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Water quality in the culture of crustacean larvae and postlarvae : effects of microbial environment and use of closed recirculation systems

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Water quality in the culture of crustacean larvae and postlarvae: effects of microbial environment and use of closed recirculation systems

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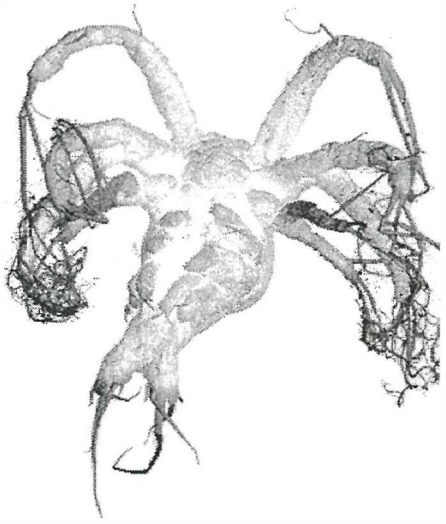

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SUMMARY

Present studies focus on the microbial control of penaeid larval cultures and on the use of recirculation systems for crustacean postlarva culture.

A first set of trials (Chapter 2) evaluated the beneficial effect of a single dose of live microalgae, microalga exudates, and probiotic bacteria from microalga growth cultures on the larviculture of penaeid first feeding larval stages feeding upon artificial microdiets. Results indicate that the presence of live microalga cells improves survival, development and growth as opposed to the addition of either water where the microalga cells were filtered out or water with microalga culture enrichment nutrients. Beneficial and detrimental effects were observed as early as the first two larval development stages. A second set of trials (Chapter 3) investigated the effects of different initial water quality treatments where microbiological composition changes were induced through mechanical, chemical and biological water treatment methods. The treatments produced significant effects on the larva capacity to survive and moult into the next larval stages as early as during first two larval stages.

In Chapter 4, the feeding appendages and digestive tract of penaeid $Nauplius_5$ and $Zoea_1$ larval stages are described with respect to their ability to ingest and excrete bacteria. Results from histological sections, scanning electron microscopy and *in vivo, in situ* real time observation of live fluorescent stained bacteria suggest bacterial colonisation may start as early as $Nauplius_5$. At this stage they already have an anal pore and antiperistalsis movements occur which are capable of bringing bacteria present in the water column into the digestive tract.

The effects of two water exchange régimes were tested in the growout of two decapod crustacean postlarvae species (*Callinectes sapidus* and *Litopenaeus vannamei*) (Chapter 5). The pattern of ammonia and nitrite accumulation under continuous water recirculation was compared to the patterns under standard batch water exchange procedures. Both methods were tested at three different daily percentage renewal rates. Survival and growth using continuous recirculation were similar to results obtained with traditional batch water exchange.

The effect of initial stocking density of two penaeid postlarvae species (*Litopenaeus vannamei* and *Farfantepenaeus brasiliensis*) under closed recirculation conditions is evaluated in Chapter 6. Results indicate a stocking density between 200-300 early postlarvae as optimal.

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To the memory of

Fernando e Rosália

For never giving up

CONTENTS

CONTRIBUTING MATERIAL

1 peer reviewed paper – journal of Microbiological Methods
1 oral communication and full paper in VI ISAN Proceedings
5 poster and abstracts presented in the 7 CCDM, V ISAN, LARVI'2001 and WAS China 2002

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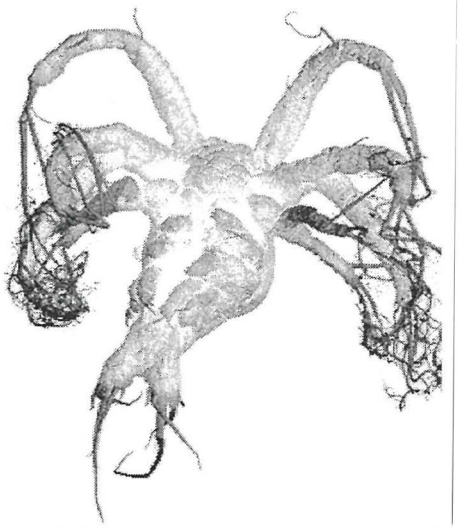
CHAPTER 1
General Introduction



INTRODUCTION

The general demand for animal protein and the market for luxury seafood items keep growing, supplied in part by the expanding aquaculture industry. Crustacean aquaculture is a fast growing industry with an average increase of production of 9.5% each year, over the last decade (FAO 2001). More species are being cultured, productivity is rising and socially, more jobs are expected to be generated all over the world (New 1997). This growth has been mainly supported by advances in scientific knowledge and technology, both in the fields of biology (genetics, nutrition and pathology) and engineering (culture systems design and water treatment). Culture of penaeid shrimp alone, accounts for up to 84% of the world crustacean aquaculture production and has provided an important source of income for developing countries in the tropic regions (FAO 2001).

Although considerable progress has been made during the past few years, the production of larvae and juveniles is still one of the main bottlenecks for several species. The problems seem to reside in the poor reproducibility in terms of survival, growth and quality.There are several factors that determine larval viability, including the nutrition and genetics of the larvae, and the biological and physio-chemical culture environment, which are in turn influenced by several conditions (Fig. 1). Manipulation of these conditions may increase the probability of producing viable larvae, and therefore, increase the reproducibility of larviculture. It is clear that manipulation of one of the three factors may only have limited effect. However, a strategy to increase larval resistance to infection, achieve microbial control, and maintain constant conditions should improve viability of larvae (Fig. 1).

The present work addresses the mechanisms of water quality control, both at the hatchery and nursery phases of decapod crustacean aquaculture, in order to develop efficient strategies to increase larval and postlarval viability. Investigations focused on the strategies for microbial control (chapters 2 - 4) and strategies to maintain constant conditions (chapters 5 - 6).

Strategies for microbial control

Poor reproducibility in terms of survival, growth and quality in larviculture suggests that there is a lack of control of at least one factor. Nutritional factors and egg quality may be ruled out as the principal cause, because the lack of reproducibility is often manifested in replicate tanks with full sibling groups that are given the same treatment. This, however, does not mean that nutritional factors and egg quality are optimal. In intensive cultivation of crustaceans mass mortality of larvae is another major restriction for regular production of high quality postlarvae. This is most likely due to opportunistic bacteria, because there are few reported incidences of specific pathogens (Dr. Gomez-Gil pers. com., Munro *et al.* 1995; Skjermo and Vadstein 1999). In an attempt to overcome the microbial problems, many research laboratories and commercial hatcheries disinfect the water before use and/or apply antibiotics as a standard procedure (Brown 1989; Sorgeloos 1994). Both methods tend to destabilize any balance of bacterial populations, and, in the long run they only provide a temporary solution to the problem. In addition, the findings of increased frequency of anti-biotic resistant bacteria present in or close to aquaculture production facilities (Torsvik *et al.*

1988; Husevag *et al.* 1991; Karunasagar *et al.* 1994; Samuelsen *et al.* 1994; Andersen and Sandaa 1994; Kerry *et al.* 1995; Tendencia and de la Pena 2001), and the recent discovery of the transfer of antibiotic resistance from the bacterial flora of domestic animals to the human microflora (Nikolich *et al.* 1994), have resulted in an increasing public concern on the use of antibiotics by the aquaculture industry. Moreover, an industry that makes prophylactic use of antibiotics cannot be regarded as sustainable. The establishment of a sustainable solution to the microbial problems in larviculture is therefore of utmost importance.

The intensive larval rearing process is characterized by the exposure of larvae to considerable stresses of a chemical, physical and biological nature. In addition, some of these stressors occur in an intermittent manner or as pulses, which may amplify their negative effects. High loads of organic matter and bacteria are introduced with the live feed, and high densities of larvae and live feed organisms will, due to defecation, result in a heavy load of organic matter and bacteria in the tanks. A substantial proportion of this organic matter will often occur in a patchy manner. It is well known from fundamental ecological community principles that irregular supplies of organic matter tend to destabilize the bacterial community and select for opportunistic and pathogenic bacteria (e.g., Andrews and Harris 1986). These conditions, in turn, produce conditions that may be detrimental to the larvae (Vadstein *et al.* 1993).

According to Vadstein (1997), microbial control cannot be regarded as absolute, but is more a question of probabilities. This is evident if one considers the three interacting factors that are required for the development of conditions that ensure good viability of the larvae (Fig. 1). Therefore, in the development of tank microbial communities, one should consider both deterministic and stochastic factors (Moriarty and Body 1995; Verschuere *et al.* 1997). Deterministic factors have a well-defined dose-response relationship. For a given value of a stochastic factor, a probability range of responses can occur. According to Verschuere *et al.* (1997), deterministic factors influencing the microbial development in aquaculture systems include salinity, temperature, oxygen concentration, and quantity and quality of the feed. They argue that these combined environmental factors create a habitat in which a selected and well-defined range of microbes is able to proliferate. The development of a microbial community in aquaculture systems is, however, also influenced by stochastic phenomena: chance favours organisms which happen to be in the right place at the right time to enter the habitat and to proliferate if the conditions are suitable (Moriarty and Body 1995).

This theoretical concept has been experimentally supported by Verschuere (1997), who monitored the community level physiological profiles of the emerging microbial communities in the culture water of *Artemia* juveniles in three identical culture series. Although completely identical from a zootechnical point of view, the culture water of the three series showed clearly distinct microbial communities developing in the first days of the experiment. The same concept may be valid for the microbial communities developing in the culture water and on the inner and outer surfaces of eggs and larval organisms (Verschuere *et al.* 2000). Obviously, due to the heterogeneity of the microbial distribution in the air and water, in feeds, and on surfaces, the stochastic factors are very important in the colonization of aquacultural environments (Moriarty and Body 1995; Verschuere *et al.* 1997; Verschuere *et al.* 2000).

The idea that both environmental conditions and chance influence the emergence of microbial communities opens opportunities for the concept of probiotics as biological conditioning and control agents. Instead of

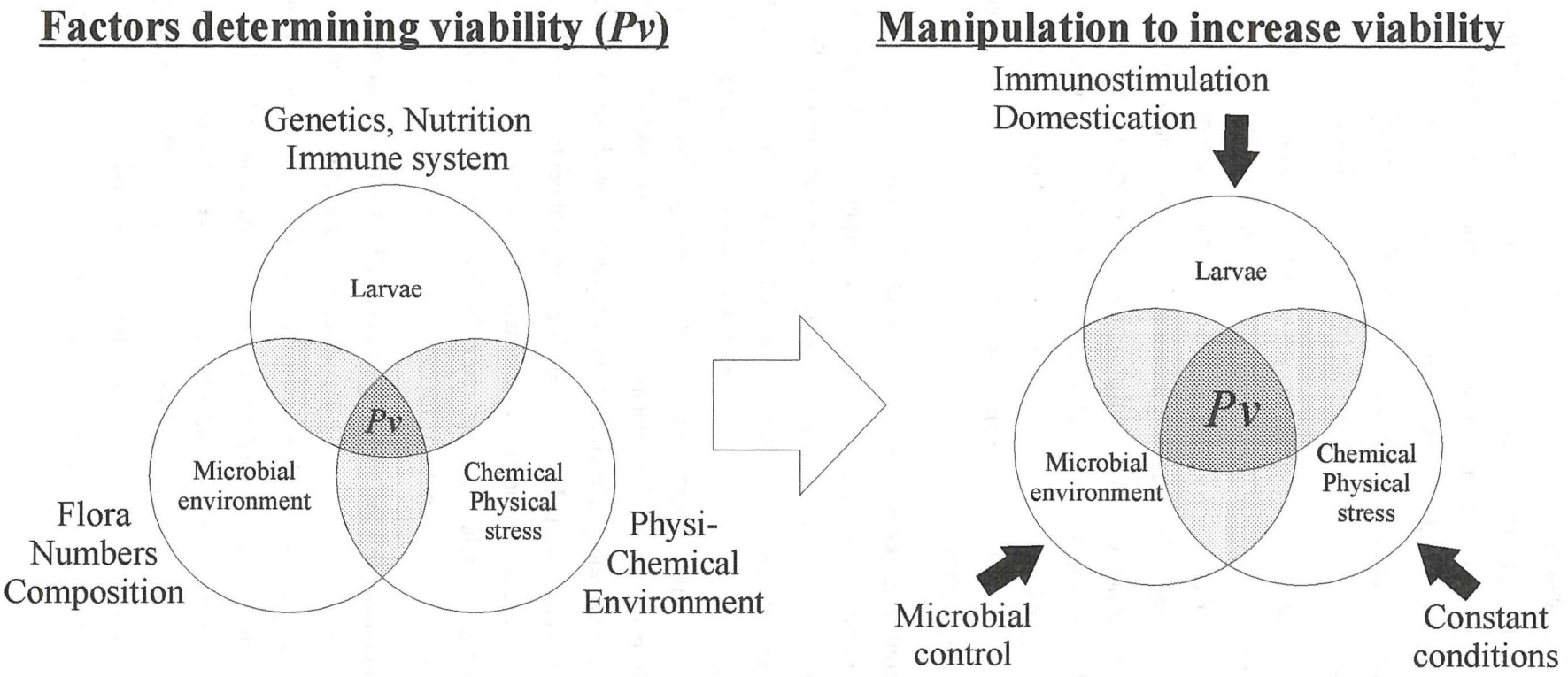


Figure 1.1 – The three factors of relevance for the probability of viable larvae (*Pv*) and conditions that influence these factors; the probability of viable larvae (*Pv*) may be increased by using methods that push the circles towards each other. Lists represent examples of such methods (adapted from Vadstein *et al.* 1993, Vadstein 1997).

Immunostimulation, Domestication

Improvement of larval resistance against bacteria
1) Stimulation of general immune system
2) Modulation of maternal immunity

Family selection
1) Broodstock selection
2) Improved broodstock nutrition
3) Adaptation to available feeds

Microbial Control

Non selective reduction of bacteria
1) Surface disinfection of eggs and larvae
2) Reduction in input of organic matter
3) Removal of organic matter
4) Grazer control of bacterial biomass

Selective enhancement of bacteria
1) Selection of desirable bacteria
2) Addition of selected bacteria to tanks
3) Incorporation of selected bacteria in feed

Constant conditions

Constant water quality culture conditions
1) Use of zero exchange recirculation systems

allowing spontaneous primary colonization of the rearing water by bacteria accidentally present, the water could be preemptively colonised by the addition of probiotic bacteria. It is suggested that in the case of preemptive colonization of rearing environments with emerging microbial communities, a single addition of a probiotic culture may suffice to achieve colonization and persistence in the host and/or in the ambient environment, provided that the probiotic cultures are well adapted to the prevailing environmental conditions (Verschuere *et al.* 2000). When the host or its environment already carries a well-established and stable microbial community, it is much more probable that the probiotic will have to be supplied on a regular basis to achieve and maintain its artificial dominance (Verschuere *et al.* 2000; Olafsen 2001). It is not likely, however, that improved microbial control may be achieved by finding "ideal probiotics". Various stages and situations may call for different approaches, such as antagonism, competition, bacteriocin production, immune stimulation, or health promotion. Thus, it is likely that the use of a selected mixture of beneficial strains may prove more effective in different situations and more stable over time (Olafsen 2001).

Vadstein *et al.* (1993) proposed a general strategy for microbial control that takes into account both the ecological conditions and the various aspects described in Figure 1. The strategy, which does not rely on the prophylactic use of antibiotics, is based on the concurrent use of three different elements (Fig. 1). Application of this strategy in fish larval rearing has, so far, produced good results (Vadstein *et al.* 1993; Salvesen and Vadstein 1995; Skjermo *et al.* 1996; Skjermo *et al.* 1997).

To achieve improved microbial control in crustacean larval rearing systems, information is needed on microbes interaction with live food organisms, on how seasonal changes in microflora composition of natural seawater may affect larviculture output, on bacterial colonisation factors, and on host regulation of the adherent microflora. A brief description of each of these investigation subjects is presented next, together with an introduction to the chapters of the thesis that focused on these themes.

Microalgal control of bacterial populations

Microalgae represent a repetitive source of bacterial inoculation into the larval culture systems, both in terms of number and composition (Muroga *et al.* 1987; Perez-Benavente and Gatesoupe 1988; Nicolas *et al.* 1989; Keskin *et al.* 1994; Olsen *et al.* 1999). In addition to their nutritional support, microalgae may thus have a significant quantitatively and qualitatively impact on the microflora composition of the water and the larvae (Tubiash *et al.* 1965; Bell and Lang 1974; Kellam and Walker 1989; Skjermo and Vadstein 1993; Bergh *et al.* 1994; Salvesen *et al.* 1999; Salvesen *et al.* 2000). Algae cultures rarely include *Vibrio* spp. (Prieur 1982; Lewis *et al.* 1988), however the contribution in bacterial numbers from other genera is considerable and may approximate to 1×10^8 CFU per supplementation to one litre of bivalve larval culture (Murchelano and Brown 1969; Prieur and LeRoux 1975; Salvesen *et al.* 2000). Bacterial growth and composition of the flora in the water depends on both the algal species and state of growth of the algae. According to Salvesen *et al.* (2000), higher levels are associated with slow-growing microalgae when expressed on a "per volume" basis, whilst on a "per algal cell" basis, variation in the bacterial load is related to species. These authors relate the difference to an effect of a higher excretion of organic products from senescent algae together with the decomposition of dead cells, which provide substrate for bacterial growth. Changing chemical and physical environmental factors can, in general, alter the chemical composition of the

Table 1.1 – Studies on the bacteria flora of crustaceans with emphasis on the digestive tract bacterial community. "+" sign stands for detailed gut microflora composition.

Species	Stage	Gut	Authors
Litopenaeus aztecus	Adults	+	(Williams and Rees 1952)
Litopenaeus aztecus	Adults		(Vanderzant *et al.* 1971)
Litopenaeus aztecus	Adults	+	(Dempsey *et al.* 1989)
Litopenaeus aztecus	Adults		(Dempsey and Kitting 1987)
Litopenaeus setiferus	Adults		(Hood *et al.* 1971)
Litopenaeus setiferus	Adults	+	(Dempsey *et al.* 1989)
Litopenaeus setiferus	Adults		(Dempsey and Kitting 1987)
Litopenaeus setiferus	Adults		(Christopher *et al.* 1978)
Litopenaeus vannamei	Adults	+	(Moss *et al.* 1996)
Litopenaeus vannamei	Adults		(Christopher *et al.* 1978)
Litopenaeus vannamei	Adults	+	(Dixon *et al.* 2001)
Litopenaeus vannamei	Adults	+	(Moss *et al.* 2000)
Litopenaeus stylirostris	Adults		(Christopher *et al.* 1978)
Litopenaeus stylirostris	Adults	+	(Dixon and Moss 2002)
Litopenaeus merguiensis	Adults	+	(Oxley *et al.* 2002)
Fenneropenaeus indicus	Larvae	+	(Hameed 1993)
Fenneropenaeus indicus	Larvae	+	(Singh 1986)
Fenneropenaeus indicus	Larvae	+	(Pillai and Jayabalan 1993)
Fenneropenaeus indicus	Adults	+	(Kumar and Dube 1990)
Marsupenaeus japonicus	Larvae	+	(Yasuda and Kitao 1980)
Penaeus monodon	Larvae	+	(Tanasomwang and Ruangpan 1995)
Penaeus monodon	Adults	+	(Leaño *et al.* 1998)
Penaeus monodon	Adults	+	(Jayabalan and Pillai 1994)
Penaeus monodon	Adults	+	(Lavilla-Pitogo *et al.* 1992)
Various penaeids	Adults		(Alvarez 1983)
Pandalus borealis	Adults	+	(Ridley and Slabyj 1978)
Homarus sp.	Eggs		(Harper and Talbot 1984)
Portunus trituberculatus	Larvae	+	(Suzuki *et al.* 1990)
Callinectes sapidus	Adults	+	(Huq *et al.* 1986)
Neptunus sanguinolentus	Adults	+	(Kumar and Dube 1990)
Atergatis floridus	Adults	+	(Sugita *et al.* 1987c)
Macrocheira kaempferi	Adults	+	(Ueda *et al.* 1989)
Macrobrachium rosenbergii	Larvae		(Colorni 1985)
Macrobrachium rosenbergii	Adults		(Anderson *et al.* 1989)
Palaemon paucidens	Adults	+	(Sugita *et al.* 1987a)
Upogebia stellata	Adults	+	(Pinn *et al.* 2002)
Acartia tonsa	Adults	+	(Sochard *et al.* 1979)
Acartia clausi	Adults		(Nagasawa *et al.* 1985)
Limnoria tripunctata	Adults	+	(Zachary and Colwell 1979)
Various copepods	Adults		(Huq *et al.* 1983)
Boeckosimus affinis	Adults	+	(Atlas *et al.* 1982)
Several crustaceans	Adults	+	(Ueda *et al.* 1988)
Several crustaceans	Adults	+	(Ueda *et al.* 1995)
Several crustaceans	Adults	+	(Sugita *et al.* 1987b)

microalgae (Reitan *et al.* 1994). Variation in these factors may also have an effect on the growth of bacteria associated with the microalgae.

Bacteria, may have a stimulating effect on the algae through decomposition of organic metabolites or through the production of stimulative substances for algal growth (DeLucca and McCracken 1978; Riquelme *et al.* 1988). Microalgae, on the other hand, may promote and/or inhibit bacterial growth by the production of organic exudates and toxic metabolites, an effect that has been extensively reported (Table 1.2). For example, it has been shown that the marine alga *Delisea pulchra* inhibits fouling by the production of furanones which interfere with the N-Acyl-Homoserine Lactone (AHL) mediated expression of bioluminescence, swarming motility and exoenzyme synthesis in different bacterial species (Kjelleberg *et al.* 1997). Misciattelli *et al.* (1998) demonstrated that the production of AHL analogues or mimics is more widespread amongst the algae, and that *Vibrio harveyi* virulence can be controlled using this mechanism.

The trend to substitute live microalgae for inert microdiets in shrimp larviculture as a move towads better efficiency and cost-effectiveness has not yet achieved complete replacement with satisfactory results. This lead the present investigation, to examine the factors behind the success and necessary use of live microalgae as a conditioning factor before the use of artificial microdiets (chapter 2). Baceuse inert microdiets can be made similar to the nutritional profile of microalgae, it was hypothesised that the bacteria and/or nutrients present in the microalgae culture water, regulate the shrimp larviculture bacterial environment and are, therefore,responsible for the production success when microalgae are used.

Seasonal fluctuations on microbial water quality

Physio-chemical properties of seawater are well known to change with seasons, especially in temperate regions. Seasonal fluctuations in bacterial numbers, which occur on a regular annual basis following the collapse of phytoplankton blooms (Blight *et al.*1995), are typical of coastal temperate regions, but should also be similar to fluctuations occurring in tropical regions as a result of monsoons, cyclones or storms (Richardson 1997). In all situations these fluctuations may stimulate bacterial blooms of opportunistic bacteria, together with high numbers of viral particles (Bergh *et al.* 1989).

Mortalities of caged or wild fish and shellfish can occur as a result of phytoplankton blooms for a number of reasons listed in Table 1.3 with examples. Although most of the reported examples refer to fish, a few mention crustaceans and their respective larval stages, and there is no reason why crustaceans should not suffer from similar adverse effects of phytoplankton blooms. Recent literature present some reports with harmful bacteria/microalgae blooms as the primary cause of mortality in shrimp culture ponds (Smith 1996) and hatcheries (Misciattelli *et al.* 1998). Smith (1996) suggests that blooms of oscillatoriales were the primary cause of disease outbreaks during 4 consecutive years at Australian shrimp farms. He provided evidence to support this hypothesis and indicates a water-soluble, heat-labile toxin as the link between shrimp mortalities and blooms of planktonic or benthic oscillatoriales. He further suggests that sub-lethal levels of toxins weakened the shrimps, possibly by a neurotoxic effect causing reduced feeding behaviour and impaired immune system, including observations of *Artemia* nauplii. As a result, shrimps may be prone to secondary infections by pathogenic bacteria (Lightner 1978).

Table 1.2 – Research on control of marine bacterial populations by algal exudates.

