapter IV**), ammonia and nitrite levels increased exponentially over time and exceeded the recommended levels of 0.42 mg L^{-1} TAN during the static culture phase, due to the high stocking density (Cobo *et al.,* in press). Implementing a continuous feeding strategy and increasing the quantity of microalgae being fed, allowed to reduce the values of total ammonia nitrogen (TAN) and nitrite by 27% and 63% for the highest stocking densities. This reduction could be explained by the capacity of the microalgae to absorb several nitrogenous compounds (Conway and Harrison, 1977; Paslow *et al*., 1985; Suttle and Harrison, 1988). During the recirculating culture phase, ammonia and nitrite levels were lower, indicating that the recirculation units allowed to maintain the water quality parameters within recommended levels. It has to be considered that in this study water quality measurements were not recorded for every single tank and treatment, but that nitrite and nitrate levels were measured of both the influent and effluent of the biofilters to merely verify the performance of the experimental recirculating units. Final values of ammonia, nitrate, total suspended solids were lower in the RAS than those measured in the TAS (**Chapter V)**. Nitrite on the other hand was higher in the RAS than in the TAS, showing the building-up of nitrite during the second step of the process of nitrification. Normally, the two steps of the reaction are carried out one after the other. However, the rate of reaction of the first step (ammonia oxidizing to nitrite) is higher than the second step (nitrite oxidizing to nitrate) and therefore an increase of the nitrite was observed.

Water quality conditions differed in the trials with umbrella *Artemia* (**Chapter VII)**. In those experiments both TAN and nitrite levels were higher in the RAS than in the TAS. The high TAN levels could be due to an increase in temperature in these experiments. Temperature has long been recognized as a factor affecting ammonia excretion by marine invertebrates. An increase in temperature resulted in progressive increases in ammonia-N excretion as was documented for *F. chinensis* between 15 and

25 °C (Chen and Nan, 1993) and *M. japonicus* between 15 and 35 °C (Chen and Nan, 1993). Alcaraz *et al*. (1997) observed that the increase of water temperature and the subsequent rise of the metabolic rate of *L. setiferus* postlarvae further increased ammonia and nitrite accumulation by the organisms. In our study, an increase in TAN values was registered with the increase of water temperature from 32 °C to 34 °C. In that way, the sudden mortality occurring at PL1 stage in that experiment could partly be explained by the high susceptibility to the increased ammonia level during and just after moulting to PL stage. Ostrensky and Wasielesky (1995) considered postlarvae as a transitional stage in terms of sensitivity to ammonia. Similarly our own experiments demonstrated that the PL1 stage was more sensitive to ammonia than the mysis stage of *L. vannamei* (Cobo *et al*., *in press*).

In general, the 1 m³ experimental recirculating units showed their effectiveness in controlling water quality parameters, allowing the intensive production of *L. vannamei* larvae.

Stocking density and water recirculation rate

Operating an intensive culture system with reliable output is a major challenge. Among others, production success depends on parameters such as shrimp stocking density, water quality and feed management. Too high or too low stocking densities can result in reduced efficiency or even complete failure (Garza de Yta *et al*., 2004). Establishing optimal water exchange rates in a culture system is necessary to maintain good water quality and thus healthy organisms without affecting the surrounding environment.

The study of the effects of water exchange practices and stocking density in shrimp culture has received considerable attention for several penaeid species at postlarval and juveniles stages (Arnold and Reid, 1992; Arnold *et al.*, 2006; Williams *et al.*, 1996; Palomino *et al.*, 2001; Decamp *et al.*, 2007; Mishra *et al*., 2008; Neal *et al.*, 2010; Sanchez-Zazueta *et al*., 2011; Coman *et al.*, 2004). In contrast to the rearing of fish larvae, stocking density is a factor which has attracted relatively little attention in shrimp larviculture. Even in TAS, few studies on stocking densities and water renewal

strategies have been carried out for penaeid larvae (Cook and Murphy, 1969; Emmerson and Andrews, 1980; Vinatea and Andreatta, 1997). This is even more so for studies on stocking density and water recirculation rates in RAS (Truong *et al.*, 2009). In this study three experiments were conducted over time to evaluate the effects of stocking density and water recirculation rate on the survival and growth performance of *L. vannamei* larvae reared in an experimental RAS (**Chapter IV**). Initially a static system was applied from Nauplii 5 to Zoea 3 stage; thereafter, larvae were transferred to a RAS to be reared up to postlarvae stage. Tested stocking densities were as high as 750, 1000, 1500 and 2000 larvae L^{-1} and water recirculation rates varied from 250, 500, 750 and 1000% day^{-1}. Also the interaction between both factors was examined. In a subsequent experiment, stocking densities of 1000 and 2000 larvae L^{-1} were tested with recirculation rates of 500 and 1000% day^{-1}. The first set of experiments showed a generally negative trend for survival and growth with increasing larval density during the static culture phase. This was also observed by several authors in the performance of juvenile shrimp (Palomino *et al*., 2001; Sookying *et al*., 2011; Ray and Chien, 1992). Emmerson and Andrews (1981) observed that in *Penaeus indicus* larvae the development time increased and the average survival and growth decreased with increasing stocking density. When testing different stocking densities (150, 300, 450, 600, 750 and 900 larvae L^{-1}), Truong *et al*. (2009), found that the larviculture of *P. monodon* in a RAS, gave the best results as for survival and bacterial count at a stocking density of 450 larvae L^{-1}.

In contrast, during the recirculating culture phase, larval performance was less affected by stocking density, although survival were significantly reduced at densities of 1500 and 2000 larvae L^{-1}. Nevertheless, the final larvae L^{-1} in the RAS was 5 to 8 higher than in TAS. Our results suggest that increasing water recirculation rate does not improve larvae survival or growth. In the second experiment with the increase of water recirculation rate the survival significantly decreased. Vinatea and Andreatta (1997) observed the same effect for *F. paulensis* in which increasing water renewal negatively affected survival and larvae performance. These authors also evidenced higher survival and better larval performance when using the stocking density of 200 larvae L^{-1} compared to the 300 larvae L^{-1}. The results of our study suggest that an initial stocking

density of 1000 larvae L^{-1} combined with a water recirculation rate of 500% day^{-1} is the most effective for intensive larval production in a RAS.

In **Chapter V and VII** results were verified at pilot scale, 1 m^3 tanks. A simple compact prototype RAS was used with an initial stocking density of 1000 larvae L^{-1} and applying a water recirculation rate of 500% day^{-1}. The high-density culture was compared with a TAS using a stocking density of 100 larvae L^{-1}. Survival in the first set of experiments (**Chapter V**) averaged 62 ± 8% and 50 ± 7% for the TAS and RAS, respectively. Dry weight was reduced in the RAS; however the total biomass was over five times higher than the biomass produced in the TAS. With an optimized feeding schedule, applying umbrella-stage *Artemia* (**Chapter VII),** survival increased to 71 ± 5% for the RAS. In terms of larval output, 710 PL L^{-1} were produced in the RAS compared to 74 PL L^{-1} produced in the TAS. Similar dry weights and length results were recorded for the larvae reared in the two culture systems. Results in terms of biomass were over 7 times higher in the RAS than biomass production in the TAS. These survival are considerably higher than the ones obtained by Truong *et al*. (2009) of 55.9±0.7% at a stocking density of 450 Nauplii L^{-1}, with a larval output of only 252 larvae L^{-1}. Our results have shown the feasibility to produce *L. vannamei* larvae at high density through the implementation of an experimental RAS at pilot scale, demonstrating its effectiveness in maximizing biomass, although in some cases with a reduction in other production parameters such as growth, survival and larval development.

Feeding strategies, feeding regimes and feed management

Understanding shrimp feeding behavior and quantification of shrimp feed consumption provides valuable information for aquaculturists to improve feed management, one of the keys to economic viability. Also, the development of effective feeding regimes for mass rearing of shrimp larvae represents one of the barriers to success. This involves the difficult task of identifying an appropriate food type, quantity, and quality for the different shrimp larval stages. There are numerous studies on feeds and feeding regimes for larval rearing of penaeid shrimp (Naessens *et al*., 1995;

Gallardo *et al*., 1995; Samocha *et al.*, 1989; Kuban *et al*., 1985; Martins *et al*., 2006; Jones *et al*., 1979; Wouters *et al*., 2009). However, very limited information is available on feeding regimes specifically for intensive culture for *L. vannamei* larvae or for culture in recirculating systems. Therefore, three experiments were performed investigating different feeding strategies during the static and recirculating culture phases in order to establish a feeding regime for the intensive rearing of *L. vannamei* larvae reared in a TAS (**Chapter IV**). In the first experiment, the standard feeding protocol of the ESPOL-CENAIM center was slightly modified, namely microalgae concentrates were used instead of fresh algae and the feeding level of microalgae and rotifers was increased to account for the high larval density. The survival of the larvae were low as of the static culture phase, resulting in a survival not higher than 45% for the densities 750, 1000 and 1500 larvae L^{-1}. In a second experiment, a continuous supply of microalgae concentrates was applied during the early larval stages (static culture phase) and higher amounts of food during the water recirculation to compensate the food loss. This resulted in a significant improvement in survival, with the best results for stocking density of 1000 (65%) and 1500 larvae L^{-1} (50%). Through the use of a higher ration of microalgae concentrates and the continuos supply by automatic feeders, larval survival in the third experiment increased even at the highest stocking density of 2000 larvae L^{-1}. The survival of 60% at a density of 1000 larvae L^{-1} (approximately 5-10 times higher than the stocking density applied in TAS) is lower than the survival usually obtained in TAS. However, the resulting larval output (larvae L^{-1}) was approximately 10 times higher than that in TAS. Biomass yields in the RAS were also 4-6 times higher than biomass obtained from TAS. Also, water quality improved resulting in a drop of 20 to 60% of TAN with the different stocking densities. Dividing a daily ration in multiple feedings or applying continuous feeding is believed to improve shrimp growth and reduce water quality deterioration (Velasco *et al*., 1999). This was also evident in our last experiments.

The feeding protocol elaborated in **Chapter IV** was rather complex (*e.g.* using a combination of several artificial diets), and therefore less suitable for use at larger scale. When scaling up the intensive culture to a pilot RAS ($1m^3$) (**Chapter V**), a single

artificial dry diet for each larval stage was used. Higher numbers of rotifers needed to be fed to larvae in RAS, increasing the input of contaminants such as filamentous bacteria which could contribute to the infestation of larvae and result in recurrent mortalities. However, survival obtained in RAS was similar to the ones in TAS. Again, larval output in terms of larvae L^{-1} was eight-fold in the RAS compared to TAS. Dry weight was reduced and metamorphosis to PL1 stage was retarded in larvae reared in RAS, but the total biomass was over 5 times higher than biomass production in TAS.

The positive effects of the use of rotifers on growth and larval stage index (LSI) in early zoea stages of *L. vannamei* have been demonstrated by Naessens *et al.* (1995). Rotifers have been suggested (Léger *et al*., 1992) as a suitable prey to fill up the size gap between algae (8 - 20µm) and freshly hatched *Artemia* nauplii (430 - 455 µm). However, the culture of rotifers is labour and time demanding, requiring high amounts of microalgae or artificial feeds. Moreover, they are susceptible to contamination with (filamentous) bacteria and protozoa. To overcome this problem of fouling, umbrella-stage *Artemia* was evaluated as a food source for the early stages of *L. vannamei*. The optimal feeding level for each larval stage was assessed under traditional culture conditions (**Chapter VI)**. Our observations showed the highest consumption rate of umbrella-stage *Artemia* at mysis stage, showing a two-fold increment in ingestion from zoea to mysis stages and suggesting that *L. vannamei* shifted to a raptorial feeding mode at the mysis stage. The use of umbrella-stage *Artemia* in the feeding schedule for *L. vannamei* was compared with a standard feeding regime based on a combination of rotifers and *Artemia* nauplii and with the traditional regime of only *Artemia* nauplii used in commercial hatcheries during Z2 stage. Results from this study demonstrated that the same larval performance was achieved when feeding larvae with umbrella-stage *Artemia* as with rotifers. The biomass output was lower when fed only *Artemia* nauplii. Umbrella-stage *Artemia* could therefore replace rotifers as well as *Artemia* nauplii. Moreover, umbrella-stage *Artemia* presents some advantages over rotifers and *Artemia* nauplii. They are easier to produce (only requiring a 12 h hatching process) than rotifers, have higher energy content, and are smaller and easier to capture than *Artemia* nauplii (Lavens and Sorgeloos, 1996). Including umbrella-stage *Artemia* in the feeding regime

of *L. vannamei* larvae starting from the early Z2 stage, gives good results in terms of growth and survival and fills the size gap between microalgae and *Artemia.* After evaluating the use of umbrella-stage *Artemia* as a food source for the early stages of *L. vannamei* under TAS, this prey was evaluated in larvae reared in an intensive RAS (**Chapter VII)**. The results showed that survival and growth, in terms of average dry body weight and length, were similar to those obtained in TAS and higher than those obtained in the experiments with rotifers as food source. The stocking density used in the RAS was 10 times higher than in the TAS yielding an average output of 710 larvae L^{-1} compared to only 74 larvae L^{-1} in the TAS. The results in this experiment (710 larvae L^{-1}) were also better than those obtained in the experimental recirculating system (650 larvae L^{-1}) (**Chapter IV**), and those obtained in the pilot RAS (500 larvae L^{-1}) (**Chapter V**). Dry weights were higher and consequently higher biomass was obtained.