Species	Authors
Microalgae	
Phaeocystis pouchettii	(Sieburth 1960)
Phaeocystis pouchettii	(Guillard and Hellebust 1971)
Skeletonema costatum	(Kogure *et al.* 1979)
Skeletonema costatum	(Naviner *et al.* 1999)
Skeletonema costatum	(Salvesen *et al.* 2000)
Tetraselmis suecica	(Austin and Day 1990)
Tetraselmis suecica	(Austin *et al.* 1992)
Tetraselmis chuii	(Misciattelli *et al.* 1998)
Tetraselmis sp.	(Salvesen *et al.* 1999)
Tetraselmis sp.	(Salvesen *et al.* 2000)
Tetraselmis sp.	(Olsen *et al.* 2000)
Tetraselmis sp.	(Skjermo and Vadstein 1993)
Gymnodinium nagasakiense	(Fukami *et al.* 1991)
Chaetoceros mulleri	(Salvesen *et al.* 2000)
Nannochloropsis oculata	(Salvesen *et al.* 2000)
Isochrysis galbana	(Salvesen *et al.* 2000)
Pavlova lutheri	(Salvesen *et al.* 2000)
Trichodesmium erythraeum	(Ramamurthy and Krishnamurthy 1967)
Trichodesmium erythraeum	(Ramamurthy 1973)
various	(Hornsey and Hide 1974)
various	(Bell and Lang 1974)
various	(Brock and Clyne 1984)
various	(Cole 1982)
various	(Duff *et al.* 1966)
various	(Kellam and Walker 1989)
various	(Tubiash *et al.* 1965)
various	(Murchelano and Brown 1969)
various	(Prieur 1982)
various	(Prieur and LeRoux 1975)
Macroalgae	
Delisea Pulchra	(Nys *et al.* 1995)
Delisea Pulchra	(Gram *et al.* 1996)
Delisea Pulchra	(Kjelleberg *et al.* 1997)
Polysiphonia lanosa	(Misciattelli *et al.* 1998)
various	(Reichelt and Borowitza 1984)
various	(Visco *et al.* 1987)
various	(Pesando 1990)

(Misciattelli *et al.* 1998) reported a relation between poor egg hatching and penaeid larvae survival results with the sudden increase in bacteria numbers during the spring bloom months in a North Wales laboratory scale hatchery. The water quality of the Menai Strait (North Wales, UK) varies seasonally with poor water quality being associated with the sharp increase of the colonial flagellate, *Phaeocystis pouchetti* Lagerh during the *Rhizosolenia delicatula* spring diatom bloom (Blight *et al.* 1995), and provides a good descriptive example of the interactions between microalgal and bacterial blooms. The peak abundance of *P. pouchetti* (1×10^3 bladders dm^{-3}) is paralleled by a significant decrease in the number of bacteria from 4.8×10^6 to 1.8×10^6 cells cm^{-3} (Blight *et al.* 1995). When the *P. pouchetti* bloom collapses (between May and June), bacterial levels rise again to 4×10^6 cells cm^{-3} accompanied by consecutive peaks of ciliates and dinoflagellates (Blight *et al.* 1995). The *P. pouchetti* colonies and individual cells are known to release acrylic acid, a substance with known "antibiotic" characteristics (Sieburth 1960). The clear effect on the bacterial community within the Menai Strait water is also reflected in the rearing success of penaeid shrimp with poorer results during the spring bloom period (Misciattelli *et al.* 1998). The acrylic acid should pass through the filters and selectively reduce the diversity of the bacterial community within the culture tanks in such a way as to select resistant strains. These strains may then colonise the space left by their direct competitors and establish a monospecific or less diverse opportunistic bacterial community, which was hypothesised by Skjermo and Vadstein (1999) as a detrimental factor in larvae culture. Guillard & Hellebust (1971) confirmed the production of acrylic acid by *Phaeocystis pouchetti*, found earlier by Sieburth (1960; 1961) and known to inhibit both gram-negative and gram-positive bacteria. Guillard & Hellebust (1971) reported relatively few bacteria in exponential growing *P. pouchetti* cultures, but dense bacterial populations in and around the gel material of old cultures. Guillard & Hellebust (1971) also found that when *P. pouchetti* grew in colonies, they excreted 16-64% of their photo-assimilated carbon into the medium mainly as carbohydrates of varying molecular weights. These authors estimated that as much as 7 $\mu g\ L^{-1}$ of acrylic acid, and at least 0.3 $mg\ L^{-1}$ of polysacharides can be liberated in a *Phaeocystis* bloom. This important release of nutrients and antibiotic substances is likely to have an effect on the water microflora composition.

Other examples of the relation between red tides and modulation of seawater microflora include Fukami *et al.* (1991), who found that bacteria collected during the decline phase of a *Gymnodinium nagasakiense* bloom had significant inhibitory effects on the axenic culture of both *G. nagasakiense* and *Skeletonema costatum*. Bacteria collected during the initial and peak phase of the red tide had opposite results, stimulating the growth of both microalgae. Their results suggest that natural communities of bacteria play an important role on the development and decay of algae blooms, and although no bacteria identification data is provided, it can be speculated that there is a succession of predominating bacteria species during an algae bloom. Another species of bloom forming algae was also reported to produce antibacterial substances such as the cyanobacterium *Trichodesmium erythraeum* (Ramamurthy and Krishnamurthy 1967; Ramamurthy 1973). Hawser *et al.* (1991; 1992), however, did not corroborate this finding working with extracts from several *Trichodesmium* species.

It is clear that the microflora composition of natural seawater may change seasonally. The mechanical, chemical and biological manipulation of water prior to culture will certainly further modify the microflora present in the

Table 1.3 – Species of microalgae which have formed blooms that killed marine organisms [adapted from Richardson (1997)] .

Bloom microalgae species	Species Affected		Potential causes of death	Region	Authors
Gymnodinium catenatum *Mesodinium rubrum* *Ceratium tripos* *Skeletonema costatum*	Fish, crustaceans and annelids	—	Anoxia and gill lesions	Mexico	(Altamirano 1987)
Trichodesmium thiebautii	Oysters	—	Affected pearl culture	India	(Chellam and Alagarswami 1978)
Trichodesmium erythraeum	Fish, Crabs	—	deaths	India	(Chidambaram and Unny 1944)
Gyrodinium aureolum	Several shellfish species	—	toxins	—	(Heinig and Campbell 1992)
Gyrodinium aureolum *Skeletonema costatum*	Pond-reared salmon	—	toxins	Scotland	(Jones *et al.* 1982)
Thalassiosira spp.	Pen-reared salmon	*Salmo salar*	gill lesions	—	(Kent *et al.* 1995)
Chaetoceros concavicornis	Atlantic salmon	*Salmo salar*	anoxia and gill lesions	—	(Rensel 1993)
Chaetoceros convolutus	Chinook salmon Chum salmon	*Oncorhynchus tshawytscha* *Onchorhynchus keta*	gill lesions	Alaska	(Farrington 1988)
Chaetoceros convolutus	Red king crab	*Paralithodes camtschatica*	anoxia and gill lesions	Alaska	(Tester and Mahoney 1995)
Chaetoceros concavicornis				Pacific	(Albright *et al.* 1993)
Chaetoceros convolutus				Pacific	(Albright *et al.* 1993)
Phaeocystis pouchettii				Atlantic	(Guillard and Hellebust 1971) (Sieburth 1960)
Phaeocystis sp.				UK	(Blight *et al.* 1995)
Phaeocystis sp.	Penaeid shrimp eggs/larvae	*Penaeus indicus*	Reduced hatching / survival	UK	(Misciattelli *et al.* 1998)
Ptychodiscus brevis	Red drum larvae	*Sciaenops ocellatus*	toxins	Gulf of Mexico	(Riley *et al.* 1989)
Alexandrium excavatum	Fish and crustacean larvae	—	toxins	—	(Robineau *et al.* 1991)
Cerataulina pelaica (diatom)	Fish and benthic fauna	—	anoxia	New Zealand	(Taylor *et al.* 1985)
Dinoflagellates	Atlantic herring	*Clupea harengus harengus*	toxins	—	(White 1977)
Heterosigma akashiwo		*Oncorhynchus tshawytscha*	toxins	—	(Black *et al.* 1991)
Raphidophyceae	Cage reared-salmon	—	toxins	New Zealand	(Change *et al.* 1990)
Protogonyaulax tamarenses	Marine fish larvae	—	toxins	—	(Gosselin *et al.* 1989)
Chrysochromulina polylepis	Ascidea Blue mussel	*Ciona intestinalis* *Mytilus edulis*	toxins	—	(Granmo *et al.* 1988)
Chrysochromulina cf. leadbeateri		—	toxins	Norway	(Aune *et al.* 1991)

Bloom microalgae species	Species Affected		Potential causes of death	Region	Authors
Oscillatoria corakiana	Penaeid shrimp Brine shimp	*Penaeus monodon*, *Penaeus japonicus* *Artemia salina*	toxins	Australia	(Smith 1996)
Spirulina subsalsa	Penaeid shrimp	*Penaeus stylirostris*	Potential toxins	USA	(Lightner 1978)
Pfiesteria piscicida				USA Atlantic coast	(Burkholder *et al.* 1995)
Gyrodinium nagasakiense				Japan	(Fukami *et al.* 1991)
Nitzschia pungens				Canada Atlantic Coast	(Bates 1989)
Cyanobacteria				—	(Sevrin-Reyssac and Pletikosic 1990)
Cyanobcteria, *Trichodesmium thiebautii* *Trichodesmium erythraeum*	Brine shimp marine copepods	*Artemia salina* *Macrosetella gracilis*, *Farranula gracilis*, *Clausocalanus furcatus*, *Miracia efferata*	Significant mortality induced by *T. thiebautii* extracts – toxins	Caribbean	(Hawser *et al.* 1992)
Microalgae bloom	Lingcod	*Ophiodon elongatus*	gill lesions	—	(Bell 1961)
Microalgae bloom	Several marine organisms	—	toxins	various	(Eilertsen and Raa 1994)
Microalgae bloom	Several fish species	—	toxins	USA	(Moshiri *et al.* 1978)
Microalgae bloom	Shellfish	—	anoxia	Mid Atlantic Bight	(Steimle and Sindermann 1978)
Phytoplankton bloom	Tuna fish	—	avoidance of bloom areas	Arabian Sea	(Nagabhushanam 1967)
Green microalgae	Pilchard	*Sardinops sagax*	anoxia and gill lesions	—	(Jones and Rhodes 1994)
Pyrodinium sp.				—	(Hallegraeff and Maclean 1989)
Several species	Fish	—		Mexico,	(Millán-Núñez and Loya-Salinas 1993)
				Pacific coast	(Millan-Núñez 1988)
Several species				World wide	(Richardson 1997)
Several species				—	(Hallegraeff 1993)
Several species				—	(Maclean 1993)
Several species				—	(Tomotoshi *et al.* 1989)
Several species				—	(Taylor and Seliger 1979)
Several species				—	(Steidinger 1983)

water. The present study hypothesises that such changes are an important factor determining differences in shrimp larviculture production (chapter 3).

Bacterial colonisation of the larval gut

Most studies on the bacterial flora in the digestive tracts of both shrimp larvae and postlarvae demonstrate that *Vibrio* and *Pseudomonas* are the dominant genera (Table 1.1). These studies are generally descriptive and merely report the presence/absense of certain bacterial strains, with only a few reporting bacterial abundance. Obtaining this information is time consuming and labour intensive to aquire, and does not provide answers to how the process of colonisation takes place.

According to Munro *et al.* (1994) and Ringo & Birkbeck (1999), the bacteria that colonise the fish larval gut originate either from the egg epiflora, the tank water or the live feed. Bacterial strains able to colonise the gut are adapted to the specific physical, chemical, and biotic environment, and can thus persist despite the action of bile, digestive enzymes, the host's immune system, anaerobic conditions and variations in acidity. Successful colonisation of the gut also involves competition with other bacteria for attachment sites and nutrients, and resistance to bacteriotoxins (Makridis *et al.* 2000). Colonisation studies have evaluated the uptake and ability of pathogenic bacteria to adhere to the intestinal mucus of fish (Olsson *et al.* 1992), to the intestine of the blue crab, *Callinectes sapidus* Huq *et al.* 1986), and to the oral region of copepods (Huq *et al.* 1983).

To accomplish colonisation studies, several techniques have been employed, such as use of cellular lines (Olsson *et al.* 1992), direct observation with scanning electron microscopy (SEM; Huq *et al.*, 1983; Lavilla-Pitogo *et al.*, 1990), histology (Jiravanichpaisal *et al.*, 1994), and bacteria grown on ^{14}C-glucose labeled media (Reitan *et al.* 1998). Apart from the long and laborious processes involved in fixation and preparation of samples for microscopy, none of these methodologies allows observations *in vivo* or on recently killed organisms, nor do they guarantee that the observed bacteria are those inoculated or of interest. The present work describes the morphology of penaeid shrimp larvae feeding appendages and digestive tract together with *in vivo*, real time observations on ingestion and retention of bacteria in the gut of shrimp larvae using a novell fluorescent staining technique (chapter 4).

Vibrios and the culture of marine larvae

Species of *Vibrio* are commonly found in marine environments (Ruby and Nelson 1978), associated with tissues such as the light organ of cephalopods and fish (O'Brien and Sizemore 1979), and in the intestine of fish (Ramesh and Venugopalan 1989) and crustaceans (Table 1.1). Vibrios have also been found in the hepatopancreas and haemolymph of crustaceans (Sizemore *et al.* 1975; Sizemore and Davis 1985; Ueda *et al.* 1993; Ueda *et al.* 1994; Gomez-Gil *et al.* 1998). Vibrios are predominant epi- and endobionts of all development stages of marine cultured organisms such as shrimp (Yasuda and Kitao 1980; Dempsey *et al.* 1989; Hameed 1993; Tanasomwang and Ruangpan 1995). Vibrios have also been implicated in a variety of diseases, in particular species such as *V. harveyi, V. alginolyticus, V. anguillarum, V. parahaemolyticus*, and *V. penaeicida*, affecting a broad range of cultured marine organisms, from fish (Saeed 1995), to oysters (Pass *et al.* 1987), lobsters (Diggles *et al.* 2000) and shrimps (Jiravanichpaisal *et al.* 1994). Massive

mortalities of shrimp larvae associated with luminescent strains of *Vibrio spp.* have been reported in hatcheries from Australia (Pizzutto and Hirst 1995), India (Karunasagar *et al.* 1994), Philippines, (Baticados *et al.* 1990; Lavilla-Pitogo *et al.* 1990), Ecuador and Mexico (Vandenberghe *et al.* 1999).

Species such as *Vibrio harveyi* are universally present in coastal waters and the appearance of pathogenic or luminous forms is often associated with salinity, temperature or nutrient changes, some of which can be induced experimentally (Prayitno and Latchford 1995; Leaño *et al.* 1998). In shrimp larvae, luminescent vibriosis is characterized by lethargy, anorexia, muscle opacity, bacterial masses in the hemocoel, and luminosity of the larvae (Lavilla-Pitogo *et al.* 1990). Electron microscopy observations have revealed that bacteria colonize the feeding apparatus, forming bacterial plaques in heavily infected larvae. Therefore, it is highly probable that the mouth constitutes the main entrance for colonization of inner tissues (Lavilla-Pitogo *et al.*, 1990). However, the development of the infection has not yet been studied in detail. It has been suggested that the lethal condition called "$Zoea_2$ Syndrome", which induces hepatopancreatic cells to separate and atrophy, and causes disintegration of the gut epithelium, is related to a contact with pathogenic *Vibrio* bacteria cells earlier in the $Zoea_1$ stage (Juarez 1997; Pantoja *et al.* 1997). In the present study, a non-pathogenic *Vibrio harveyi* strain was used in order to understand the basic principles of larval gut colonisation avoiding the development of disease in the observed larvae (chapter 4).

Strategies to maintain constant culture conditions

Apart from the internal limitations to the expansion of aquaculture due to the botlle neck of juvenile production, there are external pressures restricting the growth of the industry. The fast development of marine aquaculture activities worlwide during the last decade, raised concerns for the impact on the coastal environment due to habitat destruction and water polution, particularly by shrimp aquaculture operations. According to Phillips *et al.* (1991) "it seems inevitable that aquaculture will have to face increasingly tight restrictions over development in many areas of the world, as concern over environmental impact and competition for resources grows". Indeed, concern over the impact of aquacultural wastes and effluents has already brought aquaculture under the scrutiny of regulatory agencies in a number of developed countries (Boyd and Clay 1998; Boyd 2001a; Boyd 2001b). Aquaculture, as other traditional agricultural operations, creates wastes that, when discharged to receiving waters, can have a detrimental impact to the local aquatic environment (Boyd 2001a). Amongst other mitigation measures, this situation has led to important advances in the development of seawater recirculation systems to reduce water discharges into the environment (Losordo *et al.* 1992).

In their simplest form, water reuse systems increase the productive capacity of water (Kg biomass L^{-1}). Recirculation systems technology is becoming widely accepted as being useful in overcoming some of the problems caused by the earlier mentioned seasonal variations in coastal waters due to algal blooms. It also assists in the minimisation of the environmental problems caused by aquaculture, such as pollution with effluent water charged with nutrients and the spread of diseases (Reinersten and Haaland 1995). In addition, modern technology enables complete control over nutrient levels, solid wastes, and physio-chemical parameters, such as temperature, dissolved oxygen, salinity and pH. Advanced recirculation systems are now

being designed to enable mariculture based inland, using artificial seawater and reducing costs associated with water pumping, pre-treatment, and license granting (Lygren and Blanquet 1997; Davis *et al.* 2002). The closed, controlled environment provides the ideal conditions for implementation of tight biosecurity measures and disease control via the manipulation of the microbial flora using probionts rather than relying on antibiotics. Recirculation conditions also assist the isolation of non-indigenous species, avoiding escape into the wild. In maturation units, recirculation of water retains pheromones which may enhance mating percentage.

The application of recirculation systems to the larviculture phase is impractical as crustacean larval stages need to feed upon microalgae or small particulated feeds on a volumetric basis, and unless feed is added regularly, or outflow if fine filtered, recirculation will create a constant dilution of available food concentration. Recirculation systems are, therefore, more suitable to use from benthonic postlarval stages onwards during the nursery growing phase. However, for an efficient implementation of closed water recirculation systems for commercial scale crustacean culture, new water management protocols that permit productions similar to those of the traditional protocols, are required. Although recirculation technology is capable of processing the excess nutrients released into the water from decaying feed, faeces and excretion, it cannot do so instantly and animals have to acclimate to higher-than-normal ammonia, and nitrite concentrations before the biofilter units start operating at full capacity. This question is addressed in chapter 5 of the present work using shrimp and crab postlarvae cultured either using continuous closed recirculation conditions or using traditional batch water exchange.

There are relatively few reports of profitable large scale recirculated production systems in operation, especially during grow out phase. Recirculating systems have generally been expensive to build, which increases the cost of producing marine animals in these systems. The higher cost of producing marine organisms in recirculating systems has caused problems in the international market when competing with traditional pond aquaculture technology. Closed recirculating production systems, will not be used on a wide scale until the total cost of producing fish, shrimp or molluscs in these systems begins to approach that for ponds and other competitive production systems. The challenge for the designers of recirculating systems is to develop systems that maximize production capacity per unit of capital invested. To do so, components used in recirculating systems need to be designed and developed to reduce the cost of the unit while maintaining reliability. One way to do so is to increase the stocking density of the animals grown, and therefore increase biomass yield at the end of the production cycle. However, survival and growth might be reduced with increasing stocking density. There is a need for information on how stocking density affects both suvival and growth in order to calculate the desired break-even point. In chapter 6 of the present study, the effect of stocking density under closed recirculation conditions on growth, survival and tank water quality is evaluated for two species of penaeid shrimp.

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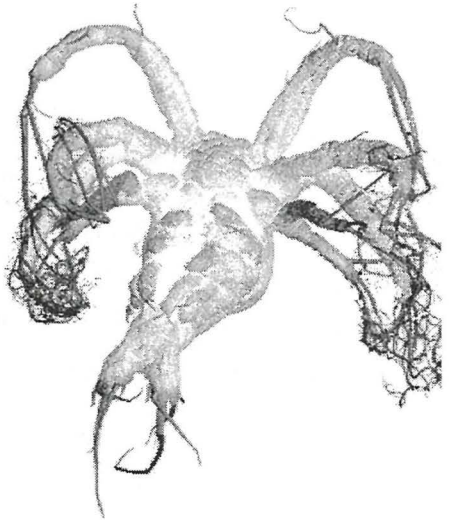
CHAPTER 2

Stimulation of first feeding *Litopenaeus vannamei* (Crustacea: Decapoda) larval stages feeding upon artificial diets by using an initial "single dose of live algae" (SDLA): effects on survival, growth and development



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INTRODUCTION

Successful penaeid larviculture depends primarily on adequate nutrition (Jones *et al.* 1997b) and on the maintenance of good water quality (Wickins & Lee 2002), both chemically and microbiologically. Most hatcheries implement feeding regimes based only on live feeds (microalgae and *Artemia* spp.) or on live feeds combined with prepared diets (Jory 1997). Formulated, off-the-shelf, microdiets (MDs) have been successful as supplements to live feeds (Jones *et al.* 1993; Jones 1998), although complete microalgae replacement has not always been consistent (Wickins & Lee 2002). Similarly, the performance of MDs for larvae of marine fish is frequently improved when they are co-fed with *Artemia* (Koven *et al.* 2001). This suggests that nutritional factors in the live food are positively influencing the ingestion, digestion and assimilation of the MD. Apart from the direct nutritional role of microalgae cells, exogenous enzymes produced by them may also contribute to the digestion process or provide factors that stimulate larval secretions or activate gut zymogens.

The positive effect of live diets upon the growth and survival of marine larvae may also be related indirectly to the modulation of microflora present in the culture water. Microalgae have a selective effect upon the bacteria present in the water (Bell & Lang 1974, Kellam & Walker 1989, Salvesen *et al.* 2000) and such effect has been shown to induce changes in the gut microflora of fish larvae (Skjermo & Vadstein 1993; Bergh *et al.* 1994).

When growing shrimp larvae upon MD, Jones *et al.* (1997b) propose the inoculation of a single dose of live algae (SDLA) during the initial culture period when the larvae first open their mouth and expose their primitive, "virgin" gut to the potential colonization by the bacteria present in the surrounding water. It was hypothesised that this algal dose conditions bacterially-variable hatchery water when artificial diets are to be used either as total or partial replacement of live feed. Indeed, based on the assessment of the effects of bacterial levels on penaeid larviculture using artificial diets, Alabi *et al.* (1997; 1999) showed that successful microalgae replacement depends on the establishment of a balanced and stable bacterial community.

Improved larviculture results using conditioning of water prior to culture with microalgae and with bacterially matured water from a recirculation system has been reported for penaeid larvae (Jones *et al.* 1998; 2000; Misciattelli *et al.* 1998). Misciattelli *et al.* (1998) propose an algal metabolite or exudate as the factor responsible for improving larval survival in cultures fed with microdiets. The authors suggest that this algal metabolite or exudate modifies the bacteria in the culture water when a single dose of live algae is presented at the initial culture stage.

The present study tests several hypothesis related to the stimulating effect of the addition of microalgae to microdiet-fed shrimp larval cultures. One hypothesis is that the initial diet has an impact on larval growth and survival, irrespective of what larvae are fed afterwards. Another hypothesis is that the beneficial effect of a SDLA is dependent on the ingestion of live, instead of dead, microalgae cells. A third hypothesis, is that the effect of the initial diet depends upon the microflora present in the culture water. The last hypothesis tested postulates that the factor responsible for improving larval survival is not solely dependent on the ingestion of microalgae cells, but that other factors might be involved, such as a) exudates and bacteria present in microalgae culture water, and b) nutrients used to enrich microalgae cultures.

MATERIALS AND METHODS

Experiments consisted on dividing the feeding regime in two periods: a first short period (< 24 h) of feeding during the moult from $Nauplius_5$ into $Zoea_1$, when larvae open their mouth and start exogenous feeding exposing the virgin gut to the outside surrounding water, and a second feeding period from $Zoea_1$ onwards. By using this division in the feeding regime, four hypothesis were tested, which are described later in this section.

Experimental animals and general experimental conditions

All *Litopenaeus vannamei* shrimp nauplii ($Nauplius_4$ - single female spawn) originated from F2 and F4 domesticated broodstock (Panama Origin), obtained from Industrias Pecis hatchery, Yucatan, Mexico. Nauplii were acclimated to temperature, salinity and pH laboratory water conditions (33-38 ‰, 28-30 °C, 7.7-7.9 pH) in each trial over a period of 1-2 h. All trials used 100 larvae L^{-1} stocking density in 1000 or 500 ml round bottom sterilized glass flasks gently aerated through a sterilised glass pipette using 0.2 µm filtered air. Shrimp larvae were subject to a 13 -11 h light-dark regime and no-water exchange. Flasks were maintained in a 28 °C thermostatically controlled water bath and left at least 12 h acclimating to bath temperature before larvae addition. All other instruments used were disinfected with chlorine and thoroughly rinsed with tap water prior to use. Microalgae were grown in autoclaved f/2 medium (Guillard & Ryther 1962) in 4 L glass jars at 20-22 °C with continuous illumination (± 150 µE m^{-2} s^{-1}) and aeration (filtered to 1 µm).

Feeding

After acclimation to laboratory conditions, $Nauplii_{3-4}$ were separated in sterile plastic containers filled with 5µm filtered seawater, and treated with 10 mg L^{-1} of EDTA. The different feed treatments were added when larvae were in the advanced $Nauplii_5$ stage, a few hours before moulting to $Zoea_1$. When more than 90% of the larvae had moulted they were transferred to spherical glass flasks (either 1000 or 500 ml) filled with 5µm filtered seawater, treated with 10 mg L^{-1} of EDTA. Five replicates per treatment were used, unless otherwise specified. In this way, it was possible to change the diet and water quality conditions available at the time of $Nauplius_5$ moulting into $Zoea_1$, and thereafter. Microdiet (Frippack CAR 30-90µm and ULTRA 80-150µm, INVE Technologies) was given 6 times per day every four h, with a ration of 1 mg L^{-1} CAR until $Zoea_2$, 1.5 mg L^{-1} CAR from $Zoea_2$ to $Mysis_1$ and 2 mg L^{-1} ULTRA from $Mysis_1$ onwards. Microdiets (MD) were prepared before each feed by dissolving in 0.2 µm fresh filtered seawater. The single dose of live microalgae treatment (SDLA) consisted of a suspension of *Chaetoceros gracilis* and *Tetraselmis chuii* microalgae (10 and 2.5 cells μL^{-1} respectively). The control treatments where larvae were fed live microalgae (posterior to the initial SDLA contact) consisted of the same species of microalgae given in different concentrations and proportions depending on the development stage of larvae. Subsequent daily adjustments were made following algal concentrations recommended by Gallardo *et al.* (1995). These concentration adjustments were done in the morning, sampling 15-20 ml using an aeration tube with a 50 µm mesh tied to one end and gentle suction. Only one flask was sampled each day.

1) Effect of first diet

Three trials were run to confirm the beneficial effects of using an initial single dose of live algae when artificial diets replace live feed either totally or partially. All trials used late $Nauplius_5$ and early $Zoea_1$ larvae, which were first fed with either a single dose of live microalgae (SDLA) or inert midrodiets (MD). Posterior feed treatments consisted on live microalgae or MD resulting in all-pair-wise combinations: (SDLA)+MD, (SDLA)+live microalgae, (MD)+MD, (MD)+live microalgae (text in brackets indicates first feed). The first (A) and second (B) trials used 1000 and 500 ml flasks, respectively, and were run simultanaeously with larvae from the same batch until $Postlarvae_2$ stages were observed (10 days). The third trial (C) used 1000 ml flasks and was run up to 12 h after first $Mysis_3$ were observed (7 days).

2) Effect of live versus dead microalgae cells

In this trial, the substitution of live microalgae cells as first feed (SDLA) was attempted using a suspension of macroalga *Spirulina platensis* dry powder. *Spirulina* has been used with sucess in the rearing of penaied shrimp larvae and is commercialised in a powder with similar particle size frequency (20 -80 μm) as the microdiet used in the present work. Since *Spirulina* is mainly used as a complementary feed, the first diet treatments included MD to increase the probability of larval survival. Therefore, the treatments tested were (SDLA+MD)+MD and (*Spirulina*+MD)+MD, whilst (SDLA)+microalgae served as a control using live feed throughout the trial (text in brackets indicates first feed). Initial "*Spirulina* +MD" treatment consisted of 1 mg L^{-1} of MD and 8 mg L^{-1} of *Spirulina sp.* dry powder, following the product's recomended concentration. The first (A) and second (B) trials used 1000 and 500 ml flasks, respectively, and were run simultanaeously with larvae from the same batch up to 12 h after first $Postlarvae_1$ were observed (9 days for the live microalgae control and 13 days for the other treatments).

Table 2.2 – Experimental design of Trial 4 using 2 factors with 2 and 4 levels each. Factors were initial water quality (artificial versus matured water) and type of feed given - 1) microparticulated + single dose of live algae (SDLA); 2) microparticulated + SDLA + daily addition of *Tetraselmis chuii* microalgae filtered water; 3) microparticulated + SDLA + daily addition of microalgae growth medium enriched water; and 4) live microalgae.

Initial Water	Feed	Daily Water Treatment	Replicate N
Matured SW	SDLA + Artificial Diet	Addition of microalgae water	5
	SDLA + Artificial Diet	Addition of nutrient water	5
	SDLA + Artificial Diet	—	5
	live algae	—	5
Artificial SW	SDLA + Artificial Diet	Addition of microalgae water	5
	SDLA + Artificial Diet	Addition of nutrient water	5
	SDLA + Artificial Diet	—	5
	live algae	—	5

3) Combined effects of first diet and initial microflora

In order to examine whether the beneficial effect of providing a SDLA to first feeding shrimp larvae was influenced by the microflora present at that time in the culture water, a selection of treatments previously tested were replicated in different water quality treatments that attempted to create different microfloras. Diet treatments included (SDLA)+MD, (MD)+MD, whilst (SDLA)+microalgae served as a control using live feed throughout the trial (text in brackets indicates first feed). The first two diet treatments were replicated in a) 5 μm filtered natural seawater, b) sterile 0.2 μm filtered natural seawater, and c) 5 μm filtered natural seawater to which a solution of a commercial probiotic (Efinol™) was added 24 h prior to the experiment with strong aeration, according to the product's recomended concentration. This trial used 3 replicates per treatment and was run in 500 ml flasks until $Mysis_3$ were observed (8 days). Differences in the pattern of total ammonia and nitrite accumulation over the experimental periods were used as indirect proof of microflora changes.

4) Artificial versus matured seawater: the role of microalgal cells, of exudates + bacteria present in the microalgae culture-water, and of nutrients used to enrich microalgae cultures

When a single dose of live algae is presented at the initial culture stage, several factors may be responsible for improving larval survival in cultures fed with microdiets. The suspension of microalgae contains a) the microalgal cells, b) exudates from microalgal cells, c) bacteria that grow in microalgae cultures and d) nutrients used to enrich microalgae cultures. Microalgal cells contribute directly to the nutrition of the larvae, whilst the other three factors modulate the microflora that eventually will establish in the culture flask. In this trial it was attempted to evaluate the effect of each factor separately, and to test them in two distinct initial microflora situations: aged seawater with a mature microbial flora and artificial seawater, freshly prepared with few bacteria.

Eight treatments were used, divided in two categories: larvae grown on "matured" seawater and larvae grown on "artificial" seawater. Each category was then further divided in four groups: (SDLA)+microalgae, (SDLA)+MD, (SDLA)+MD+"microalgae water", (SDLA)+MD+"nutrients water" (text in brackets indicates first feed). The overall experimental design is illustrated in Table 2.1.

The treatments that used "matured" seawater consisted of water that was in recirculation with no animals for at least 20 days prior to the experimental period. This water (1200 L) was strongly aerated, and constantly filtered (1 μm + activated charcoal), sterilised (U.V.) and passed through a biofilter (6 L fluidised sand bed). At the beginning of the recirculation period 10 mg L^{-1} of EDTA were added as a chelating agent.

The treatments that used "artificial" seawater consisted of water that was prepared 3-4 h prior to stocking of animals using distilled water adjusted to 39 ‰ salinity with commercial sea salts (Ocean Garden™). Although not strictly sterile, this treatment ensured that few bacteria were present at the time of stocking. It also ensured that the nutrient composition of the water was different from "matured" seawater.

The single dose of live algae (SDLA) used in this trial, consisted only of 5 cells μ L^{-1} of Chaetoceros gracilis and was given to $Nauplii_5$ in all microdiet treatments. A similar microdiet was used ("MD" CAR ULTRA FI

No1, INVE Technologies with 85 % particle size between 5-30 μm range). Controls with live feed treatment consisted of 40 and 10 cells μ L^{-1} of *C. gracilis* and *Tetraselmis chui* microalgae concentration, respectively. The concentration of *C. gracilis* was increased to 75 cells μ L^{-1} during the final two days.

Following the reported antibacterial effect of *Tetraselmis suecica* exudates (Austin *et al.* 1992; Austin & Day 1990), it was decided to work with a similar species, *Tetraselmis chuii*. One treatment consisted on filtering the microalgae cells out from the suspension and deliver the resulting water with any potential exudates and bacteria to the flasks. Only *T. chuii* cultures in exponential growth phase were used. Microalgae were filtered moments before the addition using a Whatman GF-C filter (1.2 μm). Another treatment consisted on the addition of water enriched with microalgae growth medium. Water to be used in this treatment was prepared with 70% of the recommended "Guillard f/2" growth medium concentrations (Guillard & Ryther 1962) using 35 ‰ autoclaved seawater in a 2 L flask stored at 4 °C. Both treatments were dispensed to the flasks using an initial 40 ml volume given at the beginning of the experiment and then 5 ml two times per day (11 and 23 h). These were the approximate live microalgae culture volumes delivered to the live feed treatment flasks. Since extra additions of water were to be done daily, all flasks were filled with only 850 ml at the beggining of the experiment.

Due to observations in previous trials that mortality occured in the early larval stages, and because the interest was focused on the moment when the larvae first open their mouth, this trial was finished 12 h after the first $Zoea_2$ stages were observed (6 days). Differences in the pattern of total ammonia and nitrite accumulation over the experimental periods were used as indirect proof of microflora changes.

Percentage survival, development and growth

At the end of the specified experimental periods, larvae from all treatments were killed by addition of 5 ml 37% formalin. All survivors from each flask were counted and their development stages identified individually in a zooplankton counting chamber using a stereoscopic magnifying lense. The same instrument was used to estimate total final length measuring a random sub-sample of 10 complete animals per flask replicate to the nearest μm (tip of rostrum to bifurcation of posterior end for $Zoea_2$). In the first trial, some of the few animals from the (MD)+MD treatment that survived, were partially disintegrated. This did not enable the measurement of final length, although the observation of cephalothorax characteristics enabled identification of the development stage. Average percentage survival was calculated for each treatment. Assuming that the mortality of a population is represented by an exponential decay equation, the relative mean mortality rate (also known as "intrinsic", "specific" or "instantaneous" mortality rate - number of individuals that die per unit of time and per individual alive) was calculated using the following equation:

$$\text{Relative Mortality Rate M} = \frac{Ln(N_{t_1}) - Ln(N_{t_0})}{t_1 - t_0} \qquad (2.1)$$

Where M is the average relative mortality rate between time t_0 (initial) and t_1 (final), and N_t is the number of individuals at a given time t (Causton 1977). The use of this population parameter is appropriate for comparing results from trials with different durations and/or different initial stocking densities. In the last trial, results from the artificial seawater treatments yielded very low or zero survival, which does not enable the

calculation of relative mortality. The ranking "development index" (DI) of Villegas and Kanazawa (1979) was used to estimate average inter-treatment stage of development (weights of "0", "1", "2", "3", "4", "5" and "6" were used for $Nauplii_5$, $Zoea_1$, $Zoea_2$, $Zoea_2$, $Mysis_1$, $Mysis_2$ and $Mysis_3$ stages, respectively).

Water quality analysis

It is known that there is a high specificity on the strains responsible for the nitrifying process (Wheaton *et al.* 1994). Thus, the present work used differences in the pattern of total ammonia and nitrite accumulation over the experimental periods as indirect proof of microflora changes, instead of describing the microflora composition of initial and final treatments. This is because the latter technique is laborious and time-consuming, and results are uncertain due to unsettled controversies both over bacterial strain taxonomy and bacterial identification methods (biochemical versus genetic and molecular).

Total ammonia and nitrite concentrations were measured at the beginning and end of the experimental period adapting the methods described in Parsons *et al.* (1984) to a 96 well microplate (350 µL). Sample volume used was 250 µL. For total ammonia 10 µL of phenol solution, 10 µL of nitroprusside solution and 30 µL of the oxidising solution were added. For nitrite determinations 10 µL of sulfanilamide solution and 10 µL nhaptyl solution were added. These solutions were added according to the sequence and intermediate resting periods reported by Parsons *et al.* (1984). Samples were kept at low temperature (5°C) until analysed within 2-3 hours after being collected.

Statistical analysis

All data sets were tested for departure from normality (Anderson-Darling statistic) and inter-treatment homogeneity of variance (Bartllet and Levene's statistics, depending on normal/non-normal distribution of data points) before running one-way analysis of variance (F test) with post-hoc all-pair-wise comparisons using Tukey's statistic. Non-normal relative mortality rate, final total ammonia and final nitrite concentrations data sets, were "log" transformed. When analysing length data, where there were 10 larvae measured per tank, tanks were considered a random factor. A Student-T test was used (t-value) when only two datasets were present. Percentage survival data was arcsin transformed according to Zar (1999). Non parametric data were analysed using Kruskal-Walis analysis of medians (H test), adjusting for ties followed by a Tukey-type multiple comparisons using the Nemenyi test and Dunn's standard error (SE) procedure described in Zar (1999). A Mann-Whitney test (U test) was used when only two datasets were present.

RESULTS

1) Effect of first diet

Higher survival, between 80 and 100%, from microalgae fed treatments ("SDLA + microalgae") shows larvae of extremely good quality (Fig. 2.1 A_1, B_1, C_1). These larvae were also the most advanced and larger than larvae from any other treatments (Fig. 2.1 $A_{3,4}$, $B_{3,4}$, $C_{3,4}$). Larvae from this treatment, fed exclusively on live microalgae, also presented less inter-replicate variance in all three trials, particularly in percentage survival. These larvae performed equally well when presented with an initial dose of live algae for 4-5 h, and then fed on a microdiet ("SDLA + MD"), although development was significantly delayed (Fig. 2.1 A_3, B_3, C_3). Larvae submitted to the "MD + MD" treatment presented low survival, high relative rate of mortality, and delayed development (Fig. 2.1 A, B, C).

Trial A (1000 ml – 10 days) - Larvae fed SDLA and MD reared in the 1000 ml experiment had a percentage survival above 70%, although their development was significantly delayed and thus had a shorter length than larvae fed live microalgae (Fig. 2.1 $A_{1,2,3,4}$).

Trial B (500 ml – 10 days) - Due to large inter-replicate variance in MD fed treatments, no significant differences could be calculated. However, there is a pattern of higher survival on microalgae fed larvae as opposed to MD fed larvae (Fig. 2.1 $B_{1,2}$). This experiment also had an "unfed" treatment (data not shown) where larvae were not fed and by the end of day 3 all larvae had died.

Trial C (1000 ml – 7 days) - Survival of larvae fed exclusively with microalgae ("SDLA + microalgae") was close to 100% although it was not significantly larger than survival of "SDLA + MD" fed larvae. Development and final length, however, were significantly larger for larvae fed exclusively with microalgae. Larvae fed exclusively with MD showed poor results with large variance. In this treatment there were not enough larvae from all replicates to represent final length

Total ammonia in the MD-fed treatments of trials 1 and 3 was significantly higher than microalgae-fed treatments (Fig. 2.2 A_1, B_1, C_1). In trial 3, this pattern was significant from day 4 onwards (Fig. 2.2 C_1). Nitrite concentration, on the other hand, did not present any significant differences between treatments with the exception of trial one, where it was higher in microalgae-fed treatments (Fig. 2.2 C_2).

Total ammonia accumulated linearly until day 7 (Fig. 2.2 $A_{1,2}$), whilst nitrite concentration remained constant or increased slowly during the same period (Fig. 2.2 $B_{1,2}$). The ammonia concentration stabilised from day 7 onwards, whilst nitrite levels increased dramatically during the same period. Significant differences in final total ammonia concentration between treatments were found in all three experiments (Fig. 2.2 A_1, B_1, C_1), whilst for nitrites, the only significant differences were observed in the last experiment (Fig. 2.2 C_2). The unfed treatment flasks in the second trial (Fig. 2.2 B_1) had a significantly lower ammonia concentration, although the nitrite final concentration from this treatment was similar to all other treatments (Fig. 2.2 B_2). No clear correlation could be found between the total ammonia and nitrite concentrations and the survival and growth results.

2) Effect of live versus dead microalgae cells

Larvae fed live microalgae presented a percentage survival above 90%, a very low relative mortality rate, small variance in the development stage reached and a larger length, when compared to the MD fed treatments (Fig. 2.3 $A_{1,2,3,4}$). This indicates good quality larvae. Larvae presented with a single dose of dry macroalgae powder particles (*Spirulina platensis*) at the time of first feeding did not vary significantly from larvae fed SDLA, both in 1000 and 500 ml flasks. Larvae from 500 ml flasks died faster than 1000 ml reared larvae, which resulted in lower percentage survival (Fig. 2.3 $A_{1,2}$, $B_{1,2}$). The development stage and final length of larvae reared in 500 ml flasks was similar to those reared in 1000 ml (Fig. 2.3 $A_{3,4}$, $B_{3,4}$).

Final ammonia concentration in the live feed treatment water (SDLA+microalgae) was significantly lower than MD-fed treatments (Fig. 2.4 A_1). Nitrite concentration, on the other hand, was significantly higher (Fig. 2.4 B_1). Both ammonia and nitrite final concentrations were similar between MD fed with SDLA or *Spirulina* first feed treatments (Fig. 2.4 $A_{1,2}$, $B_{1,2}$). Final values of total ammonia and nitrite from 1000 and 500 ml flasks were similar.

3) Combined effects of first diet and initial microflora

The relative mortality rate of larvae fed exclusively on live microalgae (SDLA+microalgae) was close to zero, resulting in survival above 80% and indicating good quality larvae (Fig. 2.5 A, B). These larvae were also the most advanced and larger than larvae from any other treatments (Fig. 2.5 C, D). Larvae fed exclusively on live microalgae also presented smaller within treatment variance. Survival was higher for all SDLA-fed larvae (Fig. 2.5 A), but no differences were detected in development and growth (Fig. 2.5 C, D), irrespective of bacterial "quality" of culture water. No conclusive trend could be observed on the effect of the initial microflora. Insufficient numbers of live, complete larvae in each replicate from the "(MD in normal Water)+MD" treatment, did not enable a representative value of final length for that treatment.

Total ammonia accumulated linearly until day 7, whilst nitrite concentration oscillated or increased slowly during the same period (Fig. 2.6 A). Although the final ammonia concentration in the microalgae fed treatment was not statistically different from other treatments, it had the lowest value.

4) Artificial versus matured seawater: the role of microalgal cells, of exudates + bacteria present in the microalgae culture-water, and of nutrients used to enrich microalgae cultures

Artificial seawater treatments with artificial feed collapsed in less than 5 days, with null or very low survival (Fig. 2.7 A_2). Therefore no relative rate of mortality could be calculated for these two treatments, and neither the development nor the final average length could be assessed (Fig. 2.7 B_2, C_2, D_2). The only artificial seawater cultured larvae that survived above 10% were those fed microalgae, which gave a percentage survival comparable to those of larvae reared in matured seawater and fed artificial diets. Larvae that were cultured in matured seawater and fed microalgae gave the highest percentage survival (92.2%) with a significant lower relative mortality rate (Fig. 2.7 A_1, B_1). Similarly, they were the most advanced with a few larvae already moulting into $Zoea_3$, and were larger than all other treatments, as can be seen by the significant higher

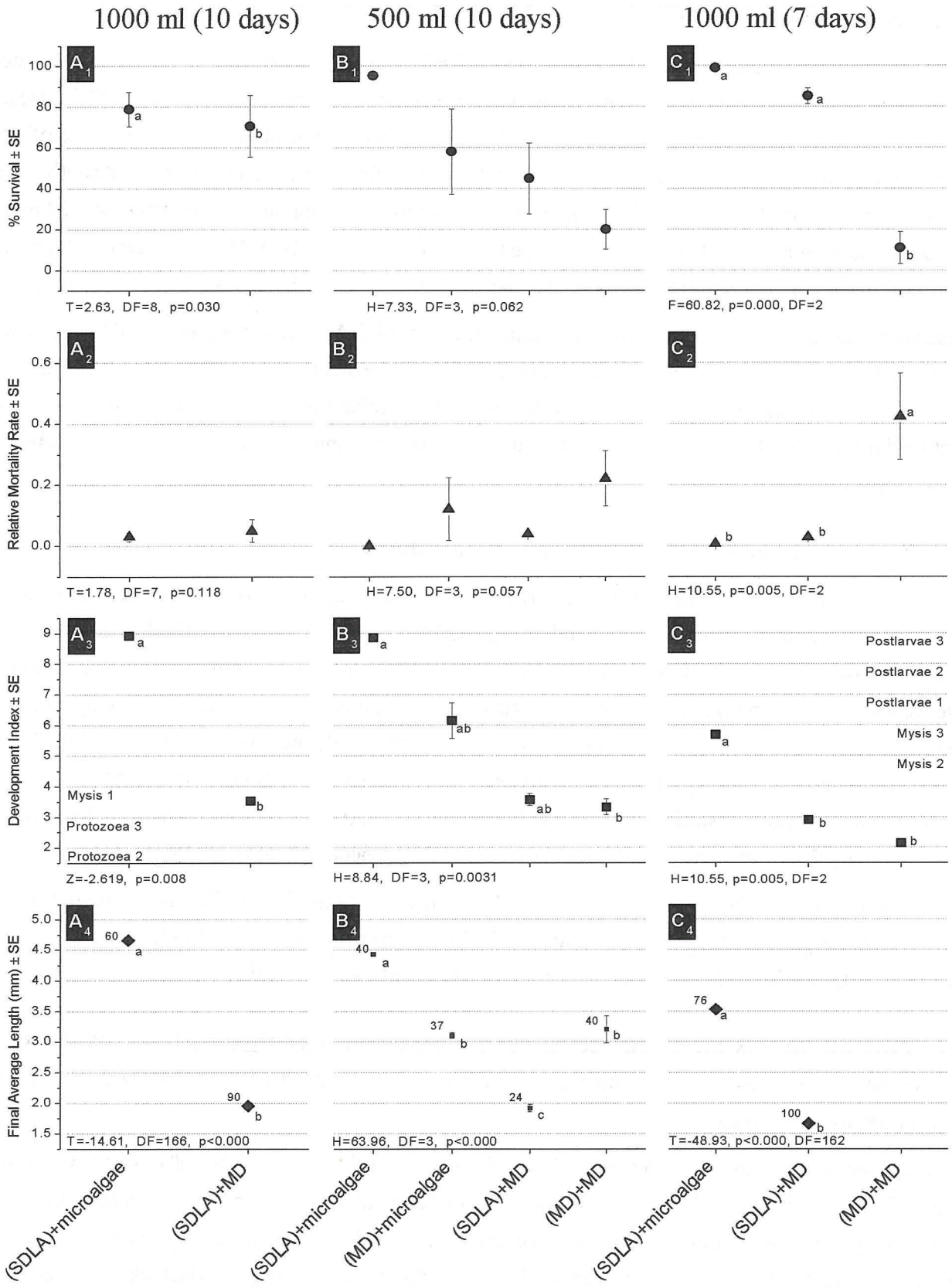

Figure 2.1 – Effect of initial diet: mean percentage survival, relative mortality rate (number of dead animals · day $^{-1}$ · animals alive^{-1}), development index and final length of *Litopenaeus vannamei* larvae in experiments using 1000 ml (**A, B**) and 500 ml (**C**) with 5 replicates to test the effect of a single dose of live microalgae (SDLA) and inert microdiets (MD) first feeding regimes with subsequent daily feeding with either live microalgae or MD. Letters between brackets indicate feed conditions when larvae first opened their mouth (initial diet). Different letters represent significant differences between treatments. Development index graphs show the larval stage with the correspondent DI line. Experiments were run for 10 and 8 days.

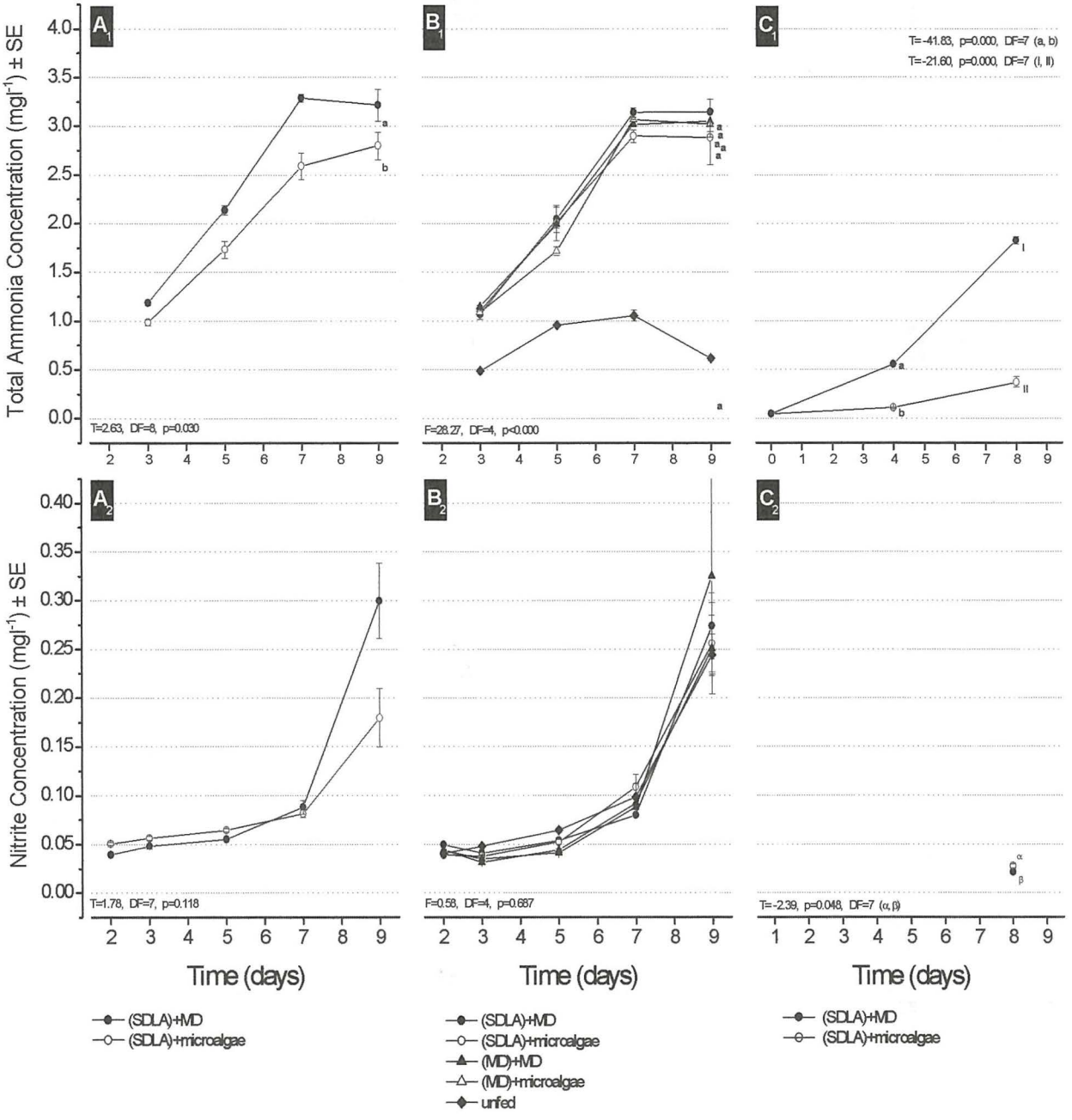

Figure 2.2 – Trial 1: Total ammonia and nitrite concentrations of three different initial and daily feed treatments. Letters between brackets indicate feed conditions when larvae first opened their mouth. Different letters represent significant differences between treatments ($p < 0.05$).

development index and final average individual length in Figure 2.4 C_1 and 2.4 D_1. Larvae cultured in artificial sea water and fed microalgae were significantly larger than larvae cultured in matured sea water and fed MD, although both shared the same development stage (Fig. 2.7 $C_{1,2}$, $D_{1,2}$).