Microbial control

RAS may not only improve water quality in terms of suspended solids and nitrogen removal, but there is evidence that they may reduce the bacterial proliferation and/or that microbial communities are more stable during culture (Suantika, 2001; Rombaut *et al*., 2001). In **Chapter V** the bacteria load in the larvae reared in the RAS was compared with the ones reared in the TAS. Bacteria load was higher in the larvae reared in the RAS than in the TAS already before the recirculation started. This could be due to the higher stocking densities, and the higher amounts of live food which is considered to be an important source of bacterial contamination (Lavens and Sorgeloos, 2000), and the higher amounts of probiotic bacteria administered to the RAS. Nevertheless, bacterial counts in the RAS did not increase with culture time and finally total bacteria load dropped at the end of the culture period. The bacterial load dropped when the recirculation started, but the levels measured were higher to those reported by Truong *et al*. (2009), when evaluating different types of RAS. These authors reported the lowest bacteria load when using a biofilter in combination with a protein skimmer and an ozone generator as components of the RAS. This could explain the lower levels obtained in their study. Bacteria load was also measured in the TAS and RAS, of the

experiment described in **Chapter VII**, again bacterial numbers were higher in the RAS than in the TAS, although not drastically higher considering the stocking densities used. As in **Chapter V,** these observations can be explained, at least partially, by the effect of live food and probiotics.

In **Chapter V**, the evolution of the microbial community in the RAS and TAS was studied by using DGGE (denaturing gradient gel electrophoresis). There is very little knowledge on the microbial ecology and its evolution during the culture period in shrimp larviculture systems and especially in intensive cultures performed in RAS. In both culture systems it was possible to identify three main groups of bacteria among the different DGGE-profiles. However, a high variability in bacterial community between systems and within systems was observed. Our results indicate that the structure of the microbial communities of the culture systems was not static but rather dynamic, different from what Suantika (2001) and Rombaut *et al*. (2001) observed during the intensive culture of rotifers in a RAS. The observations in our study suggest that the microbial community associated with the larval cultures can evolve due to the culture system practices (water exchange and recirculation), the bacterial contribution from live food and the probiotics applied. This is similar to what Vandenberghe *et al.* (1998) and Hisbi *et al*. (2000) described, namely that the flora associated with larvae of *F. chinensis* and *P. monodon,* respectively, is not very stable and is influenced by the bacterial flora of the administered food and by the environment.

Quality/Condition of the larvae originating from the different culture systems

A variety of larval quality indicators and assessment methods has been described and reviewed (Tackaert *et al*., 1989; Fegan, 1992; Samocha and Lawrence, 1992; Naessens *et al*., 1995; Racotta *et al*., 2003). The search and establishment of universal criteria to assess larval quality is a major concern at both research and production level. Common practice is to evaluate the physiological condition of the larvae by determining their ability to withstand stress conditions (temperature, pH, osmotic shocks or certain chemicals). Another parameter to assess the physiological condition is larval performance during culture (survival and growth). Morphological characteristics such as

muscle size in the sixth abdominal segment, gill development and overall pigmentation are also claimed as valid criteria for the evaluation of postlarval quality. Resistance to pathogens is also considered a criterion for larval quality as it is used to test prophylactic and curative treatments and in the study of the host-factors influencing the expression of bacterial virulence (Saulnier, *et al.*, 2000; Alabi *et al.*, 1999; Roque *et al.*, 1998). In our study a bath challenge test with a known pathogen was used to evaluate differences in larval fitness of larvae reared in TAS and RAS. On several occasions (**Chapter V and VII)**, larvae from the two culture systems were subjected to this challenge test. No difference in survival was observed when both groups of larvae were exposed to pathogenic bacteria, demonstrating that the type of culture system and the difference in stocking densities did not influence the physiological condition of the larvae. Survival upon challenge were similar with those reported by Pico (2004), when exposing PL3 *L. vannamei* to a bath challenge with the 246 CENAIM strain at a concentration of 1 $x10^7$ CFU. Similar and higher values in survival were obtained in our work when compared to Alabi *et al.* (1999) who reported survival within the range of 6.5 to 79% when challenging Z3 of *P. indicus* with different strains of *V. harveyi*.

It is agreed that the quality criteria mentioned above indeed provide information on the animals' quality or condition at the time of determination, but a possible significant relationship with the posterior performance in grow-out conditions is still being questioned (Samocha and Lawrence, 1992; Naessens *et al*.,1995; Racotta *et al.*, 2003). It has been also demonstrated by Rees *et al*. (1994) that neither size nor growth are reliable criteria for postlarval quality. Therefore, a nursery trial under laboratory conditions was performed to establish the quality/condition of the larvae originating from RAS and TAS. During the nursery phase, the performance of the larvae from the RAS did not differ from the ones culture in the TAS. In our study, higher survival (89%) was obtained compared to the 73% and 63% that Rodríguez *et al.* (2011) reported in a nursery experiment (PL2 - PL25) testing the application of β-1,3/1,6- glucans as a health management strategy.

Ideally, it would have been adequate to evaluate larvae produced under TAS and RAS conditions during grow out in ponds; however, it was difficult to count with

sufficient PL from the TAS to have enough replications for a statistically sound analysis. Although this study was not included within the framework of this thesis, a grow out experiment was carried out at the Experimental Station of ESPOL- CENAIM in Palmar (Santa Elena Province, Ecuador), 5 km from the National Aquaculture and Marine Research Center, "EDGAR ARELLANO M." (ESPOL- CENAIM). In this trial postlarvae from RAS were stocked in earthen ponds to evaluate their performance during a grow-out culture period. Direct stocking of 12 PL25 m^{-2} was replicated in 9 0.25 ha earthen ponds. The grow-out trial was carried out during the cold/dry season following the protocol used in the experimental station. After 149 days individual shrimp wet weight (g), total yield (kg ha^{-1}) and survival (%) were measured. Despite a severe white spot syndrome virus (WSSV) epidemic from day 62 until day 90 in all nine ponds and the adverse environmental conditions during the cold dry season, a relative high survival (59.6 ± 6.3%) and yield (658 ± 90 kg ha^{-1}) were recorded (Table 1).

Table 1. Harvest results from a 149-days grow-out experiment in earthen ponds stocked with *L. vannamei* larvae originating from a high density recirculating aquaculture system (RAS). Means and standard deviation of weight, survival and yield are presented

Pond	Weight (g)	Survival (%)	Yield (kg ha^{-1})
B1	9,6	61,51	705,27
B6	8,84	64,76	689,03
B8	8,84	61,69	656,56
B3	8,2	45,88	452,74
B4	9,25	57,63	642,14
B9	10,14	62,34	761,18
B2	9,42	60,19	683,62
B5	8,94	67,64	728,71
B7	9,09	54,86	600,65
Mean	9,15	59,61	657,77
Standard deviation	0,55	6,34	90,26

The survival obtained in our experiment are comparable to the 69±12.45% that Rodríguez *et al.* (2011) obtained in a grow-out experiment during the cold/dry season

with stocking density of 10 PL m^{-2}, when testing the application of probiotics and β-1,3/1,6- glucans as a health management strategy for cultured shrimp. Results from the challenge test, performance during nursery culture under laboratory conditions and grow-out culture under field conditions give strong evidence that qualified larvae can be produced at high density in a RAS. Moreover, considering that a culture system can only be successful if it is cost-effective, the RAS also displays advantages compared to the TAS from an economical point of view. The investment cost and operational cost for intensive *L. vannamei* larval culture using a RAS could be reduced by 25%. Savings are mainly on labour cost, investment cost and feed cost.

In general, the results of this thesis indicate that *L. vannamei* larvae could be raised at a high density using a recirculating system. Some of the problems and/or disadvantages inherent to the traditional culture of shrimp larvae can be solved by the use of a recirculating aquaculture system.

Conclusions and suggestions for further research

We have contributed to the optimisation and intensification of *L. vannamei* larvae rearing through the implementation of a recirculating aquaculture system. The results indicate that the proposed culture technique allow the production of higher quantities of larvae per liter, higher biomass, good quality PL, while reducing investment and operating costs, increasing biosecurity and being more environmentally sound. Further research and development is needed to fine tune and improve this culture technique so that it can be scaled-up and ready to be applied, ensuring consistent output, at commercial shrimp hatcheries.

The most important ***conclusions*** from this research are summarized below:

- *Litopenaeus vannamei* showed to be a resistant species to ammonia at early larval stages. Mysis sub-stages were the most tolerant larval stage to ammonia. For the production of *L. vannamei* ammonia levels should not exceed 0.42 mg L^{-1} TAN, equivalent to 0.06 mg L^{-1} NH_3-N.

- The super-intensive culture of *L. vannamei* larvae was feasible through the implementation of an experimental recirculating system (RAS) at stocking densities up to 2000 larvae L^{-1}.

- Stocking density had a significant ($p<0.05$) negative effect on survival, though this could be partly overcome by improving the feeding regime.

- Water recirculation rates higher than 500% day^{-1} negatively affected survival, length and biomass.

- Continuous feeding by means of automatic feeder and increased concentration of microalgae as a feeding strategy improved the survival and performance of *L. vannamei* reared at high stocking density in an experimental recirculating system.

- The combination of a stocking density of 1000 larvae L^{-1} and a water exchange rate of 500% day^{-1} consistently presented higher mean survival (39, 55 and 60%) and biomass output (0.32, 0.31 and 0.42 kg m^{-3}) compared to the other treatments.

- The feasibility to intensively produce *L. vannamei* larvae in a pilot RAS (1 m^3) was demonstrated: An initial stocking density of 1000 larvae L^{-1}, with larvae continuously fed high concentrations of microalgae and with a water recirculation rate of 500% day^{-1}, allowed to produce a 5 times higher output (larvae L^{-1}) than in a traditional culture systems (TAS).

- Under traditional culture conditions, umbrella-stage *Artemia* demonstrated to be a promising food source for *L. vannamei* larvae from the early Z2 sub-stage onwards, as a replacement of rotifers and the traditionally used *Artemia* nauplii.

- *L. vannamei* larvae were succesfully raised at 1000 larvae L^{-1} in a RAS, replacing rotifers by umbrella-stage *Artemia,* producing a 7-fold higher biomass than in the TAS.

- Compared to the TAS, the use of a RAS can contribute to a 42% saving on the total investment costs and a saving up to 30% in the running costs, mainly due to reduced labour and depreciation costs per larviculture production cycle.

- The condition of larvae reared intensively in a RAS with umbrella-stage *Artemia* in the feeding regime, was not significantly different ($p<0.05$) to those reared in TAS. This was not the case when challenged to a pathogenic bacterium neither during a nursery culture under laboratory conditions.

Suggestions for further research

- In the current study, tolerance of *L. vannamei* larvae to ammonia as a major toxic nitrogen compound was investigated. However, it is also necessary to investigate the tolerance limits for nitrite and the combined effect of ammonia and nitrite on *L. vannamei* larvae.

- A better understanding of the hydrodynamics within the system and the effect of the addition of other components to the recirculating system (*e.g.* protein skimmer, hydrocyclone, ozone generator) will contribute to further fine-tuning and improve *L. vannamei* larval rearing at high densities in RAS.

- A thorough qualitative and quantitative study of the microbial community in intensive RAS culture systems and within the different components of the recirculating unit could be elaborated.

- Future developments in the field of larval nutrition and larval feed development will probably allow partial or total replacement of live food (microalgae, *Artemia*) in order to simplify the intensive culture protocols. Further improvements in the feeding regime, feeding practices and feed rations may increase performance and profitability.

- It may be interesting to integrate biofloc technology into intensive larval rearing systems for *L. vannamei*. This could have two-fold purposes: 1) serve as food source and, 2) control the water quality.

- In the current study, the intensive *L. vannamei* larviculture in a RAS was performed from Nauplii 5 until Postlarvae 1-2. It would be interesting to verify whether the whole larval rearing phase (PL10 - PL12) can be achieved at high stocking densities in a RAS.

- The feasibility of intensive culture of *L. vannamei* larvae in a RAS at laboratory and pilot scale was demonstrated in this study. Results obtained need to be verified and validated through evaluation under large-scale commercial production conditions.

CHAPTER IX

REFERENCES

REFERENCES

Aguirre G., Ascencio F. & Pandalai S.G. (2000) Infectious disease in shrimp species with aquaculture potential. *Recent Research developments in Microbiology* **4**, 333-348.

Al Azad S., Chong V.C. & Vikineswary S. (2002) Phototrophic Bacteria as Feed Supplement for Rearing *Penaeus monodon* larvae. *Journal of the World Aquaculture Society* **33**, 158-168.

Alabi A.O., Jones D.A. & Latchford J.W. (1999) The efficacy of immersion as opposed to oral vaccination of *Penaeus indicus* larvae against *Vibrio harveyi*. *Aquaculture* **178**, 1–11.

Alcaraz G., Chiappa-Carrara X. & Vanegas C. (1997) Temperature tolerance of *Penaeus setiferus* postlarvae exposed to ammonia and nitrite. *Aquatic Toxicology* **39**, 345-353.

Alcaraz G., Chiappa-Carrara X., Espinoza V. & Vanegas C. (1999) Acute toxicity of ammonia and nitrite to white shrimp *Penaeus setiferus* postlarvae. *Journal of the World Aquaculture Society* **30**, 90-97.