Overall, matured sea-water provided better larviculture results. Daily addition of live microalgae as feed attained higher larval survival and growth than daily addition of MD. The addition of microalgal filtered water and water with nutrients from microalgal growth media did not result in any clear effect.

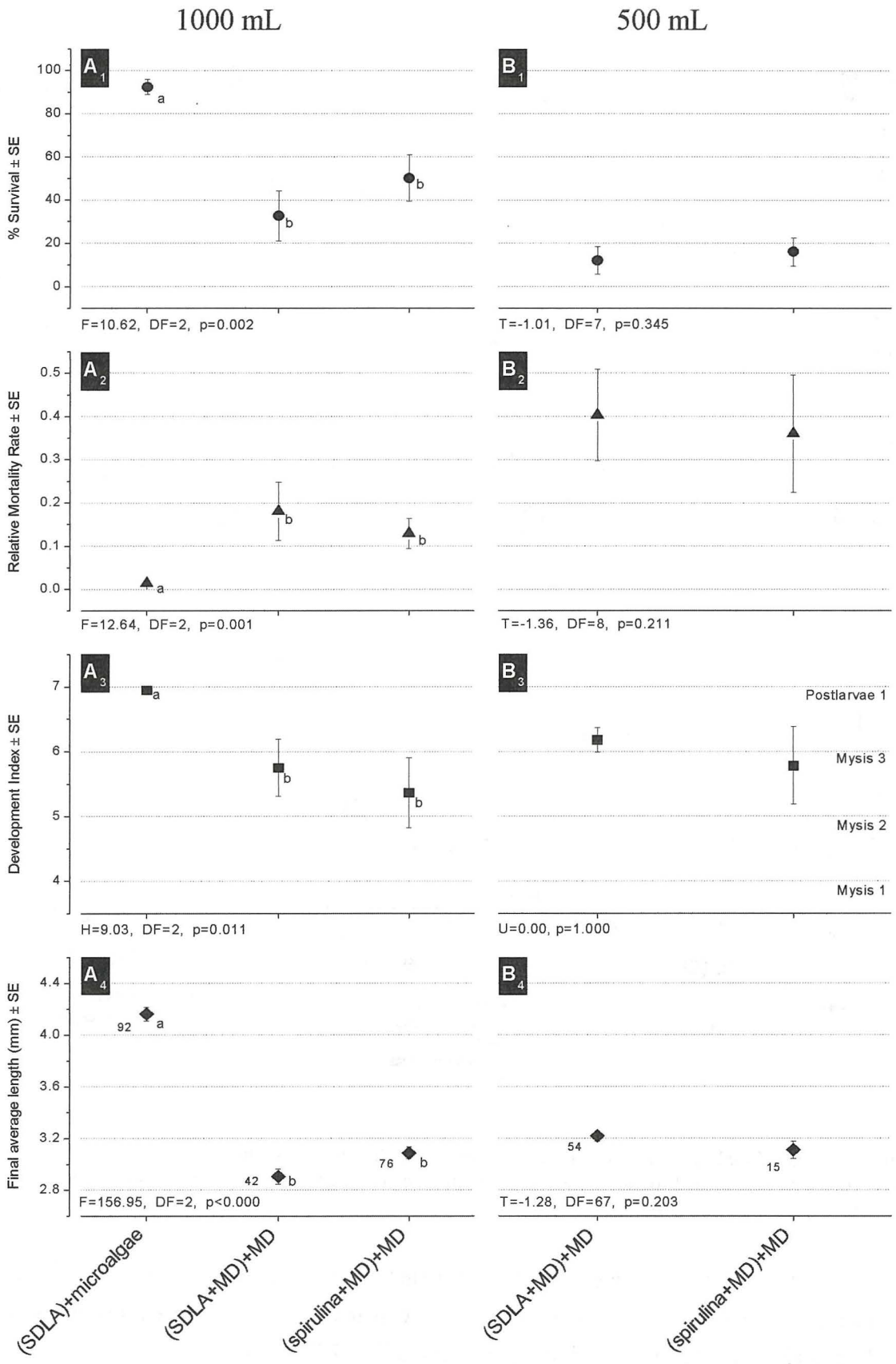

Figure 2.3 – Effect of live versus dead microalgae cells: mean percentage survival, relative mortality rate (number of dead animals · day^{-1} · animals alive^{-1}), development index and final length of *Litopenaeus vannamei* larvae from an experiment to test the substitution of a single dose of live microalgae (SDLA) with a dry algae product (*Spirulina sp.*). Letters between brackets indicate feed conditions when larvae first opened their mouth (initial diet). Different letters represent significant differences between treatments ($p < 0.05$). Development index graphs show the larval stage with the correspondent line. The experiment was run in 1000 mL (**A**) and 500 mL (**B**) flasks with 5 replicates for 9 days.

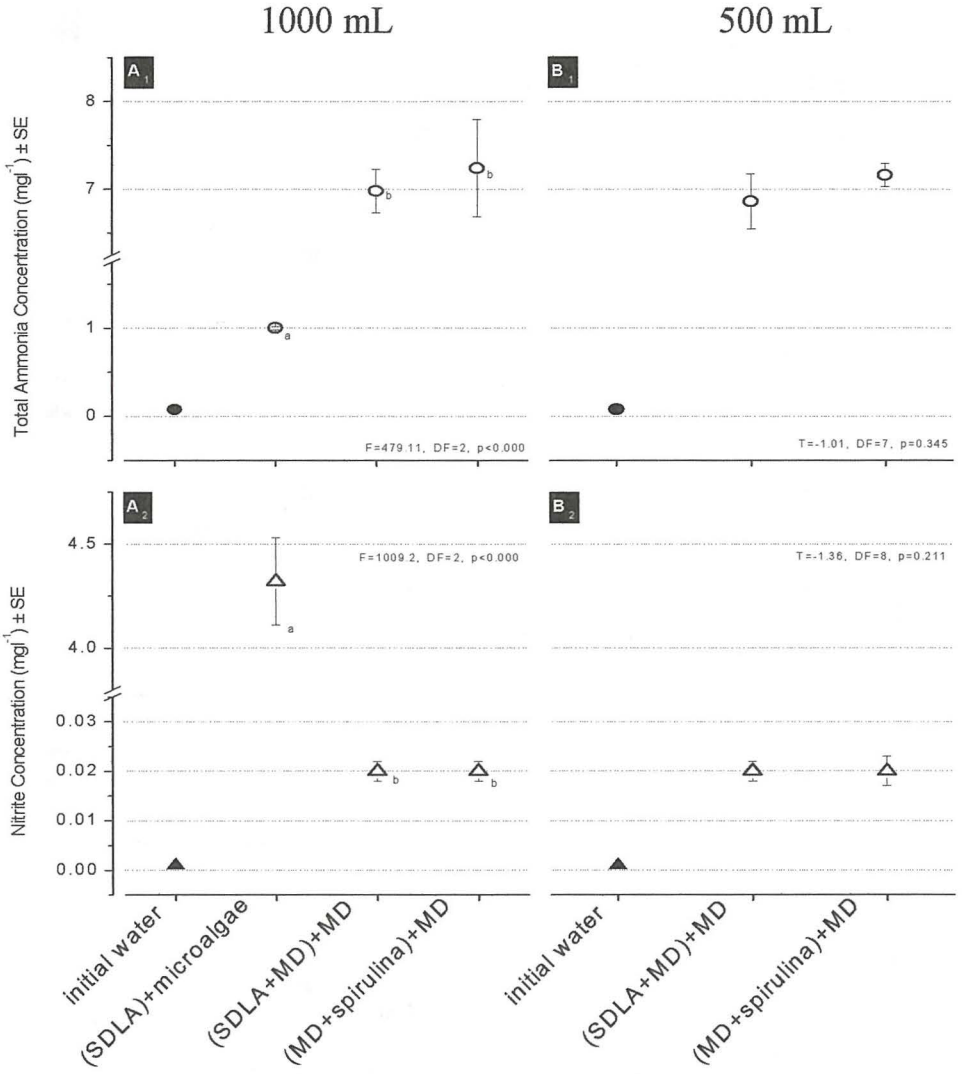

Figure 2.4 – Trial 2: mean initial and final water total ammonia ($\mathbf{A_1}$, $\mathbf{B_1}$) and nitrite ($\mathbf{A_2}$, $\mathbf{B_2}$) concentrations in experiments using 1000 ml and 500 ml to test the effect of the SDLA substitution by a dry algae product. Letters between brackets indicate feed conditions when larvae first opened their mouth. Different letters represent significant differences between treatments ($p < 0.05$). Experiments were run for 9 days with live diet control and 13 days with MD treatments.

The general pattern of total ammonia and nitrite concentrations accumulation in this experiment was similar between the matured and artificial seawater (Fig. 2.8 $A_{1,2}$, $B_{1,2}$). Total ammonia accumulated to higher concentrations in the water of artificial sea water treatments (Fig. 2.8 A_1, B_1). Nitrite showed an inverse pattern with higher accumulation in matured sea water treatments, with the exception of the "(SDLA)+MD" treatment (Fig. 2.8 $B_{1,2}$). The key difference observed in both matured and artificial seawater treatments was between artificial versus live feed, with the live feed condition having significantly lower total ammonia and higher nitrite build up when compared to all the artificial feed treatments (Fig. 2.8 $A_{1,2}$, $B_{1,2}$). Between artificial feed treatments, there was a higher build up of both total ammonia and nitrite in the flasks where "extra nutrient water" was added, reaching higher concentrations, especially for ammonia (Fig. 2.8 $A_{1,2}$, $B_{1,2}$). Such results were closely associated with the levels of total ammonia and nitrite in the water that was added to the treatments "(SDLA)+MD+microalgaeWtr" and "(SDLA)+MD+nutrientsWtr". The "microalgae water" treatment had a total ammonia concentration similar to those of the distilled water used to prepare the artificial water and the matured sea water (Fig. 2.8 C_1). The nitrite concentration, on the other hand, was five times higher than any other type of water (Fig. 2.8 C_2). The "nutrients water" presented an exactly opposite

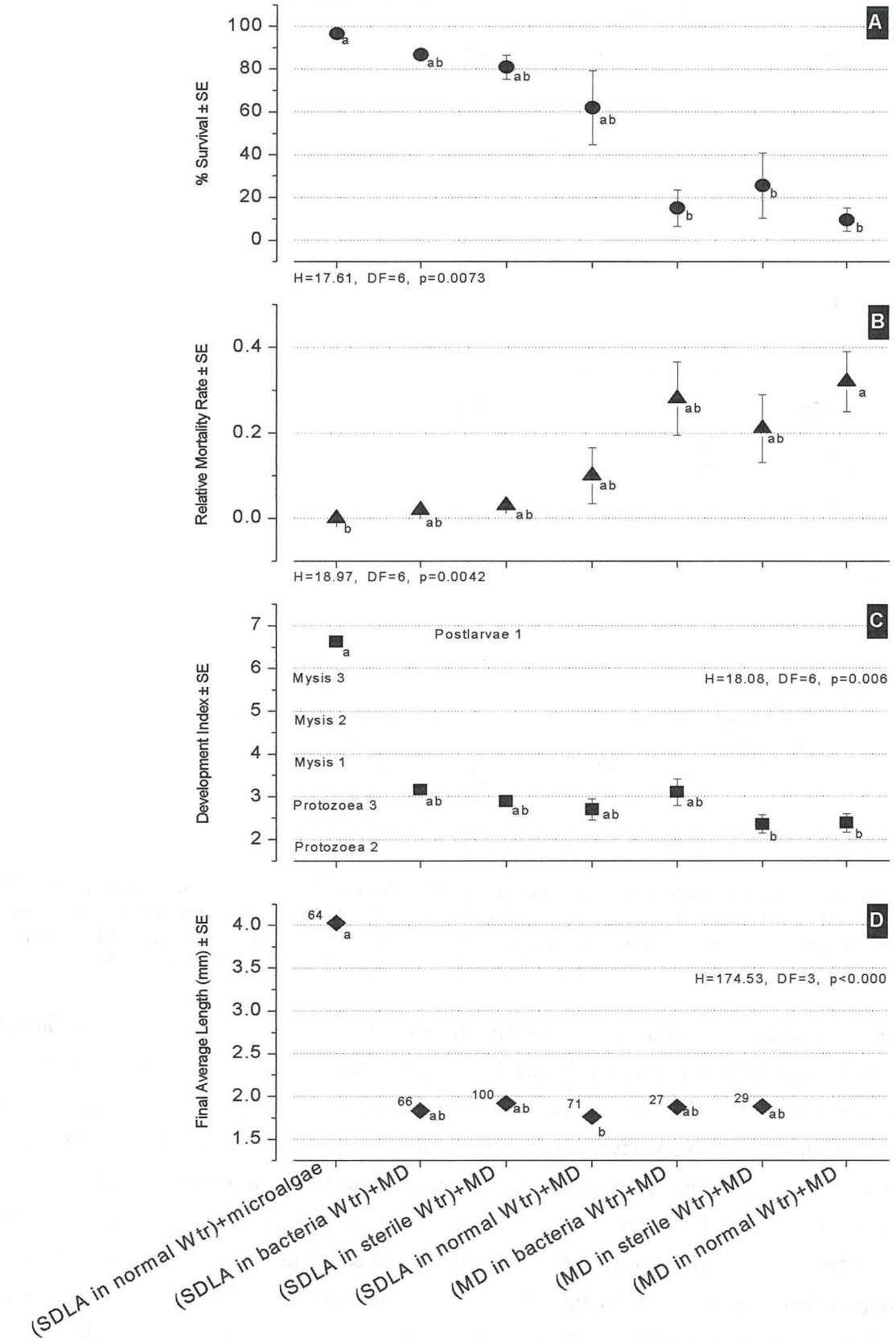

Figure 2.5 – Combined effects of first diet and initial microflora: mean percentage survival, relative mortality rate (number of dead animals · day^{-1} · animals alive^{-1}), development index and final length of *Litopenaeus vannamei* larvae subject to different initial microflora in the culture water and several feed combinations using a single dose of live microalgae (SDLA) and inert microdiets (MD) as first and daily feeds. Letters between brackets indicate feed conditions when larvae first opened their mouth. Different letters represent significant differences between treatments ($p < 0.05$). Development index (DI) graphs show the larval stage with the correspondent DI line. Experiment was run 500 ml flasks, with 3 replicates for 8 days.

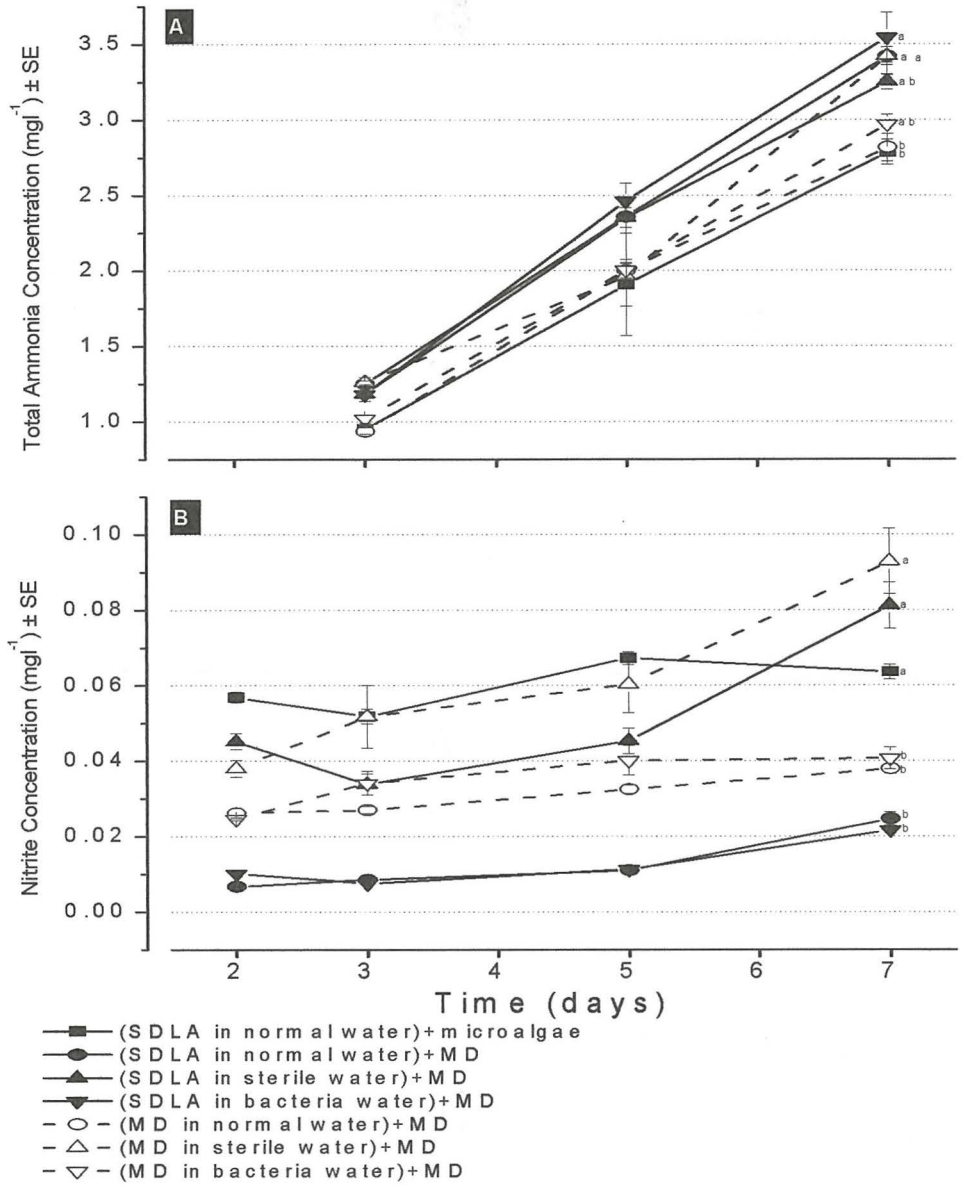

Figure 2.6 – Trial 3: evolution of culture water total ammonia ($\mathbf{A_1}$, $\mathbf{B_1}$, $\mathbf{C_1}$) and nitrite ($\mathbf{A_2}$, $\mathbf{B_2}$, $\mathbf{C_2}$) concentrations. Letters between brackets indicate feed conditions when larvae first opened their mouth. Different letters represent significant differences between treatments ($p < 0.05$).

pattern with extremely high total ammonia concentrations (36 mg L^{-1}) and a nitrite concentration equivalent to those of the distilled water used to prepare the artificial water and the matured sea water (Fig. 2.8 C_1, C_2). The cumulative total ammonia and nitrite that was added to the flasks through the addition of "microalgae" and "nutrients water" over the experimental period, is represented by stars in Figure 2.8 D_1 and 2.8 D_2. These values were calculated using the artificial and matured sea water initial ammonia and nitrite concentrations plus the daily dose multiplied by the experiment days (Fig. 2.8 C_1, C_2). For ammonia such predicted values were proportional, but below the final observed ammonia concentrations of the treatments where "microalgae" and "nutrients water" was added (Fig. 2.8 $A_{1,2}$ D_1). For nitrite the predictive values are inversely proportional to those observed (Fig. 2.8 $B_{1,2}$ D_2).

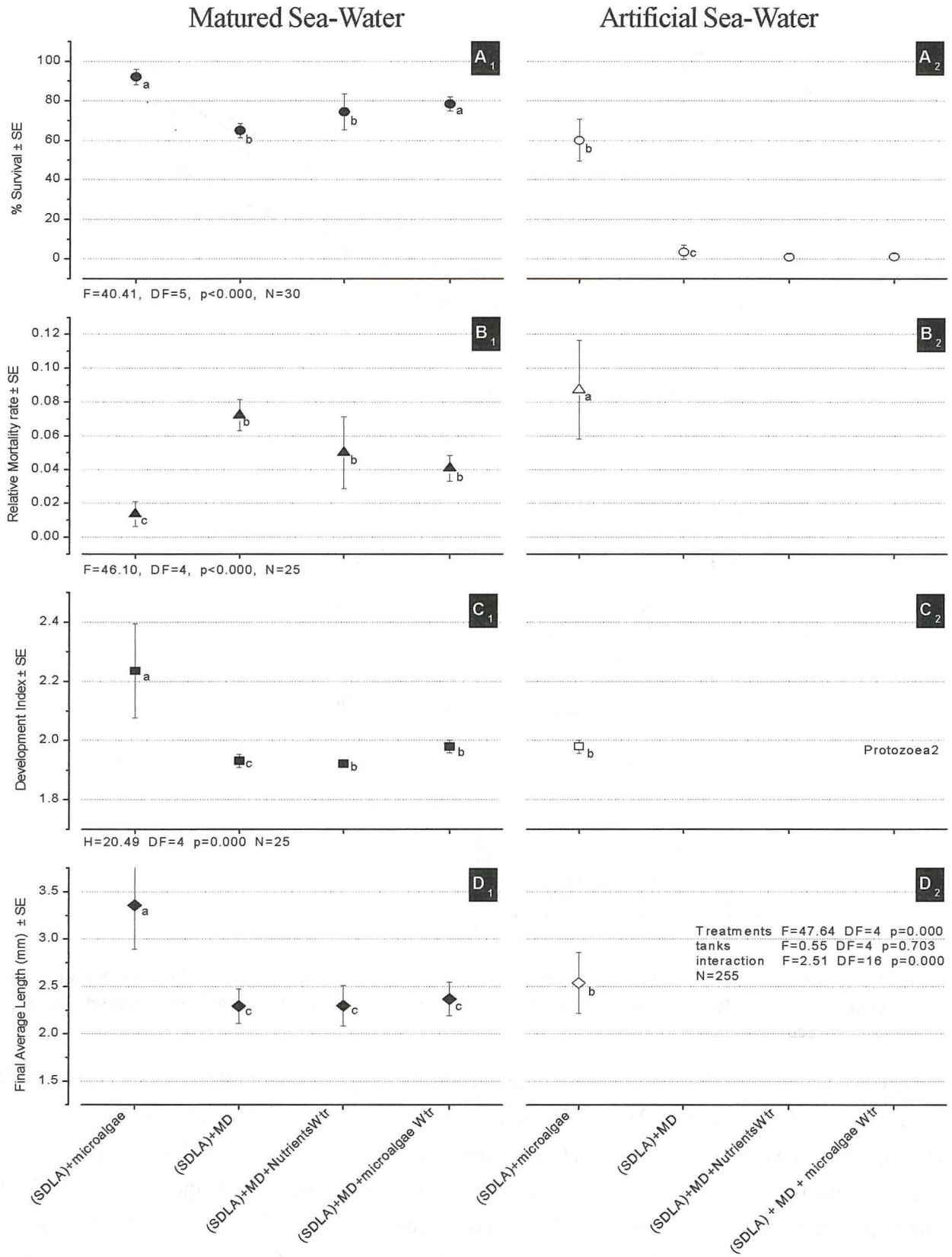

Figure 2.7 – Artificial versus matured seawater: the role of microalgal cells, of exudates + bacteria present in the microalgae culture water, and of nutrients used to enrich microalgae cultures: (**A**) mean percentage survival, (**B**) relative *mortality rate (number of dead animals · day*$^{-1}$ · animals alive^{-1}), (**C**) development index and (**D**) final length of *Litopenaeus* vannamei larvae exposed to 4 initial diet treatments in either matured or artificial seawater. Full symbols represent matured seawater; hollow symbols represent artificial seawater. Treatments details are presented in Table 2.1. Letters between brackets indicate feed conditions when larvae first opened their mouth. SDLA - single dose of live microalgae; MD - inert microdiets. Different letters represent significant differences between treatments ($p < 0.05$). The experiment was run in 1000 ml flasks with 5 replicates for 5 days.