Aneshansley E., Timmons M.B. & Colesante R.I. (2001) Survival and Growth of Walleye *Stizostedion vitreum* Fry as Affected by Water Recirculation, Feed Transition Age, and Stocking Density. *Journal of the World Aquaculture Society* **32,** 89–95.

APHA, AWWA, WPCF (1992) *Standard Methods for the Examination of Water and Wastewater*. 18th edn. American Public Health Association, American Water Works Association, and Water Pollution Control Federation, Washington, D.C.

Arellano E. (1990) Guías Técnicas en el Cultivo de Larvas de Camarón. Facultad de Ingeniería Marítima y Ciencias del Mar. ESPOL. Guayaquil-Ecuador.

Arellano E. (1993) Investigación bioquímica y nutricional del camarón. In: *En Memoria de Edgar Arellano M. once años dedicados a la investigación y desarrollo de la acuicultura en el Ecuador* (ed. by J. Calderón & S. Sonnenholzner), pp. 231. Escuela Superior Politécnica del Litoral. Facultad de Ingeniería Marítima y Ciencias del Mar. Centro Nacional de Acuicultura e Investigaciones Marinas "Edgar Arellano M." San Pedro de Manglaralto, Ecuador.

Arnold C.R. & Reid B. (1992) The intensive culture of Penaeid shrimp Penaeus vannamei Boone in a Recirculating Raceway System. *Journal of the World Aquaculture Society* **23**, 146-153.

Arnold C.R., Reid B. & Brawner B. (1990) High density recirculating grow-out systems. In: *Manual on red drum aquaculture*, pp. 182-184. Texas A&M Sea Grant College Program, Texas, USA.

Arnold S.J., Sellars M.J., Crocos P.J. & Coman G.J. (2006) Intensive production of juvenile tiger shrimp *Penaeus monodon*: An evaluation of stocking density and artificial substrates. *Aquaculture* **261**, 890-896.

Atwood H.L., Bruce J.W., Sixt L.M., Kegl R.A., Stokes A.D. & Browdy C.L. (2005) Intensive zero-exchange shrimp production systems-incorporation of filtration technologies to improve survival and growth. *International Journal of Recirculating Aquaculture* **6**, 45-53.

Barón Sevilla B., Bückle L.F. & Hernández M. (2004) Intensive Culture of *Litopenaeus vannamei* Boone 1931, in a recirculating seawater system. *Ciencias Marinas*, 179-188.

Bauman R.G.H. & Jamandre D. (1990) A practical method for determining quality of *Penaeus monodon* (Fabricius) fry for stocking in grow out ponds. In: *Proceedings of the Aquactech conference on Technical and Economic aspects of shrimp farming* (ed. by M.B. New, H. de Saram & T. Singh). Publication Infofish, Kuala Lumpur.

Bauman R.G.H. & Scura E. (1990) Determining the quality of *Penaeus monodon* postlarvae. In: *Book of Abstracts*. World Aquaculture 1990, June 10-14. Halifax, Nova Scotia, Canada.

Beard T.W., Wickins J.F. & Arnstein D.R. (1977) The breeding and growth of *Penaeus merguiensis* de Mann in laboratory recirculation systems. *Aquaculture* **10**, 275–289.

Bergheim A., Drengstig A., Ulgenes Y. & Fivelstad S. (2008) Dominating systems for production of Atlantic Salmon smolt in Europe. *Proceedings of the Aquacultural Engineering Society' s Fourth Issues Forum*. 59-72 pp.

Biedenbach J.M., Smith L.L., Thomsen T.K. & Lawrence A.L. (1989) Use of the nematode *Panagrellus redivivus* as an *Artemia* replacement in a larval penaeid diet. *Journal of the World Aquaculture Society* **20**, 61–71.

Blancheton J.P. (2000) Developments in recirculation systems for Mediterranean fish species. *Aquacultural Engineering* **22**, 17-31.

Bovendeur J., Eding E.H. & Henken A.M. (1987) Design and performance for high-density culture of the African catfish, *Clarias gariepinus* (Burchell 1882). *Aquaculture* **63**, 329-353.

Bower C.E. & Bidwell J.P. (1978) Ionization of ammonium in seawater: effects of temperature, pH and salinity. *Journal of the Fisheries Research Board of Canada* **35**, 1012-1016.

Boyd C. & Tucker C.S. (1992) *Water quality and pond soil analyses for aquaculture*. Alabama Agricultural Experiment Station, Auburn University, Alabama. 183 pp.

Bray, W. A. and A. L. Lawrence. 1992. Reproduction of Penaeus species in captivity. In: *Marine Shrimp Culture: Principles and Practices* (ed. by A. W. Fast & L. J. Lester), pp. 93-170. Elsevier Science Publishers, Amsterdam, The Netherlands.

Browdy C.L. (1998) Recent developments in penaeid broodstock and seed production technologies: improving the outlook for superior captive stocks. *Aquaculture* **164**, 3-21.

Brunvold L., Sandaa R.-A., Mikkelsen H., Welde E., Bleie H. & Bergh, O. (2007) Characterisation of bacterial communities associated with early stages of intensively reared cod (*Gadus morhua*) using denaturing gradient gel electrophoresis (DGGE). *Aquaculture* **272**, 319–327.

Buikema A.L. Jr., Niederlehner B.R. & Cairns J.K. Jr. (1982) Biological monitoring. Part IV- Toxicity testing. *Water Research* **16**, 239-262.

Carroll P., Watanabe W.O. & Losordo T.M. (2003) Microbead media offers low cost, efficiency in RAS biofilters. *Global Aquaculture Advocate* **6**, 36-37.

Chang C.F, Su M.S., Chen H.Y. & Liao I.C. (2003) Dietary β-1,3-glucan effectively improves immunity and survival of *Penaeus monodon* challenged with white spot syndrome virus. *Fish and Shellfish Immunology* **15**, 297-310.

Charmantier G. (1998) Ontogeny of osmoregulation in crustaceans: a review. *Invertebrate Reproduction & Development* **33**, 177-190.

Chen J.C. & Chin T.S. (1987) Joint action of ammonia and nitrite on tiger prawn *Penaeus monodon* postlarvae. *Journal of the World Aquaculture Society* **19**, 143-148.

Chen J.C. & Chin T.S. (1988) Acute toxicity of nitrite to tiger prawn, *Penaeus monodon* larvae. *Aquaculture* **69**, 253-262.

Chen J.C. & Chin T.S. (1989) Effect of ammonia at different pH levels on *Penaeus monodon* Postlarvae. *Asian Fisheries Science* **2**, 233-238.

Chen J.C. & Kou T.T. (1996a) Nitrogenous excretion in *M. rosenbergii* at different pH levels. *Aquaculture* **144**, 155-164.

Chen J.C. & Kou T.T. (1996b) Effects of temperature on oxygen consumption and nitrogenous excretion in *Macrobrachium rosenbergii*. *Aquaculture* **145**, 295-303.

Chen J.C. & Kou Y.Z. (1992) Effects of ammonia on growth and molting on *Penaeus japonicus* juveniles. *Aquaculture* **104**, 249-260.

Chen J.C. & Lai S. H. (1993) Effects of temperature and salinity on oxygen consumption and ammonia-N excretion of juvenile *Penaeus japonicus* Bate. *Journal of Experimental Marine Biology and Ecology* **165**, 161-170.

Chen J.C. & Lei S.C. (1990) Toxicity of ammonia and nitrite to *Penaeus monodon* juveniles. *Journal of the World Aquaculture Society* **21**, 300-305.

Chen J.C. & Lin C.Y. (1991a) Lethal effects of ammonia on *Penaeus chinensis* Osbeck juveniles at different salinity levels. *Journal of Experimental Marine Biology and Ecology* **156**, 138-148.

Chen J.C. & Lin C.Y. (1991b) Lethal doses of ammonia on *Penaeus chinensis* larvae. *Bulletin of the Institute of Zoology, Academia Sinica* **30**, 289-297.

Chen J.C. & Lin C.Y. (1992) Effect of ammonia on growth of *Penaeus penicillatus* juveniles. *Comparative Biochemistry and Physiology* **101**, 443-447.

Chen J.C. & Nan F.H. (1993) Changes of oxygen consumption and ammonia-N excretion by *Penaeus chinensis* Osbeck at different temperature and salinity levels. *Journal of Crustacean Biology* **13**, 706-712.

Chen J.C. & Sheu T.S. (1990a) Effect of ammonia at diferent pH levels on larval *Penaeus monodon*. *Journal of Fisheries Society Taiwan* **17**, 59-64.

Chen J.C. & Sheu T.S. (1990b) Effect of ammonia at different pH on *Penaeus japonicus* postlarvae. In: *The second Asian Fisheries Forum* (ed. by R. Hirano & I. Hanyu), pp. 61-68. Asian Fisheries Society, Manila, Philippines.

Chen J.C. & Tu C.C. (1990) Acute toxicity of nitrite to larval *Penaeus japonicus*. *Journal of the Fisheries Society of Taiwan* **17** (4), 277-287.

Chen J.C., Liu P.C. & Lei S.C. (1990) Toxicity of ammonia and nitrite to *Penaeus monodon* adolescents. *Aquaculture* **89**:127-137.

Chien Y.H. (1992) Water Quality Requirements and Management for Marine Shrimp Culture. In: *Proceedings of the Special Session on Shrimp Farming* (ed. by J. Wyban), pp.144-152. World Aquaculture Society, Baton Rouge, LA USA.

Chin T.S. & Chen J.C. (1987) Acute toxicity of ammonia to larvae of the tiger prawn, *Penaeus monodon*. *Aquaculture* **66**, 247-253.

Chun K.H. & Shing C.K. (1986) Feeding behaviour of the shrimp, *Metapenaeus ensis*, on *Artemia* nauplii. *Aquaculture* **58**, 175-184.

Chythanya R., Karunasagar I. & Karunasagar I. (2002) Inhibition of shrimp pathogenic vibrios by a marine Pseudomonas I-2 strain. Aquaculture 208, 1-10.

Cobo M.L., Sonnenholzner S., Wille M. & Sorgeloos P. (2012) Ammonia tolerance of *Litopenaeus vannamei* (Boone) larvae. *Aquaculture Research*, in press.

Colt J.E. & Armstrong D.A. (1981) Nitrogen toxicity to crustaceans, fish, and molluscs. In: *Proceeding of the Bio-Engineering Symposium for Fish Culture* (ed. by L.J. Allen & E.C. Kinney), pp. 34-47. Fish Culture Section of the American Fisheries Society, Bethesda, MD, USA.

Coman G.J., Crocos P.J., Preston N.P. & Fielder D. (2004). The effects of density on the growth and survival of different families of juvenile *Penaeus japonicus* Bate . *Aquaculture* **229**, 215-223.

Conway H. & Harrison P. (1977) Marine diatoms grown in chemostats under silicate or ammonium limitation. IV. Transient response of *Chaetoceros gracilis*, *Skeletonema costatum* and *Thalassiosira grmidu* to a single addition of a limiting nutrient. *Marine Biology* **43**, 33-43.

Cook H.L. & Murphy M.A. (1966) Rearing penaeid shrimp from eggs to postlarvae. In: *Proceedings 19th Annual Conference Southeast Association Game Fish Community*, pp. 283-288.

Cook H.L. & Murphy M.A. (1969) The culture of larval penaeid shrimp. *Transactions of the American Fisheries Society* **98**, 751-754.

Couch J.A. (1974) An enzootic nuclear polyhedrosis virus of pink shrimp: Ultraestructure, prevalence, and enhacement. *Jounal Invertebrate Pathology* **24**, 311-331.

Dall W., Hill B.J., Rothlisberg P.C. & Staples D.J. (1990) The Biology of the Penaeidae. In: *Advances in Marine Biology*, vol. 27, pp. 1–489. Academic Press, London.

D'Abramo L.R., Perez E.I., Sangha R. & Puello-Cruz A. (2006) Successful culture of larvae of *Litopenaeus vannamei* fed a microbound formulated diet exclusively from either stage PZ2 or M1 to PL1. *Aquaculture* **261**, 1356-1362.

D'Souza F.M.L. & Loneragan N.R. (1999) Effects of monospecific and mixed-algae diets on survival, development and fatty acid composition of penaeid prawn (*Penaeus* spp.) larvae. *Marine Biology* **133**, 621-633.

D'Souza F.M.L., Lecossois D, Heasman M.P., Diemar J.A., Jackson C.J. & Pendrey R.C. (2000) Evaluation of centrifuged microalgae concentrates as diets for *Penaeus monodon* Fabricius larvae. *Aquaculture Research* **31**, 661-670.

Davis D.A. & Arnold C.R. (1998) The design, management and production of a recirculating raceway system for the production of marine shrimp. *Aquacultural Engineering* **17**, 193 –211.

Davis D.A. & Arnold C.R. (1998) The design, management and production of a recirculating raceway system for the production of marine shrimp. *Aquacultural Engineering* **17**, 193 –211.

Decamp O., Conquest L., Cody J. & Forster I. (2007) Effect of shrimp stocking density on size-fractionated phytoplankton and ecological groups of ciliated protozoa within zero-water exchange shrimp culture systems. *Journal of the World Aquaculture Society* **38**, 395-406.

Del Campo L.M., Ibarra P., Gutierrez X. & Takle H. (2010) Utilization of sludge from recirculation aquaculture systems. *Nofina report* **9**/2012. 73 pp.