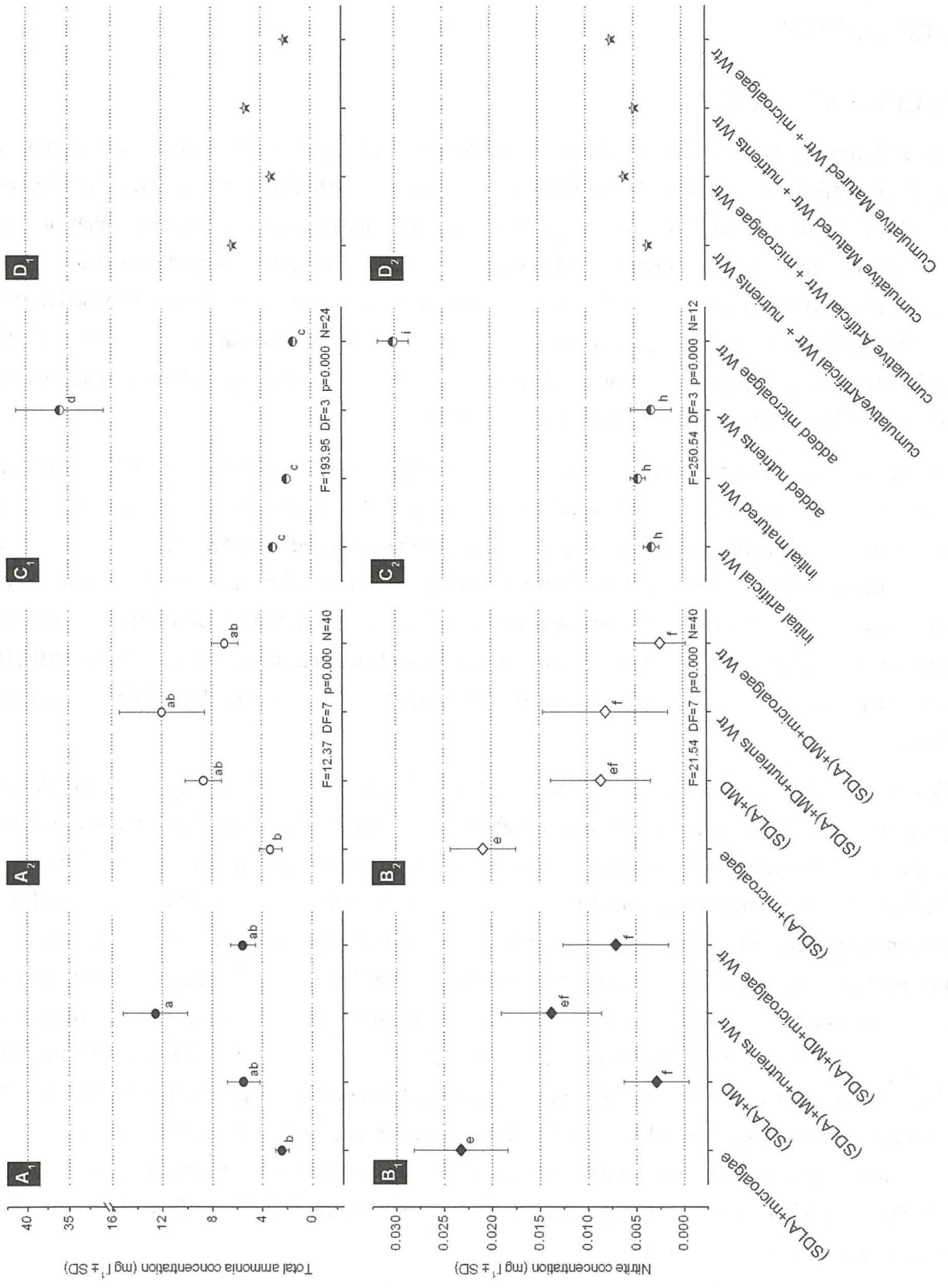

Figure 2.8 – Trial 4: final mean total ammonia ($\mathbf{A}_{1,2}$, $\mathbf{C}_1$, $\mathbf{D}_1$) and nitrite ($\mathbf{B}_{1,2}$, $\mathbf{C}_2$, $\mathbf{D}_2$) concentrations in the water of eight experimental treatments plus concentrations in the distilled water used to prepare artificial seawater, and the two "extra water" treatments – water from *Tetraselmis chuii* cultures filtered and water enriched with 70% microalgae growth medium (Guillard). Full symbols represent matured seawater; hollow symbols represent artificial seawater (**A, B**). Stars represent calculation of total or cumulative ammonia and nitrite added to the "extra water" treatments depending on initial matured and artificial water concentrations, plus concentrations of "microalgae" and "nutrients water" added (**D**). Letters between brackets in the labels indicate feed conditions when larvae first opened their mouth, or initial feed. Different letters in the graphics represent significant differences between treatments ($p < 0.05$). Experiments were run for 5 days.

DISCUSSION

1) Effect of first diet

Survival, development and final length results from this set of experiments confirm the importance of the presence of microalgae when the larvae first open their mouths and initiate exogenous feeding after moulting into $Zoea_1$. Factors present in the microalga cells and/or the microalga culture water must play an important role in initial bacterial colonisation of the digestive tract and/or stimulation of digestion and absorption. MD ingestion rates in gilthead seabream larvae increased up to 120% when the fish were exposed to the visual and chemical stimuli of various concentrations of *Artemia* nauplii. The free amino acids alanine, glycine and arginine and the compound betaine were identified from the *Artemia* rearing medium as metabolites, which stimulated this larval response (Kolkovski *et al.* 1997).

Increased variable variance from treatments using MD can be explained by different MD particle sizes and nutrient contents due to leaching. Although the probability of encounters between larvae and particles should be the same for all larvae, the actual size of particles encountered varies, as does nutrient and energy content with time. A feeding regime using 6 meals per day every 4 h, attempted to minimise such situation. Variance within the same treatment may also be due to subtle differences in aeration rate and therefore, sedimentation rate of inert food particles. Algae replicates do not show such marked variance, probably due to the fact that some alga species are highly mobile (*Tetraselmis sp.*) and therefore permanently available for larvae to feed on them.

Final values of both ammonia and nitrite were below lethal levels reported for *L. vannamei* and other penaeid species (Cochard *et al.* 1996; Chen & Tu 1990). Ammonia is one of the end products from the degradation of uneaten MD particles, MD leached nutrients, larval faeces and also from the larva excretion. Nitrite will result from the oxidation of ammonia molecules. The observation of the first two trials (Fig. 2.2 A, B) general pattern of total ammonia and nitrite accumulation indicates that the bacteria responsible for the first step of the nitrification process are established from day 7 onwards. This follows the observation that ammonia concentration decreases with a concomitant increase in nitrite concentration, irrespective of water or feed treatment. Since no food was added to the "unfed" treatment (Fig. 2.2 B_1), all larvae died by day three and the rise in ammonia concentration was significantly lower than in all other treatments, indicating that ammonia accumulation is mainly due to the addition of feed. The nitrite concentration of the "unfed" treatment, however, increased exponentially from day 7 onwards as in all other treatments, suggesting that the development of the *Nitrosomonas spp.* bacterial community was independent of the observed high ammonia concentration and feed addition.

The observed changes in time for total ammonia and nitrite concentrations were probably related to increased protein concentration due to MD leaching. Such proteins may be degraded by heterotrophic bacteria resulting in higher total ammonia levels in the MD-fed treatments. It also suggests the existence of a different water microflora composition, as indicated by the differences in nitrite concentrations, most certainly due to the presence of a nitrifying bacterial community in the "(SDLA) + microalgae" treatment. Independent of the production method, most MD for larvae of commercially important marine species have some degree of leaching, normally reported as characteristic dissolution time constant (Dra. Ruth Pedroza

pers. com.). The frequent addition of MD to the culture water will therefore provide plenty of extra readily available nutrients which can be used by bacteria present in the water. The rapid colonisation of the exterior MD surface by bacteria has been reported (Muir & Sutton 1994). Such conditions may trigger the rapid proliferation of opportunistic bacterial strains that normally have restricted opportunities for growth and therefore can adversely affect larval cultures (Olafsen 2001). A similar situation may have developed in the "(MD) + MD" treatment, where abundant nutrients and the lack of bacterial control provided by microalgae may have resulted in the growth of a harmful microflora.

2) Effect of live versus dead microalgae cells

Results from the live diet control confirm the excellent larval quality with a percentage survival above 90% to Postlarvae1. When larvae from the same batch were fed with MD, results dropped significantly. The substitution of SDLA as first feed by dry *Spirulina* did not improve MD use, but provided similar results. These results were replicated in trials A and B simultaneously, suggesting that both factors "live microalgae cells" and "microalgae culture water" can be substituted by dry microparticulate macroalgae.

Ammonia accumulated to higher concentrations in the MD-fed treatments due to diet leaching. The microalgae-fed treatment build up less ammonia since microalgae use this nutrient to grow. Nitrite also accumulated in MD-fed treatments, although this accumulation was higher in the microalgae-fed treatment. The addition of either a SDLA or inert macroalgae particles as the initial feed did not alter the final total ammonia and nitrite concentrations in any MD-fed treatments. This indicates similar nitrifying bacterial communities in both treatments. Any early differences in bacterial communities induced by the addition of either live microalgae or inert macroalgae particles may have been diluted over the 9 days experimental period.

From these results it is possible to conclude that the beneficial effect of a SDLA as first feed is mainly related to nutrition. This means that it is the nutrients in the microalgae cells that are important and not so the fact that the cells are alive, moving and continuously leaching exudates that may or may not condition the surrounding microflora. However, the experimental period lasted long enough after the initial feed treatment (9 days) to mask any early effect, and final results may have result from a convergence due to similar feed conditions (MD-fed). Furthermore, since a negative control treatment "(MD)+MD" is lacking, it is difficult to assess how much "beneficial" effect was attained with the use of either a SDLA or inert macroalgae particles as first feed.

3) Combined effects of first diet and initial microflora

This experiment was run in order to determine whether changes in larviculture output when larvae are first fed with SDLA or MD, are due exclusively to the nutritional quality of the microalgae versus MD, or whether there is a synergistic effect between initial feed type and bacteriological conditions of the culture water.

Survival, development and growth results from larvae fed microalgae exclusively confirm good larval quality. All other treatments fed with MD after the initial feed, used larvae from the same batch but had lower results (Fig. 2.5). Since larvae fed microalgae reached a significantly more advanced larval stage and had a signifi-

cantly larger length, all MD fed larvae had their growth and development compromised. However, MD fed larvae first fed with SDLA showed better survival than those first fed with MD, indicating that in spite of slower growth and development, these larvae were not close to the survival tolerance limit. Indeed, survival was higher in SDLA first fed larvae, but no differences were detected in development and growth, irrespective of bacterial "quality" of culture water. Similarly, larvae that were fed MD exclusively had lowest survival and were significantly delayed in development and growth when compared to larvae fed microalgae, but no differences due to the bacterial "quality" of the initial water could be observed. The induced differences in bacterial initial water quality were difficult to observe through the ammonia-nitrite accumulation patterns. For example, total ammonia and nitrite accumulated faster in sterile water as the nitrifying bacterial community had to start from zero, but remaining treatments were difficult to interpret. This may indicate that differences present initially were lost during the long culture period (8 days). Perhaps clearer results could be obtained if the experiment was run for a shorter period.

4) Artificial versus matured seawater: the role of microalgal cells, of exudates + bacteria present in the microalgae culture water, and of nutrients used to enrich microalgae cultures

In Trial 4, the collapse of artificial seawater treatments that were fed artificial diets in less than 5 days cannot be attributed to the observed final concentrations of total ammonia and nitrite. The potential presence of some substances with slow dissolving rates from the commercial sea salt mix could be, in part, responsible for such poor survival. However, the short resting period after the preparation of artificial seawater with distilled water and commercial sea salt mix should ensure low initial bacteria numbers in the water treatment. This situation may have triggered a rapid exponential increase in opportunistic bacteria that eventually killed the larvae.

The process of bacterial re-colonization of the initial culture water after water treatment depends on the quantity and diversity of bacteria that survive the water treatment prior to culture and on the resilience of such microflora composition to the addition of new bacteria strains. Opportunistic pathogens are known to rapidly recolonise culture water which is sterilised (Baticados & Pitogo 1990; Alabi *et al.* 1997). The introduction of new bacteria can have several sources, such as: bacteria attached to the shrimp nauplii, bacteria present in the MD, or perhaps and most importantly, the bacteria present in the microalgae cultures that are used to feed the larvae. However, healthy nauplii, hatched under controlled culture conditions, have, in general, a carapace free of bacteria (pers. observ. - chapter 4). Most microdiets are fabricated in conditions that greatly reduce mesophilic bacterial populations, although the resulting diets are not sterile and may contain dormant bacteria spores (Dr. Ruth Pedroza pers. com.). Such factors minimize the potential for a significant contribution from those two sources. Large microalgae culture facilities, on the other hand, normally have an associated bacterial community which will have an impact on the larval tanks' microflora (Kellam & Walker 1989; Salvesen *et al.* 1999; Skjermo & Vadstein 1993; Tubiash *et al.* 1965; Bell & Lang 1974). These bacterial strains are selected by the specificity of the enrichment mediums used, and microalgae exudates specific to each species which promote or restrict growth of certain bacterial strains. Such control of bacterial populations by microalgal exudates has been extensively reported (Sieburth 1960;

Duff *et al.* 1966; Hornsey & Hide 1974; Kogure *et al.* 1979; Brock & Clyne 1984; Reichelt & Borowitza 1984; Austin *et al.* 1992; Naviner *et al.* 1999).

Present observations suggest that artificially prepared seawater has a deleterious effect on larval survival. The addition of microalgae as feed to the artificial seawater treatment, however, resulted in 60% survival indicating that the lethal effect was mitigated by the presence of microalgae cells and/or microalgal culture water. Kumlu and Jones (1995) demonstrated that the nutrition supplied by 10 – 15 algal cells μL^{-1} is insufficient to support observed postlarval yields, and therefore the beneficial effect is not only due to improved nutrition. Misciattelli *et al.* (1998) further claim that the positive effect of a SDLA it is not related to the nutritional value of the microalgae cells added, nor to the probiotic bacteria associated with the algal growth culture since shrimp larvae fed on MD supplemented by algal water alone were similar to live feed controls and larval survival was equally successful when algal water filtered to 0.2 μm was added to MD fed larvae. Instead, they propose a chemical effect in algal water as the factor responsible for improving survival on artificial diet fed larval cultures, when a SDLA is given at the initial culture stage, causing a modification of the bacteria in the culture water. This algal metabolite does not appear to be bactericidal, but instead reduces or prevents swarming behaviour, exoprotease production and interferes with bioluminescence in *Vibrio harveyi* and other species of microbes, which are associated with virulence towards larval shrimp cultures (Prayitno & Latchford 1995).

In contrast, in present results, zero survival in treatments where artificial diets were complemented with microalgal filtered water or microalgal nutrient water, added at a similar rate and volumes as live microalgae-fed treatments, suggests that the microflora and any exudates present in the microalgal culture water, or the nutrients present in microalgal cultures, did not prevent larval mortality. Daily addition of extra nutrients, or extra bacteria plus potential bactericidal or bacteriostatic substances did not compensate for low water quality, unless microalgal cells were present. Present results show that there must be a beneficial effect related to the live microalgal cells themselves, probably related to their nutritional value. The daily feed with microalgae cells gave significantly better survival (92.2%), growth and development of larvae in microbiologically matured sea water when compared to larvae reared in the same water, but fed MD, with or without microalgal filtered water and nutrients from microalgal growth media.

While there was no apparent effect by the addition of either microalgal filtered water or water with nutrients from microalgal growth media upon survival, development and growth of shrimp larvae reared in matured and artificial seawater, patterns of total ammonia and nitrite accumulation did differ. The similar pattern of total ammonia and nitrite accumulation between matured and artificial seawater treatment groups indicates that the "type" of initial water quality is not as important as the diet treatments to the resulting final concentrations.

The high concentration of ammonia of the "Guillard f/2" microalgal growth medium is reduced in the presence of *Tetraselmis chuii* microalgal cells which uptake ammonia as a source of nitrogen. This high concentration of ammonia, in continuous microalgae culture methods, also stimulates the growth of a nitrifying bacterial community which contributes to the observed reduction in total ammonia concentration by oxidizing it to nitrite. These processes explain the inverted pattern of total ammonia and nitrite concentration in the

"microalgae" and "nutrients" water used for the "extra water" treatments (Fig. 2.8 $C_{1,2}$). They also explain the significant lower ammonia and higher nitrite concentrations in the "(SDLA)+microalgae" treatments.

The cumulative total ammonia and nitrite, over the experimental period, added to the flasks through the addition of "microalgae" and "nutrients" water was below the concentrations observed in those treatments, indicating that the final concentrations were also the result of other sources of production/degradation. As the experimental period (5 days) was shorter than the mean time for bacterial nitrification community development (3-5 weeks), it must be assumed that the observed differences in the build up of total ammonia and nitrite concentration are due either to the addition of these chemicals through the "extra water" supplementation, microalgae consumption, bacterial degradation of MD, and excretion by the larvae.

The "Guillard f/2" microalgal growth medium has, indeed, a strong ammonia clorure (NH_4Cl) component (Laing 1991) which is responsible for the large ammonia final concentration in the treatments where "nutrient water" was added. For the "(SDLA)+microalgae" treatments, low ammonia was most certainly due to the uptake by microalgae. The bacterial colonisation of the exterior MD surface has been reported (Muir & Sutton 1994), and for the "MD-fed" treatments, the elevated ammonia levels, higher than what was added, are probably related to the degradation by heterotrophic bacteria of the MD particles and leached proteins. Ammonia excretion (NH_3-N) during larval ontogeny has been reported to range between 0.00483 ± 0.00031 ($Zoea_1$) and 0.0193 ± 0.0051 ($Mysis_3$) $\mu g\ ind^{-1}\ h^{-1}$ (Lemos & Phan 2001) and it can be regarded as irrelevant.

All experiments

All larvae batches used in the present study were of excellent quality. When comparing between experiments using the relative mortality rate, larvae from the "(SDLA)+microalgae" treatments have an average relative mortality rate of 0.011 larvae day^{-1} larvae $alive^{-1}$. The "(SDLA)+MD" fed larvae have an average of 0.13, whilst the "(MD)+MD" larvae have an average of 0.31 larvae day^{-1} larvae $alive^{-1}$, clearly indicating a positive effect of SDLA on MD-fed larvae. These results confirm earlier suggestions that the addition of a single dose of live microalgae (10 –15 cell μL^{-1}) to MD fed penaeid larval cultures prevents culture collapse (Kurmaly *et al.* 1989; Kurmaly *et al.* 1990; Kumlu & Jones 1995; Jones *et al.* 1997a; Jones *et al.* 1997b).

Algal water alone has been shown to control microbial water quality, although present results suggest that this effect is only effective under certain conditions and may not be easily replicated. Different microbial species composition and number present in the initial culture water are likely to react differently to the controlling and selective action of microalgal exudates. Present results also show that the availability of other metabolites used in bacterial growth is critical in the selection of the microbial community. This availability depends mainly on the species and growth phase of the microalga culture used (Laing 1991). The species determines the enrichment media nutrient composition and the nutrient uptake rate, and the growth phase determines how much from the initial nutrient concentration is transferred to the shrimp larvae culture water when microalgae are added. Nutrients leached from artificial diets are also important sources of extra metabolites supporting bacterial growth.

The water from larval culture fed live microalgae is generally low in ammonia and high in nitrite, whilst water from larval culture fed MD is high in ammonia and low in nitrite. For most treatments, concentrations of both substances did not reach published lethal levels, although they may have had an effect on survival and growth at sub-lethal levels. Differences in final total ammonia and nitrite accumulation between trials are certainly related to different seawater microflora present at each trial.

The beneficial effect from adding a SDLA when using microparticulate diets still results in great economy in hatcheries, and the use of live, concentrated algae pastes could result in elimination of algal culture system.

CONCLUSIONS

The environment and available diet present when the last Nauplius stage moults from non-feeding virgin digestive tract to $Zoea_1$ feeding stage is crucial, and determines larval viability.

It is still unclear if the improvement in shrimp larviculture when larvae are fed microalgae, as opposed to the use of microdiets, is exclusively related to the nutritional value of microalgae cells and their metabolism, or if other factors such as microalgal exudates and the enrichment media used in microalgal culture also play a role.

Present results indicate that the presence of live microalgae cells improves survival, development and growth as opposed to the addition of either water where the microalgae cells are filtered out or water containing microalgal culture enrichment nutrients.

The larval response to the addition of microalgae as an initiation feed when using microdiets changes with the microflora of the water.

Beneficial and detrimental effects can be observed as early as during the first two larval development stages, confirming the crucial importance of the presence of microalgae as feed for the "first-feeding" larval stages.

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CHAPTER 3
Effects of induced changes in the microbiological composition of culture water on the survival, growth and development of first feeding *Litopenaeus vannamei* larval stages fed artificial diets

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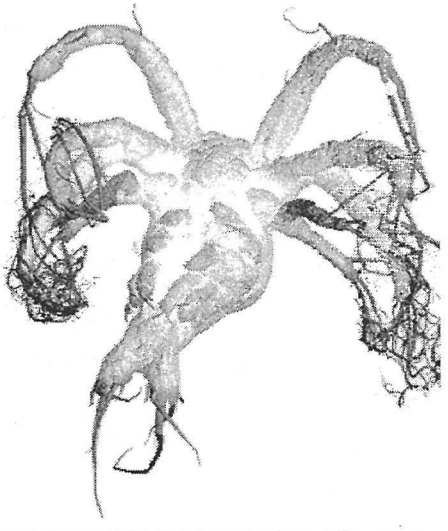

INTRODUCTION

Larviculture is still an important bottle neck in the shrimp culture industry because of the degree of unreliability of culture cycles with frequent, unexpected collapses due to various reasons, but most commonly due to disease and infestation (Wickins & Lee 2002). Massive mortalities of shrimp larvae associated with bacterial infections have been reported in hatcheries from Australia (Pizzuto & Hirst, 1995), India (Karunasagar *et al.*, 1994), Philippines, (Baticados *et al.*, 1990), Ecuador and Mexico (Vandenberghe *et al.*, 1999). This is most likely due to the proliferation of opportunistic bacteria, since there are few reported incidences of specific pathogens (Hameed, 1993; Lightner, 1993; Ruangpan & Kitao 1991; Vandenberghe *et al.* 1998, 1999). It seems that the earlier larval stages are more susceptible to bacterial infections, especially the $Zoea_2$ (Vandenberghe *et al.* 1999). As evident symptoms and death are observed in the $Zoea_2$, it is probable that the infectious process starts since $Zoea_1$ when the larvae initiate exogenous feeding activity. The ingestion of bacteria by first-feeding, recently-moulted $Zoea_1$ penaeid larval stages has been demonstrated (Soto-Rodriguez *et al.* 2002; Simões *et al.* 2002b; Simões *et al.* 2002a).

Larviculture success has also been associated to seasonal variations in water quality, due mainly to phytoplankton blooms (Alonso-Rodríguez & Páez-Osuna 2003; Wickins & Lee 2002, Misciattelli *et al.* 1998). Season fluctuations in bacterial numbers which occur on a regular annual basis following collapse of phytoplankton blooms (Blight et al. 1995) are typical of coastal temperate regions, but should also be similar to fluctuations occurring in tropical regions as a result of monsoons, cyclones or storms (Richardson 1997). According to the r-K bacteria classification gradient (Andrews & Harris 1986), the large and rapid changes in available nutrients during these events (Richardson 1997) should increase the potential for a spontaneous outbreak of opportunistic bacteria. Prayitno and Latchford (1995) report higher incidence of virulent luminescent *Vibrio* strains in shrimp larviculture tanks during, or shortly after, monsoon season.

Replacement of live by artificial foods is of major importance in commercial larval culture and the development and use of microdiets as complementary feeds, has solved problems related to the variability in the nutritional quality of the microalgae used to feed the initial larval stages (Jones *et al.* 1997; Jones 1998). However, complementary artificial feeds normally have a high degree of nutrient leaching, which sometimes leads to the accumulation of potentially toxic metabolites. The intensification of rearing strategies frequently results in the accumulation of sub-lethal concentrations of ammonia and/or nitrite (Zhao *et al.* 1998; Cheng & Chen 1994), which may adversely affect growth and survival, and may reduce the resistance of the larvae to infection from pathogens (Hameed 1995).

To partially solve the problems related to disease, fluctuations in the bacterial quality of natural seawater and accumulation of potentially toxic substances, treatment of culture water is current practice in shrimp hatcheries. Mechanical and chemical treatments include different methods ranging from sedimentation or filtration, to the use of chelating agents (elimination of heavy metals) and disinfection (ozone, chlorine, UV and antibiotics), depending on the desired level of bacterial water quality. In addition, successful modification of the microbial flora of the initial culture water through the use of biological agents, such as probiotic strains, has been shown to benefit crustacean (Nogami & Maeda 1992; Garriques & Arevalo 1995) and bivalve larval cultures (Douillet & Langdon 1993; Douillet & Langdon 1994; Riquelme *et al.* 1996; Riquelme *et al.* 2001).

Similarly, improved larviculture results using microalgal exudates or microbiologically matured biofilters to condition culture water, have been reported for penaeid (Misciattelli *et al.* 1998; Jones *et al.* 2000) and halibut larvae (Skjermo *et al.* 1997), respectively. Such mechanical, chemical and biological manipulation of initial culture water quality certainly modifies the microflora present in the water, through cell size selection, differential resistance to toxic substances and variation in efficiency of utilising available nutrients (Olafsen 2001).

Thus, distinct culture water manipulation will lead to variations in the microflora, which in turn may be responsible for differences in larval survival and growth. The first hypothesis to be tested in the present experiments was whether changes in the microbiological environment during the culture period of the initial larval stages, would result in different survival and growth of the larvae. This was achieved through the mechanical, chemical and biological manipulation of the initial culture water resulting in five different initial microfloras.

Differences in larval survival and growth due to variations in the microflora are believed to be more important during the culture period of the initial larval stages, when larvae first open their mouths and expose their primitive digestive tracts to the potential colonization by the surrounding microflora (Simões et al. 2001). The second hypothesis tested in the present experiments is that any deleterious effect of different initial microflora on larval viability will be present from the initial larval stages and therefore it should be observed on the survival and growth since the $Zoea_1$ and $Zoea_2$ stages.

Table 3.1 – Details of initial water quality manipulation for Trials 1 and 2 through changes in the physical, chemical and biological properties of sea water prior to *Litopenaeus vannamei* larviculture. Each treatment used 5 replicates. SW – sea water. "+" indicates event. Probiotic treated SW followed product's (Efinol™) instructions for dose.

Water Maturing Conditions											**Treatment**								
Volume ($L \cdot 10^2$)	**Activated charcoal**	**EDTA ($mg\ L^{-1}$)**	**U.V.**	**Skimming**	**Ozonation**	**Biofilter**	**Filtration (µm)**	**Stocked Biomass (g)**	**Days before treatment**		**EDTA ($mg\ L^{-1}$)**	**Filtration (µm)**	**Activated charcoal**	**Autoclave**	**Commercial probiotic ($mg\ L^{-1}$)**	**Days resting with aeration**	**Treatment Designation**	**Trial 2 (1000 ml flasks)**	**Trial 3 (1000 ml flasks)**
											10	1	+			2	Raw SW (1 µm)		+
											10	5	+			2	Raw SW (5 µm)	+	
											10	0.2	+	+		2	Autoclaved SW	+	+
4.5	+	10	+			+	1	0	20			1	+		5	2	Probiotic SW	+	+
4.5	+	10	+			+	1	0	20			1	+			2	Matured SW 1	+	+
0.13	+	10	+	+	+	+	50	25	40			5	+			2	Matured SW 2	+	+

MATERIALS AND METHODS

Microflora manipulation treatments

Initial water quality treatments attempted to simulate naturally occurring bacteria of raw seawater, bacteria from matured seawater systems and bacteria from a commercial probiotic mix. A control treatment with no bacteria was also used. Nauplii were not surface disinfected and were left to open their mouth and start exogenous feeding in each of the treatments water. The experiment was run in 2 consecutive years during the same seasonal period to see if any observed pattern was consistent despite specific seawater microflora differences in each year. The second experimental period was reduced in order to assess if any observed pattern of survival and growth appeared early in larval development, at the first feeding larval stages. The five different initial water microflora treatments (more details in Table 3.1) were achieved through distinct mechanical, chemical and biological manipulation of the culture water resulting in:

I) 5 µm filtered raw seawater (natural occurring bacteria)

II) 0.2 µm filtered seawater with a probiotic strain added (probiotic bacteria)

III) 0.2 µm filtered autoclaved seawater (no bacteria)

IV) 5 µm filtered matured seawater with low nutrient concentration (recirculation system selected bacteria 1)

V) 5 µm filtered matured seawater with high nutrient concentration (recirculation system selected bacteria 2 – recirculation system stocked with *L. vannamei* juveniles totalling 25 g of biomass).

Experimental animals and general experimental conditions

All *Litopenaeus vannamei* shrimp nauplii (N_4 - single female spawn) used in trials 1 and 2 were obtained from Industrias Pecis hatchery, Yucatan, Mexico and originated from F4 domesticated broodstock (Panama origin). Nauplii were acclimated to each trial specific temperature, salinity and pH laboratory water conditions (33-38 ‰, 28-30°C, 7.7-7.9 pH) over a period of 2-3 h. Nauplii were then divided in treatment groups of similar stocking density (volumetric estimates) and each group transferred to one of five 15 L plastic containers, each filled with one of the different water treatments (Table 3.1). Nauplii were left 6-20 h prior to stocking of each trial to allow moult into $Zoea_1$ stage with constant aeration. Both trials used 100 $Zoea_1$ L^{-1} stocking density in 1000 ml round bottom sterilized glass flasks, gently aerated through a sterilised glass pipette, using 0.2 µm filtered air, subjected to a 13 -11 h light-dark regime and no-water exchange throughout the entire experimental period. All other equipment was washed with chlorine and thoroughly rinsed with tap water prior to use. Flasks were maintained in a 28°C thermostatically controlled water bath and left at least 12 h acclimating to bath temperature before the addition of larvae. Natural seawater was left maturing for different periods according to the conditions described in Table 3.1 or used immediately. Both treatments used 5 flask replicates (Table 3.1). The first and second trials were run in April and May of consecutive years, respectively.

Feeding

A single dose of live algae (SDLA) of 5000 cells mL^{-1} of *Chaetoceros gracilis* plus 1000 cells mL^{-1} of *Tetraselmis chuii* was given to all treatments before $Zoea_1$ stocking. An experimental microdiet (CAR ULTRA FI No1, INVE Technologies with 85 % particle size between 5 - 30 μm range) was given at 1 mg L^{-1} ration, 6 and 4 times per day, respectively. Microdiets were prepared fresh before each feed by suspension in 0.2 μm filtered seawater.

Percentage survival, development and growth

Trials 1 and 2 were terminated 12 h after first $Mysis_1$ or $Zoea_2$, respectively, were observed. Survivors from each replicate flask were killed by addition of 5 ml of 37% formalin. They were counted, their development stages identified individually in a zooplankton counting chamber and total length measured to the nearest μm (tip of rostrum to end of telson for $Mysis_1$ and to bifurcation of posterior end for $Zoea_2$; trials 1 and 2, respectively) using a stereoscopic microscope. In trial 1 all surviving larvae were measured, whilst for trial 2 a random sub-sample of 20 animals per replicate flask was used. Average percentage survival was calculated for each treatment. Assuming that the mortality of a population is represented by an exponential decay equation, the relative mean mortality rate (also known as "intrinsic", "specific" or "instantaneous" mortality rate - number of individuals that die per unit of time and per individual alive) was calculated using equation 2.1 (Chapter 2). The ranking "development index" (DI) of Villegas and Kanazawa (1979) was used to estimate average inter-treatment stage of development (weights of "1", "2", "3", "4" were used for $Zoea_1$, $Zoea_2$, $Zoea_3$, $Mysis_1$ stages, respectively).

Water quality analysis

It is known that there is a high specificity on the strains responsible for the nitrifying process (Wheaton *et al.* 1994). Thus, the present work used differences in the pattern of total ammonia and nitrite accumulation over the experimental periods as indirect proof of microflora changes, instead of describing the microflora composition of initial and final treatments. This is because the latter technique is laborious and time-consuming, and results are uncertain due to unsettled controversies both over bacterial strain taxonomy and bacterial identification methods (biochemical versus genetic and molecular).

Total ammonia and nitrite concentrations were measured at the beginning and end of the experimental period adapting the methods described in Parsons *et al.* (1984) to the microplates with 96 350 μL wells. Sample volume used was 250 μL. For total ammonia 10 μL of phenol solution, 10 μL of nitroprusside solution and 30 μL of the oxidising solution were added. For nitrite determinations 10 μL of sulfanilamide solution and 10 μL nhaptyl solution were added. These solutions were added according to the sequence and intermediate resting periods reported by Parsons *et al.* (1984). Samples were kept at low temperature (5°C) until analysed within 2-3 hours after being collected. Non ionized ammonia fraction was calculated following the seawater ammonia dissociation model described by Khoo *et al.* (1977) using for each treatment values of pH, temperature and salinity on the day and time of total ammonia determination. Nitrates and phosphates were analysed using the standardized Hach™ kit techniques. Dissolved proteins were measured using a Pierce™BCA total protein assay kit based on the bicinchoninic acid reaction.

Statistical analysis

All data sets were tested for departure from normality (Anderson-Darling statistic) and inter-treatment homogeneity of variance (Bartllet and Levene's statistics, depending on normal/non-normal distribution of data points) before running one-way analysis of variance with post-hoc all-pair-wise comparisons using Tukey's statistic. Proportions of survival were arcsin transformed. Non-normal relative mortality rate, final total ammonia and final nitrite concentrations data sets, were "log" transformed. When analysing length data, where there were 10 larvae measured per tank, tanks were considered a random factor. A *student*-t test was used when only two datasets were present. Non parametric data were analysed using Kruskal-Wallis analysis of medians, adjusting for ties followed by a Tukey-type multiple comparisons using the Nemenyi test and Dunn's standard error (SE) procedure described in Zar (1999). A Mann-Whitney test was used when only two datasets were present. For nitrate and phosphate, despite considerable differences between treatments, the small replicate number (2 measurements per treatment) affected the statistical analysis power and no significant differences could be observed, even when using conservative non-parametric tests.

RESULTS

Percentage survival, mortality rate, development and final length

The percentage survival of larvae cultured in water from a recirculation system with a heavy organic load (Matured SW2 - treatment) in trial 1 was nil (Fig. 3.1 A_1). Similarly, trial 1 probiotic bacteria bloom treatment produced very poor percentage survival results with many replicates having zero survival (Fig. 3.1 A_1). Therefore no relative rate of mortality could be calculated for these two treatments, and neither the development nor the final average length could be assessed representatively (Fig. 3.1 B_1 C_1 D_1). The two equivalent trial 2 treatments also gave poor results, although percentage survival was not zero (relative mortality rate could be calculated) and there were enough survivors to estimate development and final average length (Fig. 3.1 A_2 B_2 C_2 D_2).

In trial 1, larvae from the "Raw" seawater treatment died at a significantly faster rate than larvae reared in "autoclaved" sea water, which explains the poorer percentage survival. Similarly, the larvae reared in "Raw" seawater only reached $Zoea_3$ stage, whilst during the same period, larvae reared in "autoclaved" sea water reached a significantly higher development stage with nearly 80% of the population already at $Mysis_1$ stage (Fig. 3.1 C_1). Such differences in larval stage distribution between treatments are further supported by final average length results, which show that larvae reared in autoclaved water grew significantly larger than larvae reared in water from a recirculation system without an organic load, which in turn grew significantly larger than "Raw" seawater treated larvae (Fig. 3.1 D_1).

In trial 2, larvae reared in water from a recirculation system with a heavy organic load (Matured SW2) died at a significantly faster rate than larvae from the "Raw", "Matured SW1" and "autoclaved" seawater treatments which resulted in a significantly lower percentage survival (Fig. 3.1 A_2 B_2). Apart from poor survival, these larvae did not manage to moult into the $Zoea_2$ stage, as almost 100% of the population reached only the $Zoea_1$ stage (Fig. 3.1 C_2), attaining a significantly smaller final average length than larvae from any other treatment (Fig. 3.1 D_2). Larvae from the probiotic bacteria bloom water treatments gave better survival when compared to "Matured SW2" larvae, and they grew significantly larger and more than half of the population managed to moult into $Zoea_2$ (Fig. 3.1 A_2 C_2 D_2). In the shorter trial 2, the induced changes in the microbiological composition of culture water, except for "Matured SW2" and "probiotic SW" treatments did not result in significant differences in percentage survival, relative mortality rate and development (Fig. 3.1 A_2 B_2 C_2). However, larvae reared in "Raw SW" reached a significantly larger final length compared to larvae from Matured SW1, Autoclaved or probiotic treated SW conditions (Fig. 3.1 D_2).

Overall and combining the information from all trials, it can be said that water from a recirculation system with a heavy organic load (Matured SW2) and water in which a probiotic bacterial bloom is induced (Probiotic SW) gave poor survival results. The use of sea water with little treatment (Raw SW), water from a recirculation system with no organic load (Matured SW1), and autoclaved water (Autoclaved SW) gave similar survival results. Differences between these treatments are only shown in terms of final average individual larval length and development stage.

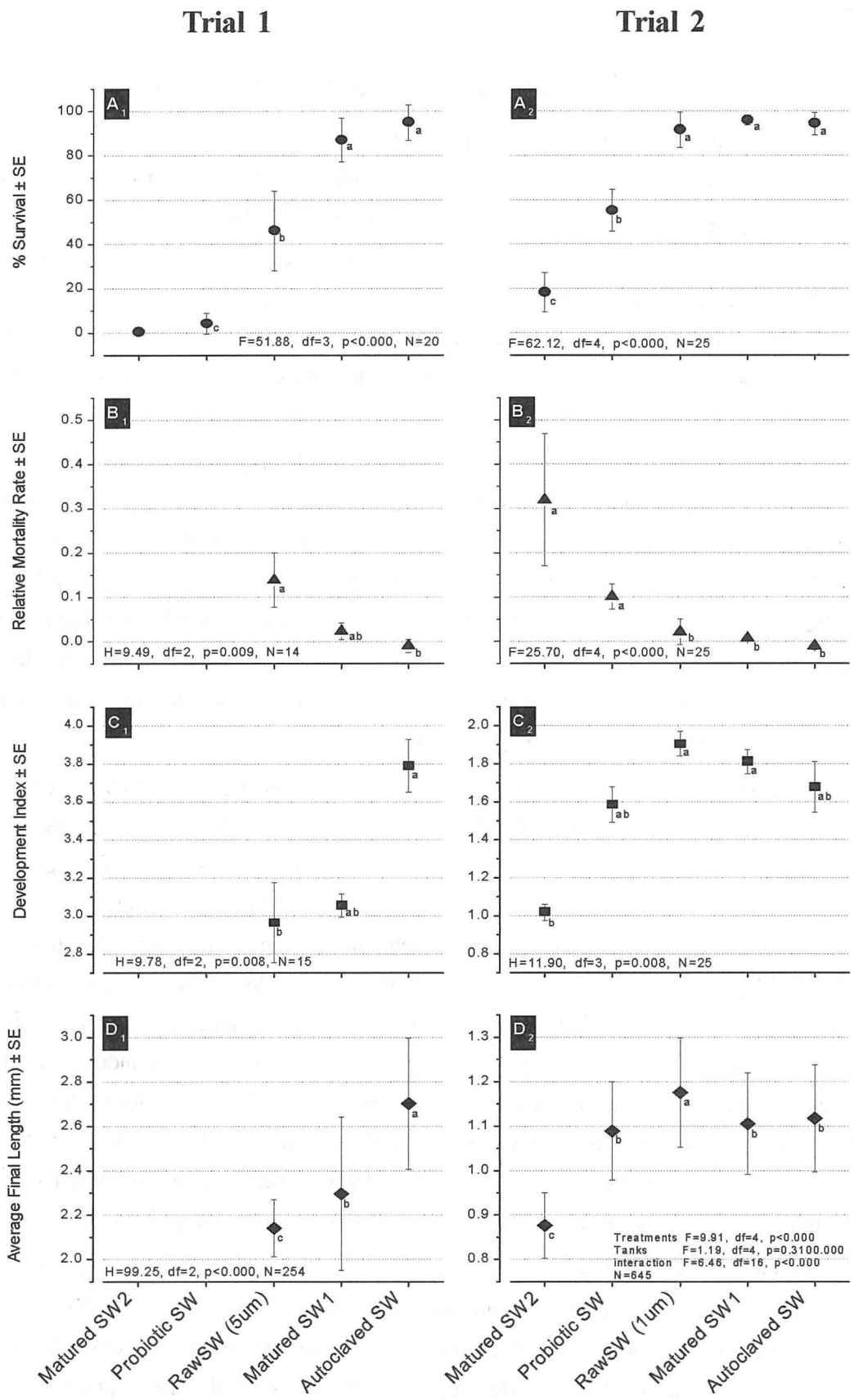

Figure 3.1 – Mean percentage survival, relative mortality rate (number of dead animals · day^{-1} · animals alive^{-1}), development index and final length of *Litopenaeus vannamei* larvae exposed to different initial culture water treatments (refer to Table 1 for treatment description). Different letters represent significant differences between treatments ($p < 0.05$). Development index (DI) graphs show the larval stage with the correspondent DI line. Experiments were run for 9 ($\mathbf{A}_{1,2,3,4}$), 6 ($\mathbf{B}_{1,2,3,4}$) and 4 ($\mathbf{C}_{1,2,3,4}$) days.

Table 3.2 – Mean non-ionised ammonia fraction ($N\text{-}NH_3$), total ammonia ($N\text{-}NH_3 + NH_4^+$), nitrite ($N\text{-}NO_2$), total proteins, nitrate ($N\text{-}NO_3$) and total phosphates (PO_4) concentrations (mg L^{-1} ± SE) at the beginning and end of the experimental periods for both trials (6 and 4 days, respectively) using several treatments of initial seawater (SW) quality (please refer to Table 3.1 for treatment description). Δ - rate of concentration change over the experimental period. Different letters represent significant differences between treatments ($p < 0.001$).

TRIAL 1	Proteins			Nitrite			Phosphate		
	day 0	day 8		day 0	day 6		day 0	day 6	
Matured SW2	63.33 ± 4.64^{b}	17.11 ± 5.46^{a}	–7.703	—	3.40 ± 0.10		—	9.88 ± 0.02	
Probiotic SW	282.22 ± 42.92^{a}	125.92 ± 4.53^{c}	–26.050	—	1.60 ± 0.10		—	1.21 ± 0.09	
Raw SW	84.75 ± 5.27^{b}	103.14 ± 6.59^{bc}	3.065	—	1.05 ± 0.05		—	2.99 ± 0.09	
Matured SW1	96.58 ± 4.93^{b}	83.64 ± 13.39^{b}	–2.157	—	1.88 ± 0.30		—	2.00 ± 0.08	
Autoclaved SW	80.19 ± 1.61^{b}	74.81 ± 9.23^{b}	–0.897	—	1.10 ± 0.10		—	2.91 ± 0.10	
TRIAL 1	**Non–ionised Ammonia fraction**			**Total Ammonia**			**Nitrite**		
	day 0	day 6	Δ	day 0	day 6	Δ	day 0	day 6	Δ
Matured SW2	0.002 ± 0.00^{a}	0.01 ± 0.00^{a}	0.001	0.18 ± 0.01	0.70 ± 0.07^{b}	0.087	4.37 ± 0.15^{a}	3.48 ± 0.14^{a}	–0.148
Probiotic SW	0.003 ± 0.00^{b}	0.00 ± 0.00^{a}	–0.001	0.13 ± 0.01	0.00 ± 0.18^{a}	–0.022	0.01 ± 0.01^{b}	0.01 ± 0.00^{b}	0.000
Raw SW	0.004 ± 0.00^{b}	0.02 ± 0.00^{b}	0.003	0.17 ± 0.01	0.98 ± 0.03^{b}	0.135	0.12 ± 0.00^{b}	0.13 ± 0.01^{b}	0.002
Matured SW1	0.004 ± 0.00^{b}	0.02 ± 0.00^{b}	0.003	0.16 ± 0.02	0.96 ± 0.04^{b}	0.133	0.14 ± 0.06^{b}	0.15 ± 0.05^{b}	0.002
Autoclaved SW	0.005 ± 0.00^{b}	0.03 ± 0.00^{b}	0.004	0.18 ± 0.01	1.14 ± 0.04^{b}	0.160	0.10 ± 0.01^{b}	0.10 ± 0.01^{b}	0.000
TRIAL 2	**Non–ionised Ammonia fraction**			**Total Ammonia**			**Nitrite**		
	day 0	day 6	Δ	day 0	day 4	Δ	day 0	day 4	Δ
Matured SW2	0.08	0.23 ± 0.01^{c}	0.038	1.75	4.85 ± 0.11^{c}	0.775	0.199	0.176 ± 0.002^{a}	–0.006
Probiotic SW	0.03	0.36 ± 0.01^{a}	0.083	0.76	7.55 ± 0.16^{a}	1.698	0.014	0.001 ± 0.003^{d}	–0.003
Raw SW	0.04	0.24 ± 0.02^{c}	0.050	0.71	5.03 ± 0.31^{c}	1.080	0.002	0.072 ± 0.007^{b}	0.018
Matured SW1	0.04	0.18 ± 0.00^{b}	0.035	0.62	3.70 ± 0.10^{b}	0.770	0.001	0.016 ± 0.10^{c}	0.004
Autoclaved SW	0.04	0.24 ± 0.00^{c}	0.050	0.77	4.96 ± 0.08^{c}	1.048	0.001	0.001 ± 0.001^{d}	0.000

Water quality

Both the initial and final total ammonia concentration and the non-ionised ammonia fraction of trial 1 treatments were lower than trial 2 treatments, with a net accumulation (Δ) generally over one order of magnitude despite trial 2 shorter duration (Table 3.2). Overall, the initial concentrations of nitrite were higher in treatments from trial 1, than in treatments from trial 2. In trial 1, the "Matured SW2" treatment gave an extremely high nitrite concentration throughout the experimental period (Table 3.2). It also presented significantly lower protein concentrations at the end of the experiment when compared to other treatments, and high final nitrate and phosphate levels. For trial 2, although the "Matured SW2" treatment nitrite concentration was below values in trial 1, it was again significantly higher than all other treatments (Table 3.2). The total and the non-ionised ammonia fraction gave concentrations higher than those in trial 1. Unfortunately, for trial 2 no data was collected for proteins, nitrate and phosphates. The probiotic bacteria bloom treatment in trial 1 showed significantly higher initial and final protein concentrations when compared to the other treatments (Table 3.2). There were substantial differences in the net accumulation of total and non-ionised ammonia fraction plus nitrite from trial 1 to trial 2 in the treatments that were dependent on water from the sea from two consecutive years (Probiotic SW, Raw SW and Autoclaved SW), with treatments from trial 1 having overall lower concentrations than the same treatments in trial 2. The net accumulation of total ammonia, non-ionised ammonia fraction and nitrite from the treatments that achieved better survival and growth in both trials, were very similar between each other (Table 3.2, Fig. 3.1).

DISCUSSION

The microflora composition of seawater is known to change over time (Blight et al. 1995, Misciattelli *et al.* 1998). Changes in the chemical and microbiological characteristics of the water are reported to have an impact on tropical aquaculture ventures (Maclean 1993). Differences in the "RawSW" treatment results between trials may be related to the specific microflora composition present at the sea at that time. In tropical coastal waters, red tides are common during certain periods (Altamirano 1987; Millán-Núñez & Loya-Salinas 1993; Hallegraeff 1993; Richardson 1997; Alonso-Rodríguez & Páez-Osuna 2003) and their *occurrence greatly unbalances normal microflora (Sieburth 1960; Guillard & Hellebust 1971; Fukami et al.* 1991). Misciattelli *et al.* (1998) related oscillations in *Fenneropenaeus indicus* larval culture output to time of the year due to seasonality of the microalgae *Phaeocystis sp.* bloom and associated bacteria in temperate, coastal waters. In some cases, dinoflagellate blooms are harmless to shrimp, such as the Peridinum balechii red tide reported by Delgado *et al.* (1996). In other cases, algal blooms can affect shrimp development (Mingyuan and Jiansheng, 1993; Cortés-Altamirano and Licea-Durán, 1999). Massive shrimp larvae mortality in postlarvae hatcheries from West Mexico has been related to the occurrence of coastal red tides produced by *Ceratium dens*, *Pseudonitzschia spp.* and *Gymnodinium catenatum* (Cortés-Altamirano and Alonso-Rodríguez, 1997). A toxin as the causative agent was ruled out by testing extracts from water and shrimp nauplii suggesting other factors were directly responsible for the massive animal losses. Production cycles in shrimp hatcheries from the North-western coast of Mexico coincide with seasonal appearance of phytoplankton blooms. During these months, most shrimp hatcheries suspend production due to poor production results, mainly associated to bacterial infections.

Good results in the present study with the "RawSW" treatment, especially in trial 2, suggests that the characteristics of the organic matter and selected bacterial population present at sea at that particular time of year had a synergistic effect upon larval health. This same water, when 0.22µm filtered to eliminate most suspended solids and sterilized by autoclaving, also yielded good survival, development and growth. This indicates an adequate chemical composition and bacterial re-colonization of the water, achieved either by bacteria attached to the shrimp nauplii, bacteria present in the artificial food, or perhaps most importantly, bacteria present in the microalgal cultures that were used to stimulate the initial larval feeding process (SDLA). Pre-sterilisation of larval culture water is not always effective as opportunistic pathogens rapidly recolonise cultures (Baticados & Pitogo 1990; Alabi *et al.* 1997). These two treatments, "RawSW" and "AutoclavedSW", plus the "Matured SW1" treatment, presented the best overall percentage survival in both trials with few differences between them, confirming that more than one initial water quality treatment may provide equally good survival. However, significant differences in larval development stage and growth in length indicate that the resulting conditions may influence larval health and nutritional state to the point of hindering their growth and development.