Dinesh K., Chan W., Lim T. & Phang V. (1995) RAPD markers in fishes: an evaluation of resolution and reproducibility. *Asia Pacific Journal of Molecular Biology and Biotechnology* **3**, 112-118.

Dooms S., Papakostas S., Hoffman S., Delbare D., Dierckens K., Triantafyllidis A., De Wolf T., Vadstein O., Abatzopoulos Th. J., Sorgeloos P. & Bossier P. (2007) Denaturing Gradient Gel Electrophoresis (DGGE) as a tool for the characterization of *Brachionus* sp. strains. *Aquaculture* **262**, 29-40.

Dore I. & Frimodt C. (1987) An illustrated guide to shrimp of the world. Osprey Books, Huntington, New York, New York. 229 pp.

Edemar R., Beltrame E. & Seiffert W. (1996) Despesca e Transporte de pós-larvas. In: *Curso internacional de Produçao de pós-larvas de camarâo marinho.* Florianópolis, Brasil. 153-156 pp.

Emerson K., Russo R.C., Lund R.E. & Thurston R.V. (1975) Aqueous ammonia equilibrium calculations: effects of pH and temperature. *Journal of Fisheries Research Board of Canada* **32**, 2379-2383.

Emmerson W.D. (1980) Ingestion, growth and development of *Penaeus indicus* larvae as a function of *Thalassiosira weissflogii* cell concentration. *Marine Biology* **58**, 65-73.

Emmerson W.D. (1984) Predation and energetics of *Penaeus indicus* (Decapoda: Penaeidae) larvae feeding on *Brachionus plicatilis* and *Artemia* nauplii. *Aquaculture* **38**, 201–209.

Emmerson W.D. & Andrews B. (1981) The effect of stocking density on the growth, development and survival of *Penaeus indicus* Milne Edwards larvae. *Aquaculture* **23**, 45-57.

FAO (2002) State of World Fisheries and Aquaculture 2002. Rome, Italy, 11-124 pp.

FAO (2006) FAO Yearbook. *Fishery and Aquaculture Statistics*. 81pp.

FAO (2010) FAO Yearbook. Fishery and Aquaculture Statistics. Aquaculture Production. 239 pp.

FAO (2012) The State of World Fisheries and Aquaculture 2012. Rome, Italy, 209 pp.

Faulk C., Kaiser J.B. & Holt J. (2007) Growth and survival of larval and juvenile cobia *Rachycentron canadum* in a recirculating raceway system. *Aquaculture* **270**, 149-157.

Fegan D.F. (1992) Recent developments and issues in the penaeid shrimp hatchery industry. In: *Proceedings of the Special Session on Shrimp Farming* (ed. by J. Wyban).World Aquaculture Society, Baton Rouge, LA, USA.

Focken U., Schlechtriem C., von Wuthenau M., García-Ortega A., Puello-Cruz A. & Becker K. (2006) *Panagrellus redivivus* mass produced on solid media as live food for *Litopenaeus vannamei* larvae. *Aquaculture Research* **37**, 1429-1436.

Franco-Nava M.A., Blancheton J.P., Deviller G. & Le-Gall J.Y. (2004) Particulate matter dynamics and transformations in a recirculating aquaculture system: application of stable isotope tracers in seabass rearing. *Aquacultural Engineering* **31**, 135-155.

Frías-Espericueta M.G., Harfush-Melendez M., Osuna-López J.I. & Páez-Osuna F. (1999) Acute toxicity of Ammonia to juvenile shrimp *Penaeus vannamei* Boone. *Bulletin of Environmental Contamination and Toxicology* **62**, 646-652.

Fromm P.O. & Gillete J.R. (1968) Effect of ammonia on blood ammonia and nitrogen excretion of rainbow trout (*Salmo gairdneri*). *Comparative Biochemistry and Physiology* **26,** 887-896.

Gallardo P., Alfonso E., Gaxiola G., Soto L.A. & Rosas C. (1995) Feeding schedule for *Penaeus setiferus* larvae based on diatoms (*Chaetoceros ceratosporum*), flagellates (*Tetraselmis chuii*) and *Artemia* nauplii. *Aquaculture* **131**, 239-252.

Gamboa-Delgado J. & Le Vay L. (2009) *Artemia* replacement in co-feeding regimes for mysis and post-larval stages of *Litopenaeus vannamei*: Nutritional contribution of inert diets to tissue growth as indicated by natural carbon stable isotopes. *Aquaculture* **297**, 128-135.

Gandy R.L. (2004) Investigations into the reproductive performance and larval rearing of the Brown shrimp, *Farfantepenaeus aztecus*, using closed recirculating systems. Ph.D. Dissertation. Department Wildlife and Fisheries Sciences, Texas A&M University, Texas, USA. 115 pp.

García-Ortega A., van Hoornyck A., Segner H., Coutteau P. & Verreth J. (1995) Effect of heat treatment on the nutritional quality if decapsulated *Artemia* cysts as food for African catfish *Clarias gariepinus* larvae. In: *Larvi' 95- Fish & Shellfish Larviculture Symposium* (ed. by P. Lavens, E. Jaspers & I. Roelants), pp. 281-284. European Aquaculture Society, Special Publication No.24, Gent, Belgium.

Garriques D. & Arevalo G. (1995) An evaluation of the production and use of a live bacterial isolate to manipulate the microbial flora in the commercial production of *Penaeus vannamei* postlarvae in Ecuador. In: *Swimming Through Troubled Water* (ed. by C.L. Browdy & J.S. Hopkins), pp. 53-59. Proceedings of the special session on shrimp farming, Aquaculture ' 95. World Aquaculture Society, Baton Rouge, Lousiana, USA.

Garza de Yta A, Rouse D.B. & Davis D.A. (2004) Influence of nursery period on the growth and survival of *Litopenaeus vannamei* under pond production conditions. *Journal of World Aquaculture Society* **35**, 357-365.

Ghanekar (2005) Prototype RAS raises larval penaeid shrimp. *Global Aquaculture Advocate* **7**, 78-80.

Gomez-Gil B. (1998) Evaluation of potential probionts for use in penaeid shrimp larval cultura. PhD Thesis, the University of Stirling, 269 pp.

Gomez-Gil B., Roque A. & Turnbull J.F. (2000) The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. *Aquaculture* **191**, 259-270.

Griffith D.R.W. (1995) Microbiology and the role of probiotics in Ecuadorian shrimp hatcheries. In: *Larvi ' 91- fish and crustacean larviculture symposium* (ed. by P. Lavens, E. Jaspers & I. Roelands), pp. 478. European Aquaculture Society, Special publication no. 24, Gent, Belgium.

Harrison K.E. (1990) The role of nutrition in maturation, reproduction and embryonic development of decapods crustaceans: a review. *Journal of Shellfish Research* **9,** 1-28.

Hazlett B.A. (1994) Alarm responses in the crayfish *Orconectes virilis* and *Orconectes propinquus*. *Journal of Chemical Ecology* **20**, 1525-1536.

Hazlett B.A. & McIay C. (2000) Contingencies in the behavior of the crab *Heterozius rotundifrons*. *Animal Behavior* **59**, 965-974.

Hirata H., Anastasios M. & Yamasaki S. (1985) Evaluation of the use of *Brachionus plicatilis* and *Artemia* for rearing prawn *Penaeus japonicus* larvae on a laboratory scale. *Memoirs of the Faculty of Fisheries Kagoshima University, Japan* **34**, 27-36.

Hisbi D., Vandenberghe J., Robles R., Verdonck L. Swings, J. & Sorgeloos P. (2000) Characterisation of *Vibrio* and related bacteria associated with shrimp *Penaeus monodon* larvae in Indonesia. *Asian Fisheries Science* **13**, 57–64.

Horowitz A., Samocha T.M., Gandy R.L. & Horowitz, S. (2001) Toxicity tests to assess the effect of a synthetic tank liner on shrimp survival and nitrification in a recirculating superintensive production system. *Aquacultural Engineering* **24**, 91 – 105

Jaime-Ceballos B., Hernández-Llamas A., Garcia-Galano T. & Villareal H. (2006). Substitution of *Chaetocers muelleri* by *Spirulina platensis* meal in diets for *Litopenaeus schmitti* larvae. *Aquaculture* **260**, 215-220.

Jayasankar P. & Muthu M.F. (1938) Toxicity of ammonia to the larvae of Penaeus indicus H. Milne Edwards. *Indian Journal of Fisheries* **30**, 1-12.

Jensen L., Wasielesky W.Jr., Cavalli R.O., Peixoto S., Silva Santos M.E. & Ballester E. (2004) Growth and survival of pink shrimp (*Farfantepenaeus paulensis*) postlarve in cages and pen enclosures. *Scientia Agricola* **61**, 332-335.

Joensen R. (2008) Recirculation of water for breeding. *Presentation at seminar of recirculation of water in aquaculture*, 27-28 February 2008, Sunndalsora, Norway.

Jones D.A., Kamarudin M.S. & Le Vay (1991) The potential for replacement of live feeds in larval culture. In: *Larvi '91-Fish and Crustacean Larviculture Symposium* (ed. by P. Lavens, P. Sorgeloos, E. Jaspers & F. Ollevier), pp. 141. European Aquaculture Society, Special Publication No. 15, Gent, Belgium.

Jones D.A., Kanazawa A. & Ono K. (1979) Studies on the presentation of artificial diets for rearing the larvae of *Penaeus japonicus* Bate. *Aquaculture* **17**, 33-43.

Jones D. A., Kanazawa A. & Rahman S. A. (1979) Studies on the presentation of artificial diets for the rearing of larvae of *Penaeus japonicas Bate. Aquaculture* **17**, 33-43.

Jones D.A., Yule A.B. & Holland, D.L. (1997a) Larval nutrition. In: *Crustacean Nutrition. Advances in World Aquaculture, Vol. 6.* (ed by L.R D'Abramo, D.E. Conklin & D.M. Akiyama), pp. 353-389. World Aquaculture Society, Baton Rouge, LA, USA.

Jones D.A., Kumlu M., Le Vay L. & Fletcher D.J. (1997b) The digestive physiology of herbivorous, omnivorous and carnivorous crustacean larvae, a review. *Aquaculture* **155**, 285–295.

Jory D.E. (2001) Manejo integral del alimento del camarón, de estanques de producción camaroneros, y principios de bioseguridad. Curso lance en acuicultura. Monterrey, México.

Juarez L.M (1997) The zoea II syndrome: a recently recognized problem in shrimp hatcheries. In: *IV Simposio Centroamericano de Acuacultura* (ed. by D.E. Alston, B.W. Green & H.C. Clifford). Asociación Nacional de Acuicultores de Honduras and the Latin American Chapter of the World Aquaculture Society, Tegucigalpa.

Juarez L.M., Moss S.M. & Figueras E. (2010) Maturation and larval rearing of the Pacific white shrimp, *Penaeus vannamei.* In: *The Shrimp Book* (ed. by V. Alday-Sanz), pp. 920. Nottingham University Press, UK.

Kanazawa A., Teshima S.I. & Sakamoto M. (1985) Effects of dietary lipids, fatty acids, and phospholipids on growth and survival of prawn (*Penaeus japonicus*) larvae. *Aquaculture* **50**, 39-49.

Kikuchi K., Iwata N., Kawabata T. & Yanagawa T. (2006) Effect of feeding frequency, water temperature, and stocking density on the growth of Tiger Puffer, *Takifugu rubripes. Journal of the World Aquaculture Society*, **37**, 13-19

Kim S.K., Kong I., Lee B.H., Kang L., Le M.G. & Suh, K.H. (2000) Removal of ammonium-N from a recirculation system using an immobilized nitrifier. *Aquacultural Engineering* **21**, 139-150.

Kir M., Kumlu M. & Erodolgan O.T. (2004) Effects of temperature on acute toxicity of ammonia to *Penaeus semisulcatus* juveniles. *Aquaculture* **241**, 479-489.

Krumis V., Ebeling J. & Wheaton F. (2001) Part-day ozonation for nitrogen and organic carbon control in recirculation aquaculture systems. *Aquacultural Engineering* **24**, 231-241.

Kuban F.D., Lawrence A.L. & Wilkenfeld J.S. (1985) Survival, metamorphosis and growth larvae from four penaeid species fed six food combinations. *Aquaculture* **47**,151-162.

Kumlu M. (1997) Feeding and digestion in larval decapods crustaceans. *Turkish Journal of Biology* **23**, 215-229.

Kumlu M. & Jones D.A. (1995) Feeding *Penaeus indicus* larvae on live and artificial diets. *Journal of the World Aquaculture Society* **26**, 406-415.

Kurmaly K., Jones D.A. & Yule A.B. (1990) Acceptability and digestion of diets fed to larval zoeas of *Homarus gammarus* and the role of dietary conditioning behavior. *Marine Biology* **106**, 181-190.

Kurmaly K., Jones D.A., Yule A.B. & East J. (1989) Comparative analysis of the growth and survival of *Penaeus monodon* Fabricius larvae from protozoea 1 to postlarva 1 on live feeds, artificial diets and on combinations of both. *Aquaculture* **81,** 27–46.

Langdon C. J., Levine D. M. & Jones D.A. (1985) Review: microparticulate feeds for marine suspension feeders. *Journal Microencapsulation* **21** , 1-11.

Lavens P. & Sorgeloos P. (1996) Manual on the production and use of live food for aquaculture. FAO Fisheries Technical Paper No. 361. FAO, Rome, Italy.

Lavens P. & Sorgeloos P. (2000) The history, present status and prospects of the availability of *Artemia* cysts for aquaculture. *Aquaculture* **181**, 397–403.

Lawrence A.L., Mc Vey J.P. & Huner J.V. (1985) Penaeid shrimp culture. In: *Crustacean and mollusk Aquaculture in the United States* (ed. by J.V Huner & E.E. Brown), pp. 127-158. Van Nostrand Reinhold, New York.

Léger P. & Sorgeloos P. (1992) Optimized feeding regimes in shrimp hatcheries. In: *Marine Shrimp Culture: Principles and Practices* (ed. by A.R. Fast & L.J Lesters), pp. 225-244. Elsevier Science Publishers Amsterdam, the Netherlands.

Léger P., Bengtson D.A., Simpson K.L. & Sorgeloos P. (1986) The use and nutritional value of *Artemia* as a food source. *Oceanographic and Marine Biology Annual Review* **24**, 521–623.

Leung P.S., Tran L. T. & Fast A.W. (2000) A logistic regression of risk factors for disease occurrence on Asian shrimp farms. In: *Economics and management of shrimp and carp farming in Asia: A collection of research papers based on the ADB/NACA Farm Performance Survey*. Bangkok: Network of Aquaculture Centres in Asia Pacific. 224pp.

Li Z-Y, He L-M., Wu J. & Chen, J.J. (2005) Study on the predominant bacterial community in prawn based on 16S rDNA PCR-DGGE Fingerprint. *Microbiology* **3**, 44-49.

Liao I.C. (1985) A brief review of the larval rearing techniques of penaeid prawns. In: *Proceedings of the First International Conference on the Culture of Penaeid Prawns/Shrimps* (ed. by Y. Taki, J.H. Primavera & J.A. LLobrera), pp. 65-78. Iloilo City, Phillipines.

Lightner D.V. (1993) Diseases of cultured penaeid shrimps. In: *Handbook of Mariculture* (ed. by J.P. McVey), pp. 393–486. CRC Press, Boca Raton.

Lightner D.V.(1996) A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp. World Aquaculture Society, Baton Rouge, Louisiana, 1996.

Lin H., Thuet P., Trilles J.-P., Mounet-Guillaume R. & Charmantier, G. (1993) Effects of ammonia on survival and osmoregulation of various developmental stages of the shrimp *Penaeus japonicus*. *Marine Biology* **117**, 591-598.

Lin Y.C. & Chen J.C. (2001) Acute toxicity of ammonia on *Litopenaeus vannamei* Boone juveniles at different salinity levels. *Journal of Experimental Marine Biology and Ecology* **259**, 109-119.

Lin Y.C. & Chen J.C. (2003) Acute toxicity of nitrite on *Litopenaeus vannamei* (Boone) juveniles at different salinity levels. *Aquaculture* **224**, 193-201.

Link W., Dixkens C., Singh M., Schwall M. & Melchinger A.E. (1995) Genetic diversity in European and Mediterranean faba bean germ plasm revealed by RAPD markers. *Theoretical and Applied Genetics* **90**, 27-32.

Liu F. (1991) Larviculture of Chinese white shrimp *Penaeus orientalis* in the People 's Republic of China. In: *Larvi '91-Fish and Crustacean Larviculture Symposium* (ed. by P. Lavens, P. Sorgeloos, E. Jaspers & F. Ollevier). European Aquaculture Society, Special Publication no. 15, Gent, Belgium.

Losordo T.M. & Westers H. (1994) System carrying capacity and flow estimation. In: Aquaculture Water Reuse Systems: Engineering Design and Management (ed. by M.B.Timmons & T.M. Losordo), pp. 9-60. Elsevier, Amsterdam.

Losordo, T.M., Masser, M., Rakocy, J., 1992. Recirculating aquaculture tank production systems, and overview of critical considerations. Southern Regional Aquaculture Center and North Carolina Cooperative Extension Service, SRAC Publication no. 451.

Lowther A. (2007) Highlights from the FAO Database on Aquaculture Statistics. *FAO Aquaculture Newsletter* **38**, 20-21

Loya-Javellana G.N. (1989) Ingestion, saturation and growth responses of *Penaeus monodon* larvae to food density. *Aquaculture* **81**, 329-336.

Lubzens E., Minkoff G., Zmora O. & Barr Y. (1997) Mariculture in Israel-past achievements and future directions in raising rotifers as food for marine fish larvae. *Hydrobiologia* **358**, 13-20.

Lubzens E., Zmora O. & Barr Y. (2001) Biotechnology and aquaculture of rotifers. *Hydrobiologia* **446/447**, 337-353.

Magallón-Barajas F., Servín R., Portilllo C., García J. & López B. (2006) Daily variation in short-term static toxicity of unionized ammonia in *Litopenaeus vannamei* (Boone) postlarvae. *Aquaculture Research* **37**, 1406-1412.

Martin J.L.M., Veran Y., Guelorget O. & Pham D. (1998) Shrimp rearing: stocking density, growth, impact on sediment, waste output and their relationships studied through the nitrogen budget in rearing ponds. *Aquaculture* **164**, 135-149.

Martins C.I.M., Eding E.H., Verdegem M.C.J., Heinsbroek L.T.N., Schneider O., Blancheton J.P., Roque d' Orbcastel E. & Verreth J.A.J. (2010) New developments in recirculating aquaculture systems in Europe: A perspective on environmental sustainability. *Aquacultural Engineering* **43**, 83-93.

Martins T.G., Cavalli R., Martion R.C., Rezende E.M. & Wasielesky W.Jr. (2006) Larviculture output and stress tolerance of *Farfantepenaeus paulensis* postlarvae fed *Artemia* containing different fatty acids. *Aquaculture* **252**, 525-533.

McIntosh D., Baijing J.B., Forward B.S., Puvanendran V., Boyce D. & Ritchie R. (2008) Culture independent characterization of the bacterial populations associated with cod (*Gadus morhua* L.) and live feed at an experimental hatchery facility using denaturing gradient gel electrophoresis. *Aquaculture* **275**, 42-50.

Menasveta P., Aranyakanonda P., Rungsupa S. & Moree N. (1989) Maturation and larviculture of penaeid prawns in closed recirculating seawater systems. *Aquacultural Engineering* **8**, 357-368.

Menasveta P., Fast A.W., Piyatiratitivorakul S. & Rungsupa S. (1991) An improved closed seawater recirculation maturation system for giant tiger prawn *Penaeus monodon* Fabricius. *Aquacultural Engineering* **10**,173–181.

Millamena O.S., Casalmir C.M. & Subosa P.F. (1991) Performance of recirculating systems for prawn hatchery and broodstock maturation tanks. *Aquacultural Engineering* **10**, 161-171.

Mishra J.K., Samocha T.M., Patnaik S., Speed M., Gandy R.L. & Ali A.M. (2008) Performance of an intensive nursery system for the Pacific white shrimp, *Litopenaeus vannamei*, under limited discharge condition. *Aquacultural Engineering*, **38** (1), 2-15.

Mock C.R. & Neal R.A. (1974) Penaeid shrimp hatchery systems. In: *FAO/CARPAS Symposium on Aquaculture in Latin America*, Montevideo, Uruguay, Nov. 26-Dec.2, 1974, 9 pp.

Mock C.R., Neal R.A. & Salser B.R. (1973) A closed raceway for the culture of shrimp. In: *Proceedings of the Fourth Annual Workshop*, World Mariculture Society. Contribution No. 363, National Marine Fisheries Service Gulf Coastal Fisheries Center, Galveston, Texas, pp. 247-259.

Mock C.R., Revera D.B. & Fontaine C.T. (1980) The larval culture of *Penaeus stylirostris* using modifications of the Galveston Laboratory technique. *Journal of the World Aquaculture Society* **11**, 102-117.

Mock C.R., Ross L.R. & Salser B.R. (1977) Design and preliminary evaluation of a closed system for shrimp culture. *Journal of the World Aquaculture Society* **8**, 335-369.

Moller T. H., Jones D. A. & Gabbot P.A. (1977) Further developments in the microencapsulation of diets for marine animals used in aquaculture. In: *Microencapsulation: new techniques and application /Proceedings of the 3rd International Symposium Microencapsulation* (ed. by T. Kondo), pp. 223-229. Tech. Inc., Tokyo.

Montaño M. & Navarro J.C. (1996) Fatty acids of wild and cultured *Penaeus vannamei* larvae from Ecuador. *Aquaculture* **142**, 259-268.

Morales V. (1993) Levantamiento larvario de camarones penaeidos. *Cartilla Pradepesca*. 38 pp.

Moriarty D. (1999) Disease Control in Shrimp Aquaculture with Probiotic Bacteria. In: *Microbial Biosystems: New Frontiers. Proceedings of the 8th International Symposium on Microbial Ecology* (ed. by C.R. Bell, M. Brylinsky & P. Johnson-Green). Atlantic Canada Society for Microbial Ecology, Halifax, Canada.

Muyzer G. & Smalla K. (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Leeuwenhoek* **73**, 127-141.

Naessens E., Cobo M.L., Van Hauwaert A., Van Horenbeeck M. & Sorgeloos P. (1995) In: *Memorias del Segundo Congreso Ecuatoriano de Acuicultura* (ed. by J. Calderón & P. Sorgeloos), pp. 101-108. Advances of the *Artemia* Project, Improvement of white shrimp *Penaeus vannamei* larval production through feeding strategies based on live and formulated feed. Escuela Superior Politécnica del Litoral, Guayaquil-Ecuador.

Neal R.A. & Mock C.R. (1976) A model closed system for aquaculture incorporating the recycling of wastes. In: *Proceedings FAO Technical Conference on Aquaculture*. Kyoto, Japan. 26 May 1976. FAO, Rome, Italy.

Neal R.S., Coyle S.D., Tidwell J.H. & Boudreau B.M. (2010) Evaluation of stocking density and light level on the growth and survival of the Pacific White shrimp, *Litopenaeus vannamei*, reared in zero-exchange systems. *Journal of World Aquaculture Society* **41**, 533-544.

New M.B. (1976) A review of dietary studies with shrimp and prawns. *Aquaculture* **9**, 101-144.

Nga B.T., Lürling M., Peeters E.T.H.M., Roijackers R., Scheffer M. & Nghia, T.T. (2005) Chemical and physical effects of crowding on growth and survival of *Penaeus monodon* Fabricius post-larvae. *Aquaculture* **246**, 455-465.

Nghia T.T. (2004) Optimization of mud crab (*Scylla paramamosain*) larviculture in Vietnam. PhD thesis, Faculty of Agricultural and Applied Biological Sciences, Ghent, Belgium. 192 pp.

Nhu V.C., Dierckens K., Nguyen T.H., Tran M.T. & Sorgeloos, P. (2009) Can umbrella-stage *Artemia franciscana* substitute enriched rotifers for Cobia (*Rachycentron canadum*) fish larvae? *Aquaculture* **289**, 64-69.

Nogami K. & Maeda M. (1992) Bacteria as biocontrol agents for rearing larvae of the crab *Portunus trituberculatus*. *Canadian Journal of Fisheries and Aquatic Sciences*. **49**, 2373-2376.

Ostrensky A. & Poersch L.H. (1992) Toxicidade aguda do nitrito na larviculturado camarao-rosa *Penaeus paulensis* Pérez-Farfante, 1967. *Nerítica curitibe* **7**, 101-107.

Ostrensky A. & Wasielesky W. Jr. (1995) Acute toxicity of ammonia to various life stages of the São Paulo shrimp, *Penaeus paulensis* Pérez-Farfante, 1967. *Aquaculture* **132**, 339-347.

Paffenhoffer G.A. (1971) Grazing and ingestion rates of nauplii, copepodids, and adults of the marine planktonic copepod *Calanus helgolandicus*. *Marine Biology* **11**, 286-279.

Palacios E., Perez-Rostro C.I., Ramírez J.L., Ibarra A.M. & Racotta I.S. (1999) Reproductive exhaustion in shrimp (*Penaeus vannamei*) reflected in larval biochemical composition, survival and growth. *Aquaculture* **171**, 309–321.

Palomino G., Contreras F., Sanchez A. & Rosas C. (2001) Density and water Exchange-dependent growth and survival of *Litopenaeus setiferus* postlarvae. *Journal of the World Aquaculture Society* **32**, 167-176.

Parado-Estepa F.D. (1998) Survival of *Penaeus monodon* postlarvae and juveniles at different salinity and temperature levels. *The Israeli Journal of Aquaculture* **50** (4), 174-183.

Paslow J., Harrison P. & Thompson P. (1985) Ammonium uptake by phytoplankton cells on a filter: a new technique. *Marine Ecology Progress Series* **25**,121-129.

Pérez-Farfante I. & Kensley B. (1997) Penaeoid and sergestoid shrimps and prawns of the world. Keys and diagnoses for the families and genera. *Mémoires du Muséum National d'Histoire Naturelle* **175,** 233 pp.

Pico C. (2004) Selección de una cepa patógena para pruebas de desafío en postlarvas de *Litopenaeus vannamei*. Tesis de Biólogo Marino. Pontificia Universidad Católica del Ecuador. Manabí-Ecuador.

Piña P., Voltolina D., Nieves M. & Robles M. (2005) Survival, development and growth of the Pacific white shrimp *Litopenaeus vannamei* protozoea larvae, fed with monoalgal and mixed diets. *Aquaculture* **253**, 523-530.

Poston H. A. & Williams R.C. (1988) Interrelations of oxygen concentration, fish density, and performance of Atlantic salmon in an ozonated water reuse system. *The Progressive Fish-Culturist* **50,** 69-76.