Water treated with commercial probiotics did not yield results as expected, probably due to high initial protein concentration and related osmoregulation difficulties and/or clogging of larvae gill lamellar surfaces by excess bacteria (Brock & Lightner 1990), reducing gas exchange (Fisher 1988). Since most commercial probiotic mixes include enrichment nutrients such as some form of available protein for the bacteria, as well as the dormant bacteria, numbers reach high densities very quickly and a bloom of bacteria occurs. In the

case of the "ProbioticSW" treatment in trial 2, high initial nitrite concentrations plus rapid accumulation of total ammonia, although below reported toxic levels (Lin & Chen 2001), may have contributed to the observed results acting synergistically with the potential high numbers of bacteria at a sub-lethal level. Bacteria that fill the necessary requisites of a successful probiotic strain are necessarily fast growing (Gomez-Gil *et al.* 2000; Verschuere *et al.* 2000) and should out-compete nitrifying bacteria for surface area attachment, as the latter are slow growing (Wheaton *et al.* 1994), and may thus reduce the ammonia and nitrite processing capacity of the natural seawater nitrifying community. Probiotic strains also produce extra ammonia from the heterotrophic consumption of the probiotic mix of enrichment nutrients, uneaten feed, larval faeces and moults. Perhaps a better method of delivering these potentially benign organisms to the culture water is through simultaneous culture with microalgae as reported by Gomez-Gil *et al.* (2002).

The high mortality plus reduced growth and retarded development of trial 1 larvae, cultured on water from a recirculation system with a heavy organic load (Matured SW2), can be explained by the high nitrite concentrations (Table 3.2) present in the water as they were above the tolerance limits reported for other species (Chen & Tu 1990; Cheng & Chen 1994). These results indicate that the biofilter unit used in the culture system with a heavy organic load had not yet reached nitrification equilibrium, or presented oscillations in its efficiency due to changes in stocking density and feeding regimes that favoured temporal high nitrite concentration. In trial 2, although both non-ionised and total ammonia and nitrite concentrations in the initial water were below reported safe levels (Lin & Chen 2001), poor results were observed, suggesting that other factors present in the water were responsible for low survival, development and growth. In trial 1, for example, the "Matured SW2" treatment showed high initial concentrations of nitrate and phosphate. These observations further confirm that water from a commercial running recirculation system with live stock needs more treatment than just 5µm activated charcoal filtration if it is to be adequate for shrimp larval rearing due to potentially toxic concentrations of water nutrients. However, the use of matured water from recirculation systems should not be discarded as it has the advantage of possessing a well developed microflora and can be used with good and repeatable results, such as the ones obtained from the "Matured SW1" treatment conditions in the present study. Care must be taken, though, to ensure fine pre-treatment before contact with larvae to reduce concentrations of ammonia, nitrite, nitrate, phosphate and suspended solids, as these may be toxic to larvae and may stimulate growth of opportunistic bacteria that normally have restricted opportunities for growth and which could become pathogenic (Olafsen 2001; Misciattelli *et al.* 1998). Most commercial biofilter units used in recirculation systems only process large concentrations of both ammonia and nitrite adequately if there are no sudden changes in stocking density and feed quantities and frequency. Hence, the use of water from recirculation systems without any organic load other than the normal dissolved organic material present in natural seawater is recommended in order to produce bacteriological "matured" seawater. Positive results for survival and growth were reported for penaeid (Jones *et al.* 2000) and halibut larvae (Skjermo *et al.* 1997), using microbiologically matured sea water obtained in such a way.

Since all larvae were fed the same diet and submitted to the same zoo-technical rearing conditions, except for the initial water quality manipulation, the wide range of significant end results in terms of survival, development and growth can be considered sufficient evidence to indicate that different microbial populations

developed in each of the five different treatments. This is further confirmed by the significant inter-treatment differences in the net accumulation of several nutrients between the beginning and end of both experimental periods, many of which are only possible due to changes in the presence and number of specific bacterial strains. Although there are important differences between both trials, the overall response pattern is the same, and differences in net accumulation of total ammonia and nitrite may be explained by the different duration of each trial. For example, survival of "Matured SW2" and "Probiotic SW" treatments in trial 2 would be as accentuated as in trial's 1 equivalent treatments if the experiment had run for as long as trial 1. The similarity between response patterns in both trials, irrespective of trial duration, also suggests that differential microflora development has an effect on larval survival, development and growth as early as the first two shrimp larval development stages, and that such effect is stronger during the initial feeding period.

When zoo-technical and water quality data from both trials are combined, it is evident that the initial water treatments did change the microflora present in the water. As the inadvertent ingestion of bacteria by first feeding, recently-moulted penaeid larval stages feeding on microalgae or microdiets has been recently demonstrated (Soto-Rodriguez *et al.* 2002; Simões *et al.* 2002a,b), it is concluded that larvae from different treatments in this study ingested distinct bacterial strains and numbers. Such differential bacterial ingestion may be the key to understanding the changes in larval survival and growth response when fed artificial diets, as bacteria may assist or hinder the digestion and assimilation of such diets in the primitive larval digestive tract. Bacteria may also assist or hinder the establishment of a protective gut lining microflora against infection by pathogenic strains. The potential for bacteria established on the larval gut to secrete extra exogenous enzymes, apart from the larva enzymes and those released by food items, together with the competitive exclusion of pathogenic bacteria, has been the main argument for the search and development of probiotic bacteria (Verschuere *et al.* 2000; Gomez-Gil *et al.* 2000).

There is little difference between aerobic cultured and wild shrimp gut microflora (Oxley *et al.* 2002). The anaerobic bacterial strains isolated from the digestive tract of cultured adult penaeid species such as *Litopenaeus vannamei* were found to produce esterases and carbohydrases, possibly useful in digestion (Dixon *et al.* 2001). Studies on fish larvae have demonstrated the active role of exogenous enzymes in the breakdown of food inside the larval gut and although some controversy still exists about the fraction of complementary enzyme activity from exogenous sources, it is well established that they have a role in initial *larval digestion and assimilation (Dabrowski & Glogowski 1977; Lauff & Hofer 1984; Kurokawa et al.* 1998). It is therefore important to understand more precisely the fate of ingested bacteria and their role in larval digestion and assimilation in order to improve the use of artificial diets.

Low larval viability is seldom found to be correlated to the presence of an obligate pathogen, but rather to the proliferation of a few opportunistic bacteria which may become harmful when present in sufficient numbers (Munro *et al.* 1995). Intensive rearing technology does not only have an impact on bacterial cell numbers, but does also affect species composition by selective enhancement of opportunistic members of the bacterial community (Salvesen 1999). After treatment the water will be more or less free of bacteria, and there is a low level of competition between species and high substrate availability per bacterium. Opportunistic bacteria with relatively high growth rates are favoured under such conditions (Andrews & Harris 1986). Many opportunistic members of the *Vibrio* and *Pseudomonas* genera have potential maximum growth

rates that are very fast and that enable them to respond and become dominant during transient periods of low competition and high nutrient availability (Salvesen 1999). High levels of opportunistic bacteria combined with a low bacterial diversity in the early developmental stages could hamper the establishment of a protective microflora.

CONCLUSIONS

Induced alterations in the microbiological composition of initial culture water quality following mechanical, chemical and biological treatment resulted in different survival and growth of the larvae.

The microflora present in initial culture water has an effect upon larvae survival and growth since the $Zoea_1$ and $Zoea_2$ stage. Therefore, the microflora present when last Nauplius stage moults from non-feeding virgin digestive tract to $Zoea_1$ feeding stage is an important factor determining future larval viability.

Enhancement of both survival and growth for larvae fed on microdiets when reared in matured seawater with low nutrient availability was validated.

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CHAPTER 4
The morphology of feeding appendages and digestive tract and observations on ingestion and retention of bacteria in the gut of first feeding larval stages of the penaeid shrimp *Litopenaeus vannamei*

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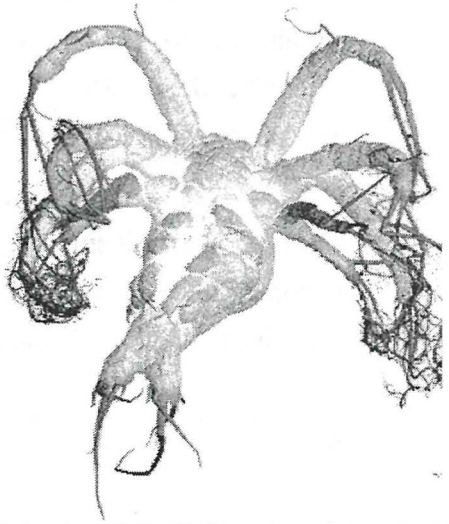

INTRODUCTION

The initiation of exogenous feeding in penaeid larvae ($Zoea_1$) has been identified as a critical phase in terms of survival, growth and development as the larvae are exposed internally to environmental microbial communities for the first time at the moult from $Nauplius_5$ to $Zoea_1$ (Alabi *et al.* 1997; Jones 1998). Exposure to the microflora present in the water at this time may have beneficial or detrimental effects on the digestive and assimilation capacity of the larvae, and thus on their overall nutrition, as well as on their ability to withstand contact with any pathogenic organisms present in the water. It has been suggested that the lethal condition called "$Zoea_2$ Syndrome", which induces hepatopancreatic cells to separate and atrophy, and causes disintegration of the gut epithelium, is related to a contact with pathogenic *Vibrio* bacteria cells earlier in the $Zoea_1$ stage (Juarez 1997; Pantoja *et al.* 1997). Anaerobic bacteria isolated from the digestive tract of adult penaeid species such as *Litopenaeus vannamei* have been found to produce enzymes, such as esterases and carbohydrases, that may supplement enzymes produced by the shrimp (Dixon *et al.* 2001). *Vibrio harveyi*, a commonly found bacterial strain present in the digestive tract of both larval and adult shrimp has also been reported to produce digestive enzymes (Soto-Rodriguez 2002). Many studies, mainly with fish larvae, have demonstrated the active role of exogenous enzymes in assisting in the breakdown of food inside the larval gut, although there is some debate regarding the ratio of enzyme activity which arises from exogenous sources (Dabrowski & Glogowski 1977; Lauff & Hofer 1984; Kurokawa *et al.* 1998).

Because bacteria established in the larval gut may secrete extra exogenous enzymes and also may result in the exclusion of pathogenic bacteria, there is a strong incentive to identify probiotic bacteria that could be administered to larvae (Verschuere *et al.* 2000; Gomez-Gil *et al.* 2000). Modification of the microbial flora of the culture water through the use of probiotics has been shown to benefit crustacean (Nogami & Maeda 1992; Garriques & Arevalo 1995) and bivalve larval cultures (Douillet & Langdon 1993; Douillet & Langdon 1994; Riquelme *et al.* 1996; Riquelme *et al.* 2001). However, the route for crustacean larval gut colonization and the time of first contact with external bacterial flora are still poorly understood. Furthermore, it is not known if shrimp larvae are capable of actively filtering bacteria from the water or whether the ingestion of bacteria is a passive mechanism simultaneous to normal feeding activities.

Lovett and Felder (1990c) observed and described antiperistaltic waves starting in the anus and travelling anteriad up to the first segment midgut trunk in the space between the peritrophic membrane and the gut epithelium of *Litopenaeus setiferus* $Zoea_3$. Bacteria that enter through the anus are less likely to be digested by enzymes present in the anterior and lateral midgut caeca of a shrimp $Zoea_1$ than those that are ingested through the mouth. It is therefore important to know when in the larval development, the mouth and anus opening occurs, as well as to investigate the existence of antiperistaltic waves in younger larval stages. To determine whether first colonisation of the gut occurs through the larval mouth or anus, and whether bacteria are capable or not of attaching to the gut epithelium is essential for the correct use and application of probiotic products

The purpose of this study was to examine ingestion of bacteria by the first feeding stage penaeid larvae and to compare it with microalgal filtration. This study also followed the movement of live bacteria inside the digestive tract of penaeid larvae in order to determine the rate of ingestion, gut retention and excretion of a

potential probiotic bacteria species of the genera *Vibrio* by $Zoea_1$ and $Zoea_2$ larval stages. To identify the moment when the larval gut becomes exposed to the surrounding microflora environment for the first time, anatomical observations were made using standard histological techniques and scanning electron microscopy.

MATERIALS AND METHODS

Test organisms for bacteria ingestion and anatomical observations

All specimens come from three independent batches of *Litopenaeus vannamei* $Nauplii_{2-3}$. All specimens were thoroughly rinsed in sterile seawater (natural 0.45 µm filtered, autoclaved seawater) on a 150 µm sterile mesh, disinfected with 10 µg ml^{-1} of active iodine (Argentyne at 10 %) for 15 min. They were rinsed again and then maintained in a white plastic container with 12 L of sterile sea water with 20 µg ml^{-1} of the antibiotic enrofloxacin (Cheminova de Mexico, Mexico) according to reported minimum inhibitory concentration (MIC, Roque *et al.* 2001) and "no-effect-concentration" (Williams *et al.* 1992). Aeration was provided through a sterile glass pipette using a hydrophobic 0.22 mm filter. Temperature was maintained at 28 ± 1 °C using a thermostatically controlled freshwater bath. $Nauplii_5$ moulted into first feeding (early or recently moulted) $Zoea_1$ stages after 36 to 48 h incubation in antibiotic treated seawater. Advanced $Zoea_1$ and $Zoea_2$ stages were fed a combination of different proportions of cells from *Chaetoceros muelleri* (60%), *Tetraselmis sp.* (30%) and *Isochrisis galbana* (10%) cultures, at an approximate concentration of 50,000 cells ml^{-1}, with no water exchange. Microalgae were grown axenically, however, samples were plated on TCBS agar (DIFCO, USA) to confirm that no *Vibrio spp.* were introduced. Prior to any observation or experimental contact with stained bacteria, animals were starved for 2-6 h in 0.45 µm filtered sterilised seawater with complete water exchanges every hour to avoid re-ingestion of faeces and debris.

Histology and preparation of samples for SEM

Samples for scanning electron microscopy were fixed, dehydrated and mounted following methodology described by Felgenhauer (1987). Histological samples were prepared for light microscopy using sections of paraffin-embedded tissues that had been stained following standard procedures for either haematoxylin-eosin-floxin general stain (without decalcification) and gram-Humberton bacterial stain (Lightner 1996).

Bacterial isolate

An innocuous *Vibrio harveyi* strain (CAIM 79), was isolated from a *Litopenaeus stylirostris* shrimp hatchery in the Gulf of Santa Clara, Sonora, Mexico (Soto-Rodriguez *et al.* 2002a) and was used throughout this study. This strain was Gram-negative, motile, positive for oxidase, fermented glucose, was sensitive to the vibriostatic compound 0/129, and used D-mannitol as a sole source of carbon. These features placed the isolate as member of the genus *Vibrio*. It tested negative for arginine "dihydrolase", and positive for ornithine and lysine decarboxylase. It was luminescent and positive for citrate and negative for Voges-Proskauer. It could not use L-arabinose and D-glucosamine . It could grow on 2.5, 6.0, 8.0 % NaCl, and at 40 °C. All these properties identified this strain as *V. harveyi* (Alsina & Blanch 1994). The strain was cryopreserved at -70 °C according to the recommendations of Gherna (1994) in an ultralow freezer (Revco Scientific, Asheville) at the Collection of Aquacultural Important Microorganisms (CAIM) at CIAD, Mazatlan Unit for aquaculture and environmental management.

Preparation of fluorescent labelled bacteria (FLB)

Cryopreserved bacteria were recovered in triptych soy broth (TSB, Bioxon, Mexico) at 30 °C and agitated at 100 rpm in a reciprocal shaking bath for 20-24 h. The broth was centrifuged at 8,000 rpm for 6 min at 15 °C, and the resulting bacterial pellet was re-suspended in 9.5 ml of sterile seawater by strong agitation. The methodology of Soto-Rodriguez *et al.* (2002b), was used to label bacteria with 5-4,6-dichlorotriazin-2-yl aminofluorescein (DTAF, Sigma Chemical Co. D-0531, St. Louis). DTAF was dissolved in sterile phosphate buffer saline to give a concentration of 0.5 mg ml^{-1} and served as the staining solution. A 0.5 ml aliquot of this solution was then added to 9.5 ml of the bacterial suspension and incubated for 2 h at 30°C under constant agitation at 90 rpm in total darkness. For malachite green stained bacteria, 0.5 ml aliquot of a 1 mg ml^{-1} malachite green solution was added to 9.5 ml of the bacterial suspension and incubated for 1 h at room temperature. At the end of incubation periods, cultures were centrifuged and the pellet re-suspended in sterile seawater. This procedure was repeated 3 to 6 times until an unstained suspension was observed. Final viable bacteria density in the suspension was estimated by serial dilution of samples in sterile saline solution (2.5 % NaCl) and plating in thiosulphate-citrate-bile salts- sucrose agar (TCBS, Difco, Mexico) and triptych soy agar (TSA, Bioxon, Mexico) + 2.0 % NaCl, and incubation of the suspension for 24 h. For each bioassay, fresh FLB were prepared.

Microscopy

Fluorescent labelled bacteria samples were observed with an Olympus BX60 microscope with incident UV light (BX-FLA reflected light fluorescence attachment), BP 450-490 nm excitation filter, DM500 nm dichroic filter, and BA515 nm barrier filter. Photographs were taken with an Olympus automatic camera and a PM-20 exposure control unit, using ISO 400 Kodak colour film. Live larvae and histological sections were observed using a Carl Zeiss Axiolab microscope with achromatic objectives and digital pictures taken using a Sony SSC-DC 54A digital camera. A JEOL JSM – 531OLU scanning electron microscope was used to observe the external structure of Nauplii and $Zoea_1$. Black and white photographs were taken using the microscope exposure unit and Kodak TMAX 100 plates of 4x5 inches. Observations of live specimens were made using ice-cold seawater to slow down the swimming activity of the larvae within 5-10 min after sampling. However, photographs were taken using larvae fixed in 2-4 drops of 3% glutaraldehyde sterile sea water solution. All anatomical descriptions were made using nomenclature of Lovett & Felder (1989).

Observations of the ingestion of microalgae and bacteria

A pool of $Zoea_1$ were transferred to two 1 L round bottom sterile glass flasks. A sample of animals was taken and observed at time 0. Subsequently, microalgae (50.000 cells ml^{-1} *Chaetoceros. muelleri*) were added to one flask, and bacteria marked with malachite green (1.26×10^6 CFU ml^{-1}) were added to the other flask; samples of several larvae were removed 5, 15 and 30 min, the time assumed to be sufficient for all animals to start feeding. Animals were immediately mounted on a glass slide, excess water drained, and two drops of 3% glutaraldehyde solution added to instantly kill and immobilise larvae for latter observation. Gut fullness was assessed using an ordinal scale: 0 - empty, 1/3 - any particulate matter observed that did

not fill more than half the gut. 2/3 - any particulate matter observed that filled more than half, but less than a complete gut. 1 - gut completely full from mouth to anus.

FLB ingestion observations

The distribution, location, number and arrangement of either single monodispersed bacteria cells or bacterial colony clumps was observed in the sterile gut of larvae which never fed (early $Zoea_1$) or were previously starved for one h (late $Zoea_1$ and $Zoea_2$) presenting the larvae with several densities of FLB only or in combination with microalgae. Larvae were kept in sterilized beakers filled with 0.45 mm filtered, autoclaved seawater at room temperature without aeration. Observations were made at different times from 15 min to 12 h (as listed in Table 4.1).

FLB evacuation observations

Recently moulted $Zoea_1$ larvae were transferred into sterilized beakers (50 ml) filled with sterile seawater which contained 1.52×10^7 CFU (FLB) ml^{-1}. After 2 h, a random sample of 20 larvae was removed (t=0), fixed with glutaldehyde and observed. The remaining live animals were then transferred to clean water without FLB for 30 min, and then were transferred to clean water again. After 10 min (t=40min) another sample of 20 larvae was removed, fixed and observed immediately. A third sample of larvae was removed at t=3:30 h.

Table 4.1. Experimental conditions of fluorescent labelled bacteria (FLB) assays with *Litopenaeus vannamei* larvae. Observations were immediately after larval contact with FLB.

Larval stage	FLB (CFU ml^{-1})	Microalgae concentration (cells ml^{-1})	FLB exposure time (hours)	Volume	Number of animals
Early $Zoea_1$	1.26×10^7	50.000	0, ⅔, 3½	50 ml	±80
	1.52×10^7	—	0, ⅔, 3½	50 ml	±80
	9.7×10^6	150.000	0, ¼, 1	40 ml	±60
	9.7×10^6	—	0, ½, 1½	40 ml	±60
Advanced $Zoea_1$	6.60×10^6	—	0, ⅔, 2, 4	50 ml	±80
	1.12×10^7	—	0, 12	100 ml	±140
$Zoea_2$	8.85×10^6	—	0, ⅔	100 ml	±120
	1.40×10^7	—	0, 12	100 ml	±140

RESULTS

Nauplius$_5$ morphology

In early Nauplii$_5$, the primordial gut is present, represented by a simple tube with a clearly-defined lumen (Fig. 4.1 A, B; Fig. 4.2 A, B, D$_2$) in which there is some peristaltic activity. The gut progressively differentiates into distinct structures, forming the primordial anterior and lateral midgut caecae, the midgut trunk, and the hindgut (Fig. 4.1 A, B, C; Fig. 4.2 A, B, C, D). Yolk vacuoles were observed inside the primordials of the anterior and lateral midgut caecae (Fig. 4.1 A, B).

A mouth aperture was not observed in any specimens using either light or electron microscopy, although ventral evaginations from the midgut towards the foregut and mouth regions, difficult to observe due to the large labrum, suggested that the process was about to occur (Fig. 4.2 A). No clear mouth aperture was detected in SEM observations, although a possible invagination towards the mouth and primordial foregut can be seen (Fig. 4.3 A$_2$). When the feeding appendages emerge during the moult from Nauplius$_5$ to Zoea$_1$, some of the underlying tissue ruptures (Fig. 4.3 C$_{1,2}$); this may coincide with the formation of the mouth.

Rectal dilator muscle fibres surround the hindgut chitinised cells and typical contractions-distensions and peristaltic activity of the hindgut observed in all subsequent larval stages were also observed in advanced Nauplii$_5$ (Fig. 4.1 C). These observations were confirmed with advanced Nauplii$_5$ histological sections where a clear cellular difference between the midgut trunk epithelial cells and hindgut chitinised cells (Fig. 4.2 C, D). The anal pore in Nauplii$_5$ is evident prior to the moult to Zoea$_1$ (Fig. 4.1 C; Fig. 4.2 D$_1$; Fig. 4.3 A$_3$, B).

Zoea$_1$ morphology

Although a histological section clearly showing the mouth of a Zoea$_1$ was not seen, recently moulted Zoea$_1$ were observed feeding on microalgae (Fig. 4.4 A, B, C), and histological sections show the presence of algae in the anterior and lateral caeca, the entire midgut trunk and the hindgut (Fig. 4.5 A, B, C). SEM observations of specimens fixed at the time of light microscopy observations revealed defecation a few minutes after moulting (Fig. 4.6 C, D). The anus presents an area ornamented with short spines organized in a ridge which, together with strong and frequent peristaltic movements of the hindgut supported by rectal dilator muscle fibres, aids to pull faecal strands out (Fig. 4.6 B, C, D).

Ingestion of microalgae vs. ingestion of bacteria

No bacterial growth was observed when homogenates of treated nauplii were plated on TSA and TCBS media. Furthermore, SEM observations of Nauplii$_5$ and Zoea$_1$ showed no external bacteria attached, and histological sections revealed no bacteria. Therefore antibiotic disinfection of early Nauplii effectively eliminated all bacteria. This ensured that the bacteria stained with malachite green and with DTAF in feeding studies represented only those bacteria that were offered experimentally.