Poxton M.G., Murray K.R. & Linfoot B.T. (1982) The growth of turbot (*Scophthalmus maximus* L.) in recirculating systems. *Aquacultural Engineering* **1**, 23-34.

Prayitno S.B. & Latchford J.W. (1995) Experimental infections of crustaceans with luminous bacteria related to *Photobacterium* and *Vibrio*: Effect of salinity and pH on infectiosity. *Aquaculture* **132**, 105–112.

Provenzano A.J. Jr. & Winfield J.G. (1987) Performance of a recirculating fish production system stocked with tilapia hybrids. *Aquacultural Engineering* **6**, 15-26.

Racotta I.S. & Hernández-Herrera R. (2000) Metabolic responses of the white shrimp, *Penaeus vannamei*, to ambient ammonia. *Comparative Biochemistry and Physiology* **125**, 437-443.

Racotta I.S., Palacios E. & Ibarra A.M. (2003) Shrimp larval quality in relation to broodstock condition. *Aquaculture* **227**, 107-130.

Racotta I. S., Palacios E., Hernández-Herrera R., Bonilla A., Pérez-Rostro C.I. & Ramírez J.L. (2004) Criteria for assesing larval and postlarval quality of Pacific White shrimp (*Litopenaeus vannamei*, Boone, 1931). *Aquaculture* **233**, 181-195.

Ray W.M. & Chien Y.H. (1992) Effects of stocking density and aged sediment on tiger prawn, *Penaeus monodon*, nursery system. *Aquaculture* **104** (3-4), 231-248.

Rees J.F., Curé K., Piyatiratitivorakul S., Sorgeloos P. & Menasveta P. (1994) Highly unsaturated fatty acid requirements of *Penaeus monodon* postlarvae: an experimental approach based on *Artemia* enrichment. *Aquaculture* **122**, 193-207.

Reid B. & Arnold C.R. (1992) The intensive culture of the Penaeid shrimp *Penaeus vannamei* Boone in a recirculating raceway system. *Journal of the World Aquaculture Society* **23**, 146–153.

Rodríguez J., Cedeño R., Bayot B., Echeverría F., Teixeira J., Valladares A., Aguayo D. & Sonnenholzner S. (2011) Effects of the *Vibio alginolyticus* probiotic, β-1,3/1,6-glucans and temperature on shrimp production. *Dynamic Biochemistry, Process Biotechnology and Molecular Biology* **5**. Special Issue1. Global Science Books.

Rodríguez J., Espinosa Y., Echeverría F., Cárdenas G., Román R. & Stern S. (2007) Exposure to probiotics and β-1,3/1,6-glucans in larviculture modifies the immune response of *Penaeus vannamei* juveniles and both the survival to White Spot Syndrome Virus challenge and pond culture. *Aquaculture* **273**, 405-415.

Roiha I.S., Otterlei E. & Samuelsen O.B. (2011) Evaluating bioencapsulation of florfenicol in rotifers (*Brachionus plicatilis*). *Aquaculture Research* **42**, 1110-1116.

Rombaut G., Suantika G., Boon N., Maertens S., Dhert P., Top E., Sorgeloos P. & Verstraete W. (2001) Monitoring of the evolving diversity of the microbial community present in rotifer culture. *Aquaculture* **198**, 237-252.

Ronquillo J.D., Matias J.R., Saisho T. & Yamasaki S. (1997) Culture of *Tetraselmis tetrathele* and its utilization in the hatchery production of different penaeid shrimps in Asia. *Hydrobiologia*, **358**, 237-244.

Roque A., Turnbull J.F., Escalante G., Gomez-Gil B. & Alday-Sanz M.V. (1998) Development of a bath challenge for the marine shrimp *Penaeus* v*annamei* Boone, 1931. *Aquaculture* **169**, 283–290.

Rosenthal H. & Black E.A. (1993) Recirculation systems in aquaculture. In: *Proceedings of an Aquacultural Engineering Conference* (ed. by J.K. Wang), pp. 284-294. American Society of Agricultural Engineers, 21-23 June 1993, St. Joseph, MI, USA.

Salser B., Mahler L., Lightner D., Ure J., Donald D., Brand C., Stamp N., Moore D. & Colvin B. (1978) Controlled environment aquaculture of penaeids. In: *Drugs and food from the sea* (ed. by P.N. Kaul & C.J. Studerman), pp. 345-355. University of Oklahoma, Norman, Oklahoma, USA.

Sammouth S., Roque d'Orbcastel E., Gasset E., Lemarié G., Breuil G., Marino G., Coeurdacier J.L., Fivelstad S. & Blancheton J.P. (2009) The effect of density on sea bass (*Dicentrarchus labrax*) performance in a tank-based recirculating system. *Aquacultural Engineering* **40**, 72-78.

Samocha T.M., Uziel N. & Browdy C.L. (1989) The effect of feeding two prey organisms, nauplii of *Artemia* and rotifers, *Brachionus plicatilis* (Muller), upon survival and growth of larval marine shrimp, *Penaeus semisulcatus* (de Haan). Aquaculture 77, 11-19.

Samocha T.M. & Guajardo, H. (1998) A simple stress test for *Penaeus vannamei* postlarvae. *Aquaculture* **165**, 233-242.

Samocha T.M. & Lawrence A.L. (1992) Shrimp nursery systems and management. In: Proceeding of the Special Session on Shrimp Farming (ed. by J. Wyban), pp. 87–105. World Aquaculture Society, Baton Rouge, LA, USA.

Samocha T.M., Hamper L., Emberson C.R., Davis A.D., McIntosh D., Lawrence A.L. &Van Wyk P.M. (2002) Review of Some Recent Developments in Sustainable Shrimp Farming Practices in Texas, Arizona, and Florida. *Journal of Applied Aquaculture* **12**, 1-42.

Samocha T.M., Lawrence A.L. & Bray W.A. (1993). Design and operation of an intensive nursery raceway system for penaeid shrimp. In: CRC Handbook of Mariculture, Vol. 1. Crustacean Aquaculture (ed. by J.P. McVey), pp. 173–210. CRC Press, Boca Raton, FL, USA.

Samocha T.M., Uziel N. & Browdy C.L. (1989) The effect of feeding two prey organisms, nauplii of *Artemia* and rotifers *Brachionus plicatilis* (Muller) upon survival and growth of larval marine shrimp *Penaeus semisulcatus* (de Haan). *Aquaculture* **77**, 11–19.

San Miguel L. (1996) Caracterización de una bacteria "probiótica " en *Penaeus vannamei* y estudio *in vivo* de la interacción con una bacteria patógena. *Tesis de Acuicultor*. Escuela Superior Politécnica del Litoral. Guayaquil-Ecuador. 124 pp.

Sanchez-Zazueta E., Hernández J.M. & Martinez-Cordero F.J. (2011) Stocking density and date decisions in semi-intensive shrimp *Litopenaeus vannamei* (Boone, 1931) farming: a bioeconomic approach. *Aquaculture Research* **in press**.

Sandaa R.-A., Magnesen T., Torkildsen L. & Bergh, Ø. (2003) Characterisation of the bacterial community associated with early stages of great scallop (*Pecten maximus*), using denaturing gradient gel electrophoresis (DGGE). *Systematic and Applied Microbiology* **26**, 302-311.

Sandifer P.A. & Smith T.I.J. (1975) Effects of population density on growth and survival of *Macrobrachium rosenbergii* reared in recirculating water management systems. *Journal of the World Aquaculture Society* **6**, 43-53.

Saulnier D., Haffner P., Goarant C., Levy P. & Ansquer D. (2000) Experimental infection models for shrimp vibriosis studies: a review. *Aquaculture*, **191** (1-3), 133-144.

Schaefer H. & Muyzer G. (2001) Denaturing Gradient Gel Electrophoresis in Marine Microbial Ecology. *Methods in microbiology* **30**: 425-466.

Smalla K., Cresswell N., Mendoca-Hagler L.C., Wolters A. & Van Elsas J.D. (1993) Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. *Journal of Applied Bacteriology* **74**, 78-85.

Soares R., Peixoto S., Wasielesky W. & D'Incao F. (2006) Effect of different food items on the survival and growth of *Farfantepenaeus paulensis* (Pérez-Farfante 1967) postlarvae. *Aquaculture Research* **37**, 1413-1418.

Solórzano L. (1983) Métodos de análisis químicos utilizados en el curso Latinoamericano de post-grado: "Instrumentación y Análisis químicos de agentes contaminantes en el mar". *Boletín Científico y Técnico* **7**, 14-19.

Solórzano L. (1969) Determination of ammonia in natural waters by the phenol-hypochlorite method. *Limnology and Oceanography* **14**, 799-801.

Sookying D., D. Silva F.S., Davis D.A. & Hanson T.R. (2011) Effects of stocking density on the performance of Pacific white shrimp *Litopenaeus vannamei* cultured under pond and outdoor tank conditions using a high soybean meal diet. *Aquaculture* **319**, 232-239.

Sorgeloos P. (1980) The use of the brine shrimp *Artemia* in aquaculture. In: *The brine shrimp Artemia, Volume 3, Ecology, Culturing, use in aquaculture* (ed. by G. Persoone, P. Sorgeloos, O. Roels, & E. Jaspers), pp. 25-46. Universa Press, Wetteren, Belgium.

Sorgeloos P., Coutteau P., Dhert P., Merchie G. & Lavens P. (1998) Use of brine shrimp *Artemia* spp., in larval crustacean nutrition: A review. *Reviews in Fisheries Science* **6**, 55-68.

Sorgeloos P., Lavens P., Léger Ph. & Tackaert W. (1991) State of the art in larviculture of fish and shellfish. In: *Larvi' 91-Fish & Crustacean Larviculture Symposium* (ed. by P. Lavens, P. Sorgeloos, E. Jaspers & F. Ollevier), pp. 484-486. European Aquaculture Society, Special Publication No. 15, Gent, Belgium.

Sprague J.B. (1971) Measurement of pollutant toxicity to fish. III. Sublethal effects and "safe" concentrations. *Water Research* **5**, 245-266.

Sturmer L.N., Samocha T.M. & Lawrence A.L. (1992) Intensification of penaeid nursery systems. In: *Marine Shrimp Culture: Principles and Practices* (ed. by A.W. Fast & L.J. Lester), pp. 321- 344. Elsevier, Amsterdam.

Suantika G. (2001) Development of a Recirculation System for the mass culturing of the Rotifer *Brachionus plicatilis.* Thesis submitted in fulfillment of the requirements for the degree of Doctor (Ph.D.) in Applied Biological Sciences. Faculty of Agriculture. University of Gent, Belgium.

Suantika G., Dhert P., Nurhudah M. & Sorgeloos P. (2000) High-density production of the rotifer *Brachionus plicatilis* in a recirculation system: consideration of water quality, zootechnical and nutritional aspects. *Aquacultural Engineering* **21**, 201–214.

Suantika G., Dhert P., Rombaut G., Vandenberghe J., De Wolf T. & Sorgeloos P. (2001) The use of ozone in a high density recirculation system for rotifers. *Aquaculture* **201**, 35-49.

Suantika G., Dhert P., Sweetman E., O' Brien E. & Sorgeloos P. (2003) Technical and economical feasibility of a rotifer recirculation system. *Aquaculture* **227**, 173-189.

Subasinghe R.P. (2005) Epidemiological approach to aquatic animal health management: opportunities and challenges for developing countries to increase aquatic production through aquaculture. *Preventive Veterinary Medicine* **67**, 117–124.

Sung H.H., Hsu S.F., Chen C.K., Ting Y.Y. & Chao W.L. (2001) Relationships between disease outbreak in cultured tiger shrimp (*Penaeus monodon*) and the composition of *Vibrio* communities in pond water and shrimp hepatopancreas during cultivation. *Aquaculture* **192**, 101-110.

Sutterlin A.M., Couturier C.Y. & Devereaux T. (1984) A recirculation system using ozone for the culture of Atlantic salmon. *The Progressive Fish-Culturist* **46,** 239-244.

Suttle C. & Harrison P. (1988) Rapid ammonium uptake by freshwater phytoplankton. *Journal of Phycology* **24**, 13-16.

Tackaert W., Abelin P., Dhert P. & Sorgeloos P. (1989) Stress resistance in postlarval penaeid shrimp reared under different feeding procedures. *Journal of the World Aquaculture Society* **20**, 74-79.

Terjesen B.F., Ulgenes Y., Faera S.O., Summerfelt S.T., Brunsvik P., Baeverfjord G., Nerland S., Takle H., Norvik O.C. & Kittelsen A. (2008) RAS research facility dimensioning and design: a special case compared to planning production systems. In *Aquaculture Engineering Society issues forum proceedings*. Roanoke, Virginia, USA, July 23-24.

Teshima S., Ishikawa M. & Koshio S. (2000) Nutritional assessment and feed intake of microparticulate diets in crustaceans and fish. *Aquaculture Research* **31**, 691–702.

Thach T., Truong N., Wille M. & Sorgeloos P. (2005) Recirculation systems: Sustainable alternatives for backyard shrimp hatcheries in Asia? *Aquaculture Asia Magazine*, July-September 2005, 32-33.

Timmons M.B., Ebeling J.M.& Piedrahita R.H. (2009) Acuicultura en sistemas de recirculación. *NRACE Publication No 101-2009,* Ithaca, New York. 958 pp.

Timmons M.B., Ebeling J.M., Wheaton F., Summerfelt S. & Vinci B.D. (2002) Recirculating Aquaculture System. *NRAC Publication No 001-2002*, Ithaca, New York. 769 pp.

Timmons M.B. & Losordo T.M. (1994) *Aquaculture Water Reuse Systems: Engineering Design and Management.* Elsevier, Amsterdam.

Treece G.D. (2000). Shrimp culture. In: *Encyclopedia of aquaculture* (ed. by R.R Stickney), pp 806-868. New York, USA.

Treece G.D. & Fox J.M. (1993) Design, Operation and Training Manual for an Intensive Culture Shrimp Hatchery. Texas A & M University, Sea Grant Collection Program, Galveston. 187 pp.

Truong N., Tran B., Vu U., Thach T. & To T. (2009) Research on the larviculture of tiger shrimp *Penaeus monodon* with high stocking densities using recirculating biofilter system. In: *Larvi '09-Fish & Shellfish Larviculture Symposium* (ed. by C.I. Hendry, G. Van Stappen, M. Wille & P. Sorgeloos). European Aquaculture Society, Special Publication No. 38, Oostende, Belgium, 2009.

Tseng K. & Wu K. (1998) Culture of *Penaeus monodon* in a recirculating system. *Aquacultural Engineering* **17**, 138-147.

Tseng K.F., Su H.M. & Su M.S. (1998) Culture of *Penaeus monodon* in a recirculating system. *Aquacultural Engineering* **17**, 138-147.

van Rijn (1996) The potential for integrated biological treatment systems in recirculating fish culture-A review. *Aquaculture* **139**, 181-201.

Van Wyk P.M., Davis-Hodgkins M., Laramore C.R., Main K.L., Mountain J. & Scarpa J. (1999) Farming of Marine Shrimp in Recirculating Freshwater Systems. Florida Department of Agriculture and Consumer Services, Tallahassee, FL, 194 pp.

Vandenberghe J., Li Y., Verdonck L., Li J., Sorgeloos P., Xu H. S. & Swings, J. (1998) Vibrios associated with *Penaeus chinensis* (Crustacea: Decapoda) larvae in Chinese shrimp hatcheries. *Aquaculture* **169**, 121-132.

Vandenberghe J., Verdonck L., Robles-Arozarena R., Rivera G., Bolland A., Balladares M., Gomez-Gil B., Calderón J., Sorgeloos P. & Swings J. (1999) Vibrios associated with *Litopenaeus vannamei* larvae, postlarvae, broodstock, and hatchery probionts. *Applied and Environmental Microbiology* **65**, 2592-2597.

Velasco M., Lawrence A. L. & Castille F.L. (1999) Effect of variations in daily feeding frequency and ration size on growth of shrimp, *Litopenaeus vannamei* (Boone), in zero-water exchange culture tanks. *Aquaculture* **179**, 141-148.

Velasco M., Lawrence A.L. & Neil W.H. (2001) Comparison of survival and growth of *Litopenaeus vannamei* (Crustacea: Decapoda) postlarvae reared in static and recirculatin cultura systems. *Texas Journal of Science* **53**, 227-238.

Verschuere L., Rombaut G., Sorgeloos P. & Verstraete, W. (2000) Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews* **64**, 655-671.

Villalón J.R. (1991) Practical manual for semi-intensive commercial production of marine shrimp. Texas A&M University, Sea Grant Program, Galveston. 103 pp.

Vinatea L. & Andreatta E.A. (1997) Comparative study of continuous and static water renewal strategies in the larviculture of *Penaeus paulensis* (Pérez Farfante, 1967) associated with high stocking densities and different water renewal rates. *Aquaculture* **154**, 247-259.

Wang K. & Ma S. (1990) Advances in larval rearing techniques for *Penaeus chinensis* in China. In: *The culture of Cold-tolerant Shrimp* (ed. by K.L. Main & W. Fulks), pp. 42-48. The Oceanic Institute, Honolulu, HI, USA.

Whitfield M. (1974) The hydrolysis of ammonium ions in sea water. A theorical study. *Journal of Marine Biology* **54**, 565-580.

Wickins J.F. (1976) The tolerance of warm-water prawns to recirculated water. *Aquaculture*, **9**: 19-37.

Wickins J.F. & Lee D.O'C. (2002) *Crustacean Farming: Ranching and Culture*, Second Edition, Blackwell Publishing Ltd, Oxford, UK.

Wilkenfeld J.S. (1984) Survival, metamorphosis and growth of Penaeid shrimp larvae reared on a variety of algae and animal foods. *Journal of World Mariculture Society* **15**, 31-49.

Wilkenfeld J.S. (1992) Commercial hatchery status report: an industry panel viewpoint. In: *Proceedings of the special session on shrimp farming* (ed. by J. Wyban). World Aquaculture Society, Baton Rouge, LA, USA, pp. 71-86.

Wilkenfeld J.S., Fox J.M. & Lawrence A.L. (1981) Consumption of frozen and live *Artemia* by protozoea of *Penaeus setiferus*. *Journal of the World Mariculture Society* **12**, 250-259.

Williams A.S., Davis D.A. & Arnold C.R. (1996) Density dependent growth and survival of *Penaeus setiferus* and *Penaeus vannamei*. *Journal of the World Aquaculture Society* **27**, 107–112.

Williams R.C., Hughes S.G. & Rumsey G.L. (1982) Use of ozone in a water reuse system for salmonids. *The Progressive Fish-Culturist* **44**, 102-105.

Wouters R. & Van Horenbeeck T. (2003) Larval Shrimp Feeds, Current Status. In: *Responsible Aquaculture for a Secure Future, Proceedings of a Special Session on Shrimp Farming* (ed. by E. J. Darryl), pp. 90-109.World Aquaculture 2003, Recife, Brazil. The World Aquaculture Society, Baton Rouge, Louisiana, USA.

Wouters R., Cobo M.L., Dhont J. & Wille M. (2009) Developments in feed formulations, feeding practices and culture techniques for marine shrimp larvae. In: *The Rising Tide, Proceedings of the Special Session on Sustainable Shrimp Farming, Aquaculture 2009* (ed. by C.L. Browdy & E. J. Darryl), pp. 79-91. The World Aquaculture Society, Baton Rouge, Louisiana, USA.

Wouters R., Van Hauwaert A., Naessens E., Ramos X., Pedrazzoli A. & Lavens P. (1997) The effect of dietary n-3 HUFA and 22:6n-3/20:5n-3 ratio on white shrimp larvae and postlarvae. *Aquaculture International* **5**, 113-126.

Yúfera M., Rodríguez A. & Lubián L.M. (1984) Zooplankton ingestion and feeding behavior of *Penaeus kerathurus* larvae reared in the laboratory. *Aquaculture* **42**, 217-224.

Zherdmant M.T., San Miguel L., Serrano J., Donoso E. & Mialhe E. (1997) Estudio y utilización de probióticos en el Ecuador. *Panorama Acuícola* **2**, 28.

Zin K.Y. & Chu C.J. (1991) Acute toxicity of ammonia to *Penaeus japonicus* Bate juveniles. *Aquaculture Research* **22**, 259-263.

Zmora O. & Shpigel M. (2006) Intensive mass production of *Artemia* in a recirculated system. *Aquaculture* **255**, 488-494.

SUMMARY

SUMMARY

The production of marine crustacean species - dominated by white leg shrimp *Litopenaeus vannamei* – is expected to grow in the coming decades (FAO, 2012). This growth will require a shift toward more intensive farming practices as well as increasing numbers of good quality larvae from commercial hatcheries. Larval rearing is identified as the weak link in this process, currently already unable to provide constant supply of larvae. Hatcheries applying traditional aquaculture systems (TAS) are facing problems with the degradation of water quality and the occurrence of diseases. The development of recirculating aquaculture systems (RAS) may be very useful for the intensification of larviculture. RAS could increase the larval output thus enhancing the profitability of hatchery production, while minimizing water discharge into the environment and reducing the risk of pathogen introduction into the culture system. As such, the objective of the present work is to contribute to the development of new culture techniques for the intensive production of *L. vannamei* larvae through the use of RAS.

In the first part of the present thesis (**Chapter III**), we determined the ammonia tolerance of *L. vannamei* larvae at different developmental stages and sub-stages. Results from 24h short-term toxicity tests with *L.vannamei* larvae exposed to increasing concentrations of Total Ammonia showed a progressive tolerance to ammonia with larval development until mysis stage. A pronounced decrease in ammonia tolerance was observed at postlarval stage PL1. For *L. vannamei* larviculture, it is recommended that TAN levels should not exceed 0.42 mg L^{-1} TAN, equivalent to 0.06 mg L^{-1} NH_3-N, in order to avoid ammonia toxicity.

In **Chapter IV**, a first attempt of intensification of larval rearing techniques was made in a series of experiments through the implementation of an experimental RAS. The experiments evaluated the effects of stocking density (750 to 2000 larvae L^{-1}), feeding strategy (different concentrations of live and artificial food and continuous feeding), and recirculation rate (250 and 1000% day^{-1}) on the survival and growth of *L. vannamei* larvae. Increasing density led to increased mortality, unless the feed ration

was increased to compensate losses through the recirculation system. Compared to the standard stocking densities applied in TAS, an increase of more than 20% in survival was obtained with the density of 1000 larvae L^{-1} when artificial diet ration was increased and when a continuous supply of microalgae concentrates was provided during the early larval stages. Our results suggest that increasing water exchange rate does not improve larval survival or growth. The combination of a stocking density of 1000 larvae L^{-1} and a water exchange rate of 500% day^{-1} consistently presented the highest mean survival and biomass output. In addition, it was demonstrated that RAS were able to maintain acceptable ammonia and nitrite levels.

In a first pilot-scale experiment (**Chapter V**), a simple compact prototype RAS of 1 m^3 was used with an initial stocking density of 1000 larvae L^{-1}, applying a water exchange rate of 500% day^{-1}. The high-density culture was compared with a TAS using a stocking density of 100 larvae L^{-1}. A lower survival (50±7% in RAS versus 62±8% in TAS) was recorded for the intensive larval RAS, probably due to the infestation with filamentous bacteria coming from the live feed, especially rotifers. Dry weights were reduced, but larval output (number of larvae produced per liter) and biomass in RAS was eight-fold and five-fold of that in TAS, respectively. Bacteria load was higher in the larvae reared in the RAS compared to those reared in the TAS already before the recirculation started. During the culture period, it was possible to identify three main groups of bacteria among the different DGGE-profiles in both culture systems. However, a high variability among bacterial communities of the different systems and within systems was observed.

To overcome the problem of fouling (a problem that occurred when applying rotifers during previous experiments), in a trial described in **Chapter VI**, umbrella-stage *Artemia* was evaluated as a food source for the early stages of *L. vannamei* and its optimal feeding concentration for each larval stage was also assessed, under traditional culture conditions. The observations showed the highest consumption rate of umbrella-stage *Artemia* at mysis stage, corresponding to a two-fold increment in ingestion from zoea to mysis stage and suggesting that *L. vannamei* shift to a raptorial feeding mode at the mysis stage. Including umbrella-stage *Artemia* in the feeding regime of *L. vannamei*

larvae starting from the early Z2 sub-stage yielded good results in terms of growth and survival while filling the size gap between microalgae and *Artemia*. Therefore, this new prey item was also evaluated in the intensive larval RAS (**Chapter VII)**. The results show that survival and growth were similar to those obtained in TAS, and higher than those obtained in the experiments where rotifers were used. Survival was comparable to those obtained in TAS (71% in RAS and 74% in TAS). However, the stocking density used in RAS was 10 times higher, yielding an average output of 710 larvae L^{-1} compared to only 74 larvae L^{-1} in the TAS. Higher dry weights and higher biomass were also obtained. The condition of the larvae was estimated by means of a challenge test with a pathogenic bacterium and by monitoring postlarval performance in a subsequent nursery culture test. These tests indicated that there was no difference in condition between larvae reared intensively in a RAS (including umbrella-stage *Artemia* in the feeding regime) and larvae reared in TAS. Finally, it was calculated that the investment cost and annual production cost for intensive *L. vannamei* larviculture using RAS could be reduced by 25% as compared to the TAS.

In conclusion, the findings of this thesis demonstrate that the intensification of *L. vannamei* larviculture is feasible through the implementation of RAS, and proved the effectiveness of intensive RAS in producing high quantities of good quality larvae, maximizing biomass, controlling water quality, reducing operating costs, and increasing biosecurity.

SAMENVATTING

SAMENVATTING

De verwachting is dat de productie van mariene crustaceën – gedomineerd door de witpootgarnaal *Litopenaeus vannamei* – gestaag zal blijven groeien tijdens het volgende decennium (FAO, 2012). Deze groei zal een verschuiving vereisen naar meer intensieve teelttechnieken en zal leiden tot een toenemende vraag naar larven van goede kwaliteit. De larvicultuur (de kweek van garnalenlarven in commerciële broedhuizen) werd geïdentificeerd als de zwakke schakel in dit intensifiëringsproces; de sector is nu reeds niet meer in staat om een constante aanvoer van garnalenlarven te verzekeren. Broedhuizen die traditionele aquacultuur systemen (TAS) toepassen, worden geconfronteerd met problemen van waterkwaliteit en ziekten. De ontwikkeling van recirculatie aquacultuur systemen (RAS) kunnen zeer nuttig zijn voor de intensifiëring van de larvicultuur. RAS kunnen de larvale productie en dus ook een rendabiliteit van de broedhuizen verhogen. Ze kunnen de lozing van afvalwater in verminderen en het risico op de introductie van pathogenen in het teeltsysteem beperken. Het doel van dit onderzoek is bij te dragen tot de ontwikkeling van nieuwe teelttechnieken voor de intensieve productie van *L. vannamei* larven door het gebruik van RAS.

In het eerste deel van dit proefschrift (**hoofdstuk III**), werd de ammoniaktolerantie van *L. vannamei* larven vastgesteld in verschillende ontwikkelingsstadia en -substadia. Resultaten van toxiciteitstesten op korte termijn (24u) op *L. vannamei* larven - blootgesteld aan toenemende concentraties van ammoniak (gemeten als totaal ammoniak stikstof, TAN) - tonen een progressieve toename aan de tolerantie voor ammoniak tijdens de larvale ontwikkeling en dit tot aan het mysis stadium. Een uitgesproken vermindering in ammoniak tolerantie werd dan weer waargenomen bij het postlarvaal stadium PL1 t.o.v. het mysis stadium. Om ammoniaktoxiciteit te vermijden wordt er voor de larvicultuur van *L. vannamei* aanbevolen om het niveau 0.42 mg L^{-1} TAN niet te overschrijden. Dit komt overeen met 0,06 mg L^{-1} NH_3-N.

In **hoofdstuk IV** werd een eerste poging tot intensifiëring ondernomen tijdens drie experimenten waarin experimentele RAS werden gebruikt. De experimenten

evalueerden de invloed van de larvale densiteit (750 tot 2000 larven L^{-1}), het voederregime (verschillende concentraties levende en artificiële voeders, en continue voedering) en de snelheid van waterrecirculatie (250 en 1000% dag^{-1}) op de overleving en groei van *L. vannamei* larven. De sterfte nam toe naarmate de densiteit steeg, tenzij het voederrantsoen werd verhoogd om de verliezen door het recirculatiesysteem te compenseren. Vergeleken met de standaardpraktijk in TAS, werd in RAS bij een densiteit van 1000 larven L^{-1} een toename van meer dan 20% in overleving waargenomen indien het rantsoen aan articifiële voeding werd verhoogd en er een continue aanvoer van concentraten van microalgen werd toegediend tijdens de vroege larvale stadia. Onze resultaten suggereren dat het verhogen van de waterrecirculatie de larvale overleving of groei niet verbeteren. De combinatie van een densiteit van 1000 larven L^{-1} en een waterrecirculatie van 500% dag^{-1} leidde keer op keer tot de hoogste gemiddelde overleving en productie van biomassa. Bovendien werd aangetoond dat een RAS het niveau van ammoniak en nitriet op een aanvaardbaar peil kon handhaven.

In een eerste test op experimentele schaal (**hoofdstuk V**) werd gebruik gemaakt van een eenvoudig compact prototype RAS van 1 m3 met een initiële densiteit van 1000 larven L^{-1} en een waterrecirculatie van 500% dag^{-1}. Deze kweek met hoge densiteit werd vergeleken met een TAS met een densiteit van 100 larven L^{-1}. In het intensieve RAS werd een lagere overleving (50 ± 7% in RAS versus 62 ± 8% in TAS) bekomen, waarschijnlijk als gevolg van de besmetting met fillamenteuze bacteriën afkomstig van het levend voeder, voornamelijk van rotiferen. In het RAS was het drooggewicht lichtjes lager, maar de larvale productie (aantal geproduceerde larven per liter) en de biomassa waren respectievelijk acht en vijf maal hog+er dan in het TAS. Alvorens de waterrecirculatie aanving, was de bacteriële druk hoger in de larven geteeld in het RAS in vergelijking met het TAS. In beide teeltsystemen werden tijdens de teeltperiode drie groepen bacteriën geïdentificeerd binnen de verschillende DGGE-profielen. Er werd echter een grote variabiliteit in bacteriële gemeenschappen waargenomen zowel binnen als tussen de twee teeltsystemen.

Om het probleem van watervervuiling te vermijden (een probleem dat zich tijdens eerdere experimenten stelde door het aanwenden van rotiferen) werd in de studie

- beschreven in **hoofdstuk VI** - *Artemia* in umbrella-stadium geëvalueerd als voedselbron voor de vroege stadia van *L. vannamei*. Ook de optimale voedselconcentratie van dit *Artemia* stadium werd voor elk larvaal stadium beoordeeld; dit onder traditionele teeltomstandigheden. De waarnemingen toonden dat de hoogste inname van umbrella-stadium *Artemia* gebeurt op het ogenblik dat de larven zich in het mysis stadium bevinden; de inname is dubbel zo hoog in het mysis stadium dan in het zoea stadium. Dit suggereert dat de voedingsgewoonte van *L. vannamei* in het mysis stadium verschuift naar een gewoonte waarbij actief op dierlijke prooien wordt gejaagd. Het toevoegen van umbrella-stadium *Artemia* in het voedingsregime van *L. vannamei* larven vanaf het begin van het Z2 sub-stadium, leverde goede resultaten op in termen van groei en overleving en vult de leemte tussen de grootte van microalgen en die van *Artemia* nauplii. Daarnaast werd deze nieuwe prooi ook geëvalueerd in een intensieve RAS (**hoofdstuk VII**). De resultaten toonde een groei die vergelijkbaar was met diegene verkregen in het TAS. Ook de overleving was vergelijkbaar: 71% in het RAS tov 74% in het TAS. Aangezien de larvale densiteit in het RAS 10 maal hoger was dan in het TAS, was de gemiddelde productie 710 larven L^{-1} in het RAS in vergelijking tot 74 larven L^{-1} in het TAS. Ook het drooggewicht en de biomassa waren groter in het RAS. Het voederregime met umbrella-stadium *Artemia* resulteerde ook in een hogere groei en overleving dan die voorheen gemeten in experimenten waarbij rotiferen werden gebruikt. De conditie van de larven werd geschat door middel van een blootstellingstest aan een pathogene bacterie alsook door het volgen van de prestaties van de postlarven in een daaropvolgende kweekproef. Deze tests gaven aan dat er geen verschil was in kwaliteit tussen de larven gekweekt in intensieve RAS (inclusief met umbrella-stadium *Artemia* in het voedingsregime) en larven opgekweekt in TAS. Tot slot werd berekend dat de intensieve larvicultuur van *L. vannamei* in RAS de investering en jaarlijkse productiekosten zou kunnen verminderen met 25% ten opzichte van TAS.

Kortom, de bevindingen van dit proefschrift tonen aan dat de intensifiëring van de larvicultuur van *L. vannamei* haalbaar is via de implementatie van RAS. Ze bewezen ook de effectiviteit van intensieve RAS in het produceren van grote hoeveelheden van larven van goede kwaliteit, het maximaliseren van biomassa productie, het controleren

van de waterkwaliteit, het verminderen van operationele kosten en het beperken van het risico op besmetting door ziekten.

CURRICULUM VITAE

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Personal Data

María de Lourdes Cobo Barcia
México 905 y Argentina
Salinas, Ecuador
Tel: 593-4-2777283/593-994083836
Tel. Office: 593-4-2916120
artemiaec@yahoo.com
Place of birth: Guayaquil-Ecuador
Nationality: Ecuadorian

Higher education

1982 - 1988	Universidad de Guayaquil - Ecuador Facultad de Ciencias Naturales - Escuela de Biología. Biologist
1995 - 1997	Ghent University - Belgium Faculty of Applied Biological Sciences Master of Aquaculture

Positions held

Public Sector

Date:	2009 - present
Position:	Head Plankton Research
Location:	Escuela Superior Politécnica del Litoral (ESPOL) – Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM)
Date:	1997 - 2009
Position:	Head of Plankton & Shrimp Larviculture Laboratory Researcher Recirculation & Shrimp Larviculture Coordinator of Mollusk & Fish Production Program
Location:	Fundación CENAIM – ESPOL
Date:	1992 - 1995
Position:	Head of the Zooplankton Laboratory
Location:	ESPOL-CENAIM

Private Sector

Date:	1987 - 1990
Position:	Technical Assistant and Head of the Biological Department in the field of shrimp culture
Location:	Shrimp farms CAMOSA, CAMAVEL from the group of the Progress Bank

Publications

***Peer-reviewed publications*:**

Cobo M.L., Sonnenholzner S., Wille M. & Sorgeloos P. (2012) Ammonia tolerance of *Litopenaeus vannamei* (Boone) larvae. Aquaculture Research, *in press.*

Proceedings (National and International conferences):

Naessens E,.Cobo M.L., Van Hauwaert A., Van Horenbeeck M. & Sorgeloos P. (1993) Advances of the *Artemia* Project: Improvement of white shrimp *Penaeus vannamei* production through feeding strategies based on live and formulated feed. Memorias Segundo Congreso Ecuatoriano de Acuicultura.

Naessens E.; Pedrazzoli A.; Vargas V.; Townsend S.; Cobo M.L. & Dhont J. (1995). Evaluation of preservation methods for *Artemia* biomass and application in postlarval rearing of *Penaeus vannamei*, *in*: Lavens, P. *et al.* (Ed.) (1995). *Larvi '95: Fish & Shellfish Symposium, Gent, Belgium, September 3-7, 1995. EAS Special Publication,* 24: pp. 338-341

Van Hauwaert A, Cobo M.L., Ramos X., & Naessens E. (1995) Uso de Rotíferos y Biomasa de *Artemia* en la fase larval del camarón *Penaeus vannamei.* Memorias Tercer Congreso Ecuatoriano de Acuicultura

Wouters R., Cobo M.L., Dhont J. & Wille M. (2009). Developments in Feed Formulations, Feeding Practices and Culture Techniques for Marine Shrimp Larvae. *In***:**Browdy, Craig L., and Darryl E. Jory, editors. 2009 The Rising Tide, Proceedings of the Special Session on Sustainable Shrimp Farming, Aquaculture 2009. The World Aquaculture Society, Baton Rouge Louisiana, USA.

Publications in magazines:

Cobo M.L. & Sonnenholzner S. (2003) CENAIM study examines effects of transportation on shrimp post-larvae survival. Global Aquaculture Advocate, Vol. 6 (6), pp. 38.

Cobo M.L. & Wouters R. (2010) Prácticas de alimentación y formulación de dietas para la larvicultura de camarón. Edición 79.

Cobo M.L. (2010) Se puede substituir la *Artemia* en la alimentación de larvas de camarón? Revista Aquacultura. Edición 80. pp. 30

Cobo M.L. 2010. Sistemas de Recirculación de Agua en Programas de Producción Acuícola. Revista. Aquacultura. Edición 82.

Cobo M.L., Panchana M. & Arriagada A.(2011) Evaluación de concentrados de microalgas en la alimentación de los rotíferos. Aquacultura. Edición 85.

Sonnenholzner S., Cobo M. L., Román R. & Chang M.L. (2010) Evaluación de concentrado de microalga *Chaetoceros gracilis* en la larvicultura de *Penaeus vannamei* Revista Aquacultura. Edición 81.

Participation at scientific conferences and seminars

World Aquaculture Society Congress 2002. "Evaluation of different methods for *Litopenaeus vannamei* shrimp post-larvae quality". Beijing- China. Oral presentation

ESPOL-CIENCIA. 2002. "Evaluación de cuatro estresores para determinar la calidad de post-larvas de *Litopenaeus vannamei*. Ecuador. Oral presentation.

World Aquaculture Society Congress 2003. "Effect of Transport and Acclimation on the survival of *Litopenaeus vannamei* post-larvae". Brazil. Poster presentation.

V International Recirculating Conference. 2004. "First Attempts to produce *Litopenaeus vannamei* larvae in an experimental Recirculating System". Ranoeke, Virginia , EEUU Oral presentation.

ESPOL-CIENCIA. 2005. "Cultivo intensivo de larvas de camarón *Litopenaeus vannamei* en un sistema experimental de recirculación. Ecuador. Oral presentation.

ESPOL-CIENCIA. 2005. "Evaluación de la capacidad probiótica de cepas bacterianas para el cultivo de larvas del camarón blanco, *Litopenaeus vannamei*. Poster presentation.

II Internacional Conference of Biodiversity 2008. "Acuacultura de la concha *Spondylus* spp. como alternativa para la conservación y repoblación en la Costa Ecuatoriana". Ecuador Oral presentation.

X Ecuadorian Aquaculture Conference Congreso 2008. "Avances en la Producción de *Spondylus* spp. y de la ostra *Crassostrea gigas*". Ecuador. Oral presentation.

II Internacional Aquaculture Conference of Continental Waters 2010. Sistemas de Recirculación de Agua en Programas de Producción Acuícola. Ecuador. Oral presentation

XII Ecuadorian Aquaculture Conference & Aquaexpo. 2010. Toxicidad de Amonio en larvas de *Litopenaeus vannamei*. Ecuador. Oral presentation.

I Larviculture Conference & LarvaExpo. 2010. Cultivo Intensivo de larvas *Litopenaeus vannamei* en Sistema de Recirculación: Alimentación. Ecuador. Oral presentation.

Teaching Activities

1999 - 2005. Responsible Teacher for the courses: "Live Food Production" and "Penaeid Larviculture" in the Master Program in Marine Aquaculture Science sponsored by the Belgian Technical Cooperation (BTC), ESPOL, Ghent University, Catholic University of Leuven and Fundación CENAIM-ESPOL.