Figure 4.4 C and Figure 4.6 A, show the details of branched appendages covered with many fine setae that Zoea$_1$ uses for feeding and swimming. Results of chi-square tests at 5, 15 and 30 min showed that there was a significant association ($p<0.05$) between the degree of gut fullness (0, 1/3, 2/3, 1) and the type of feed

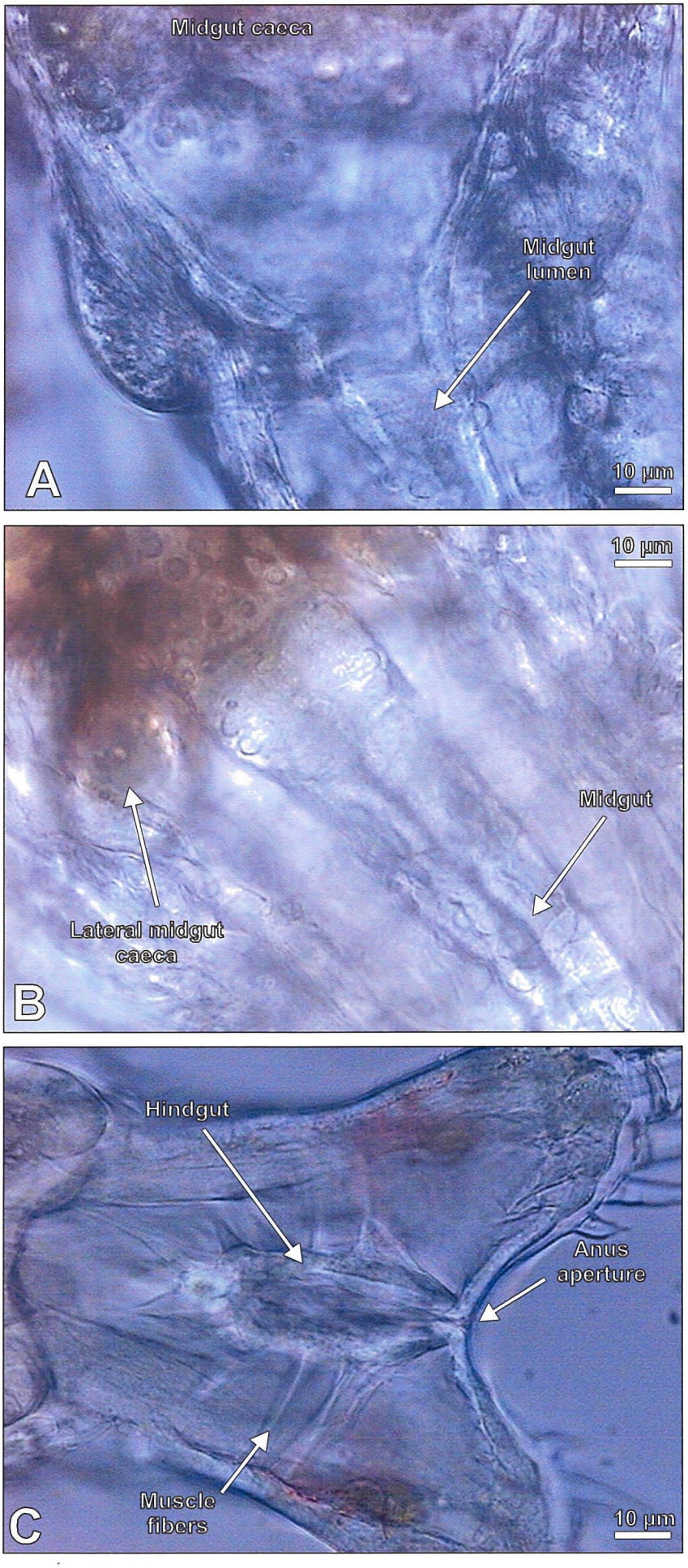

Figure 4.1 - Microphotographs of whole mounts of advanced Nauplius$_5$ of *Litopenaeus vannamei*. **A** – Anterior and lateral midgut caeca with aperture to midgut. **B** – Anterior and lateral midgut caeca, anterior gut and midgut. **C** – Hindgut detail. Note well developed muscle fibers.

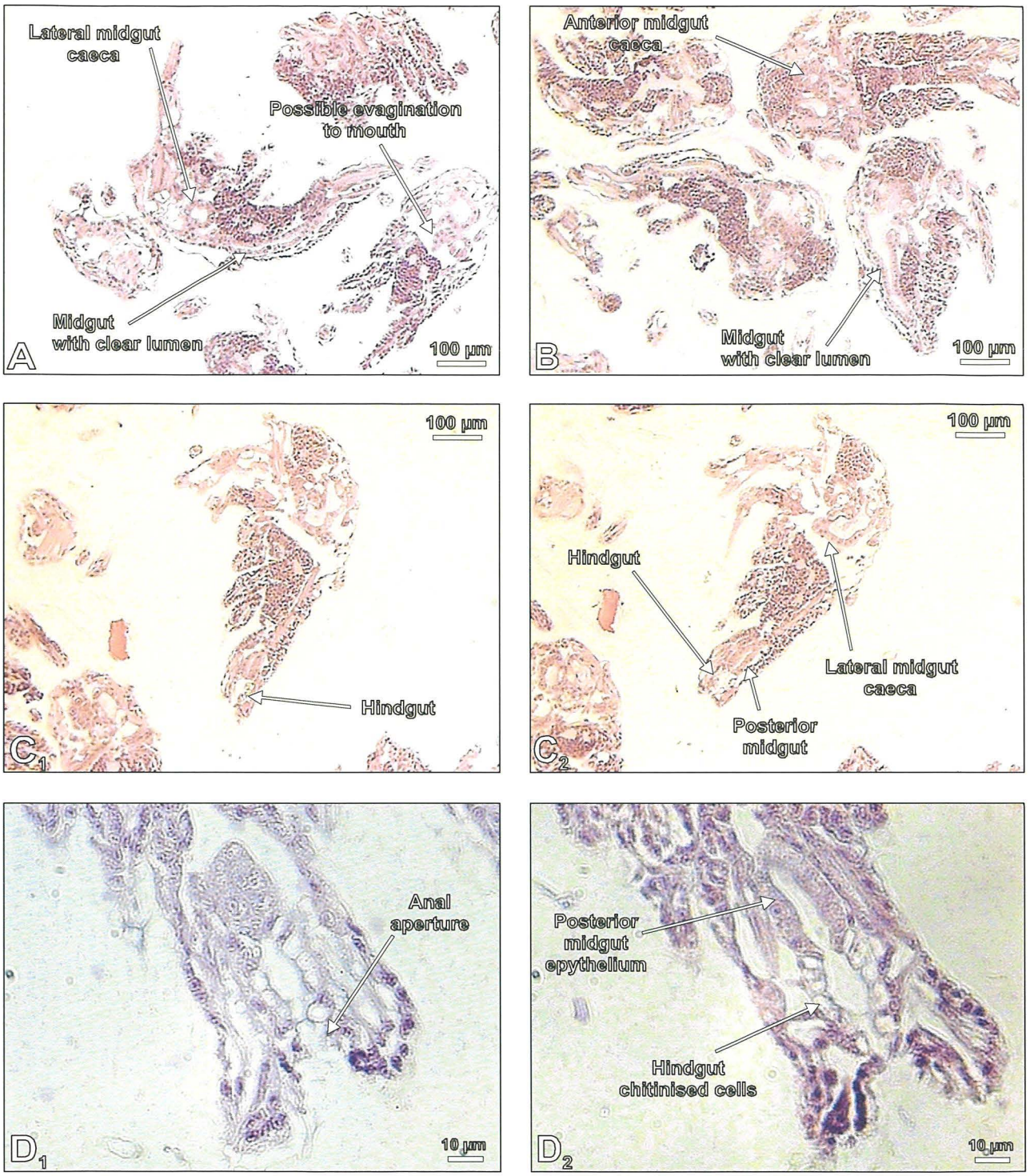

Figure 4.2 - Microphotographs of histological sections of Nauplius$_5$ of *Litopenaeus vannamei*. Photographs with the same letter represent the same individual. **A**- Sagital and transversal section of midgut and possible evagination to mouth. **B** - Sagital sections of anterior midgut caeca, possible evagination to mouth aperture, midgut trunk, hindgut and anus. **C** - Sagital and longitudinal sections of the complete gut width. **D** - Longitudinal sections of hindgut and anus aperture.

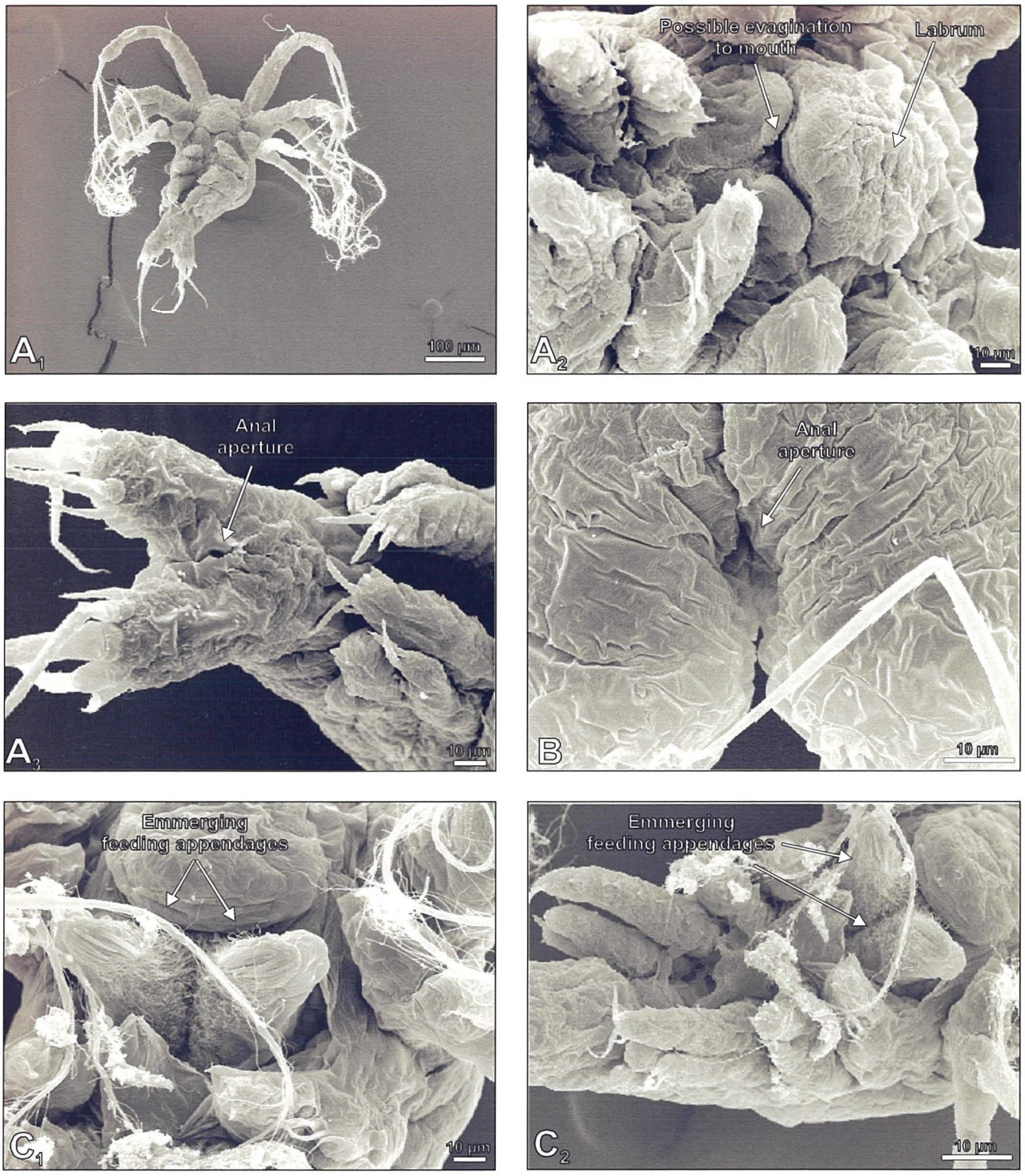

Figure 4.3 - Microphotographs of scanning electron microscopy (SEM) preparations and live advanced Nauplius$_5$ of *Litopenaeus vannamei*. Photographs with the same letter represent the same animal. **A** - Primordial mouth invagination, feeding appendages and anal aperture. **B** - Anal aperture. **C** - Advanced Nauplius$_5$ in moulting process to Zoea$_1$. Note emergence of feeding appendages from beneath the labrum.

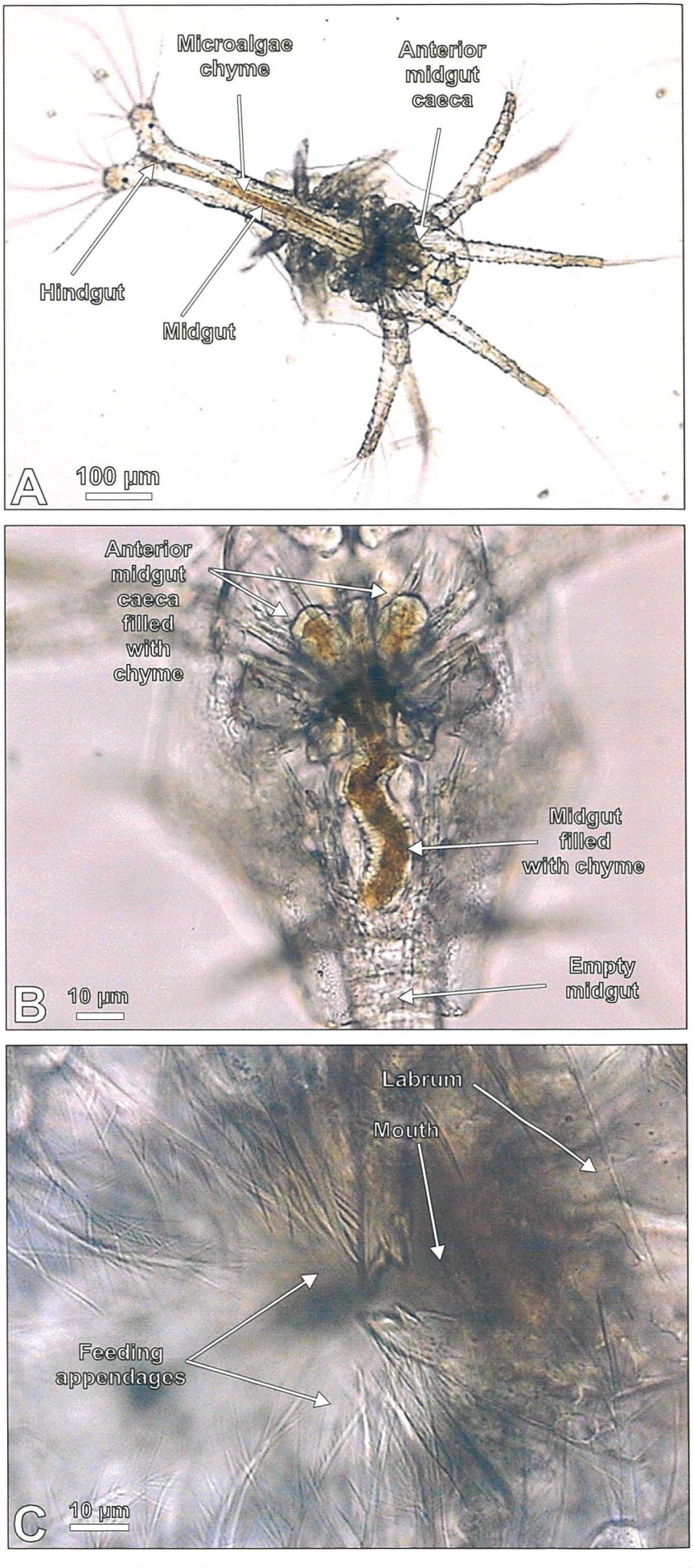

Figure 4.4 - Microphotographs of whole mounts of recently moulted $Zoea_1$ of *Litopenaeus vannamei*. **A** - Ventral view of $Zoea_1$ feeding on microalgae (*Chaetoceros muelleri* 150.000 cells ml^{-1}) for 15 min showing a completely filled digestive tract. **B** - Dorsal view of a $Zoea_1$ feeding on microalgae (*Chaetoceros muelleri* 150.000 cells ml^{-1}) for 5 min after starvation for 2 h. **C** - Ventral view of the oral region.

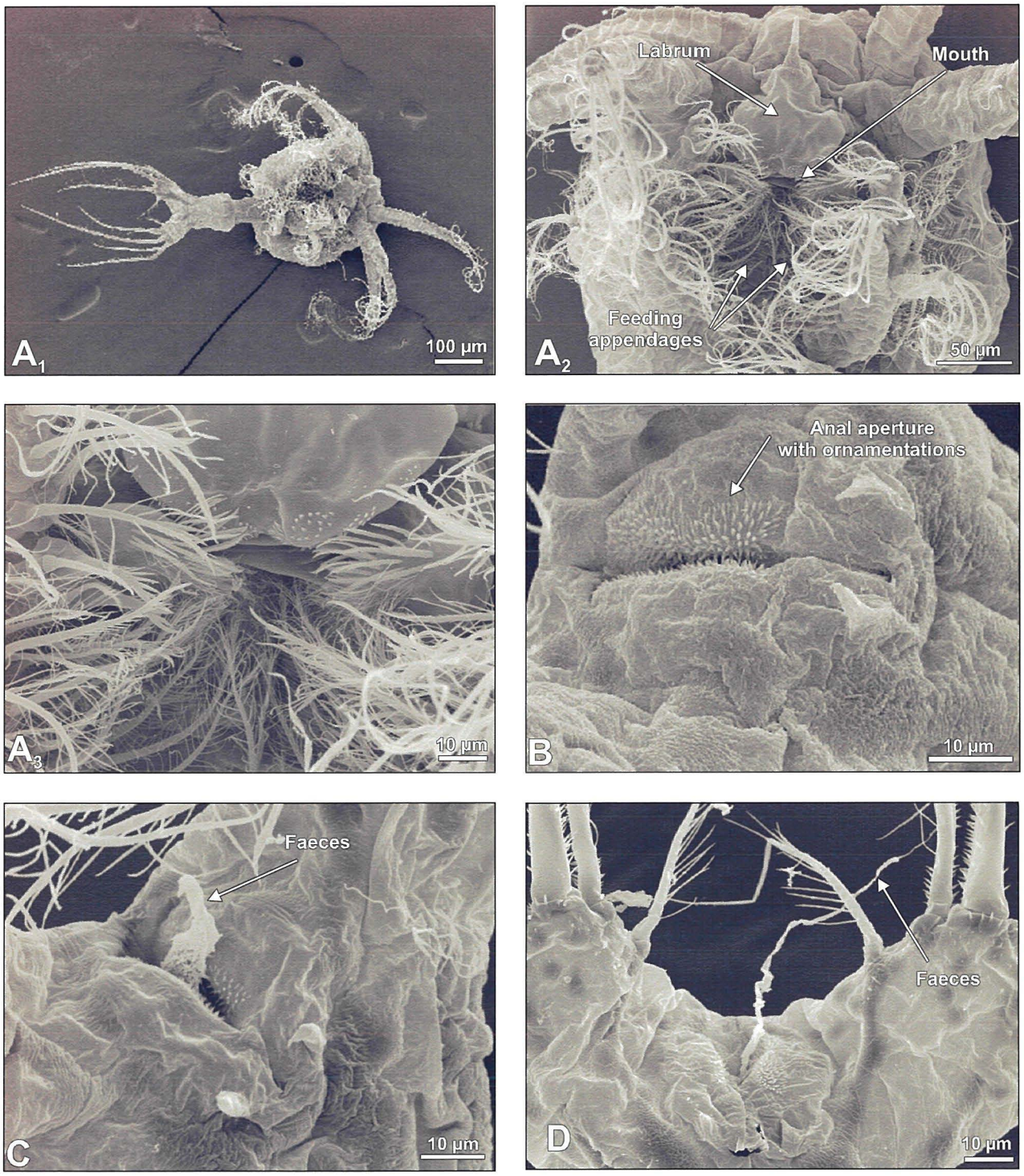

Figure 4.5 - Microphotographs of scanning electron microscopy (SEM) preparations of recently moulted *Litopenaeus vannamei* $Zoea_1$. Photographs with the same letter represent the same individual. **A** – Ventral view of the feeding apparatus and mouth of a recently moulted $Zoea_1$. **B** – View of $Zoea_1$, with detail of anus. Note ornamentations. **C** – Peritrophic membrane from a starved (never fed) $Zoea_1$. **D** – Faeces from a $Zoea_1$ fed exclusively on bacteria (9.7×10^6 CFU ml^{-1}) for 15 min.

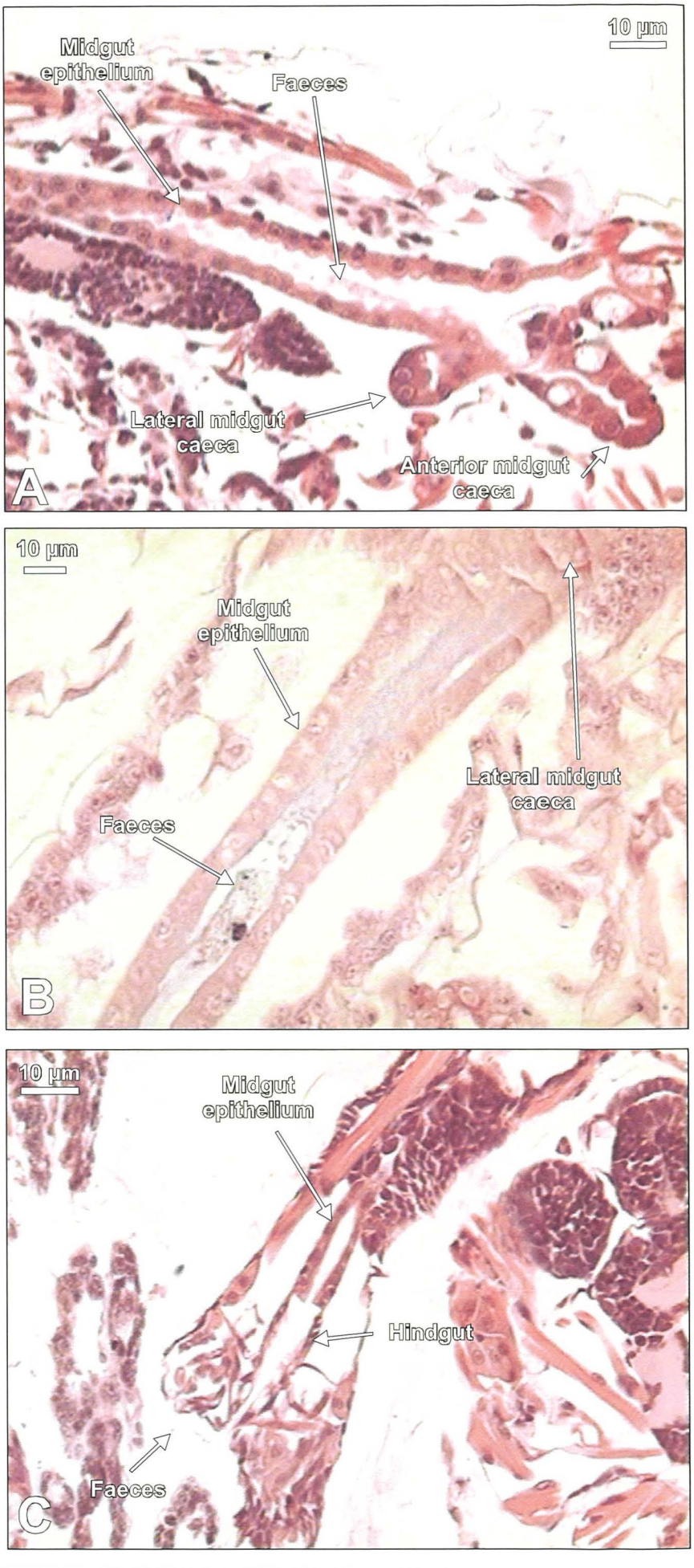

Figure 4.6 - Microphotographs of histological sections of recently moulted *Litopenaeus vannamei* $Zoea_1$. **A** - Anterior and lateral midgut caeca and midgut trunk regions of a $Zoea_1$ fed exclusively with fluorescent labelled bacteria (9.7×10^6 CFU ml^{-1}) for 15 min. **B** - Midgut trunk of a $Zoea_1$ fed fluorescent labelled bacteria (9.7×10^6 CFU ml^{-1}) and microalgae (*Chaetoceros muelleri* 150.000 cells ml^{-1}) for 30 mins. **C** - Midgut trunk and hindgut of a $Zoea_1$ fed exclusively with fluorescent labelled bacteria (9.7×10^6 CFU ml^{-1}) for 15 min.

larvae ingested (microalgae vs. bacteria), indicating that microalgae are filtered by larvae more efficiently and rapidly than bacteria (Fig. 4.7). Results of chi-square tests for microalgae fed larvae during the periods 0-5, 5-15 and 15-30 minutes, showed that the frequencies of gut fullness degree were significantly different between periods ($p < 0.05$). The opposite was true for bacteria fed larvae.

After only 5 min, more than 50% of larvae fed with microalgae had their guts filled to 2/3, whilst for larvae fed bacteria, the guts were practically empty. At 15 and 30 min, more than 85% of the larvae examined had ingested some microalgae. In contrast, bacteria were never ingested in significant quantities by 80% of the larvae examined. Compared to larvae feeding on microalgae, bacteria-fed larvae showed less gut extension and thinner faecal strands as considerable less biomass was ingested (Fig. 4.8). No bacteria-fed larvae was ever observed with the long, continuous strands of digested food that were characteristically present in the guts of larvae fed microalgae. Most faecal strands were practically empty, and although they may be filled with some individual bacteria, there was little evidence that bacteria were being actively fed upon by shrimp Zoea$_1$ larvae.

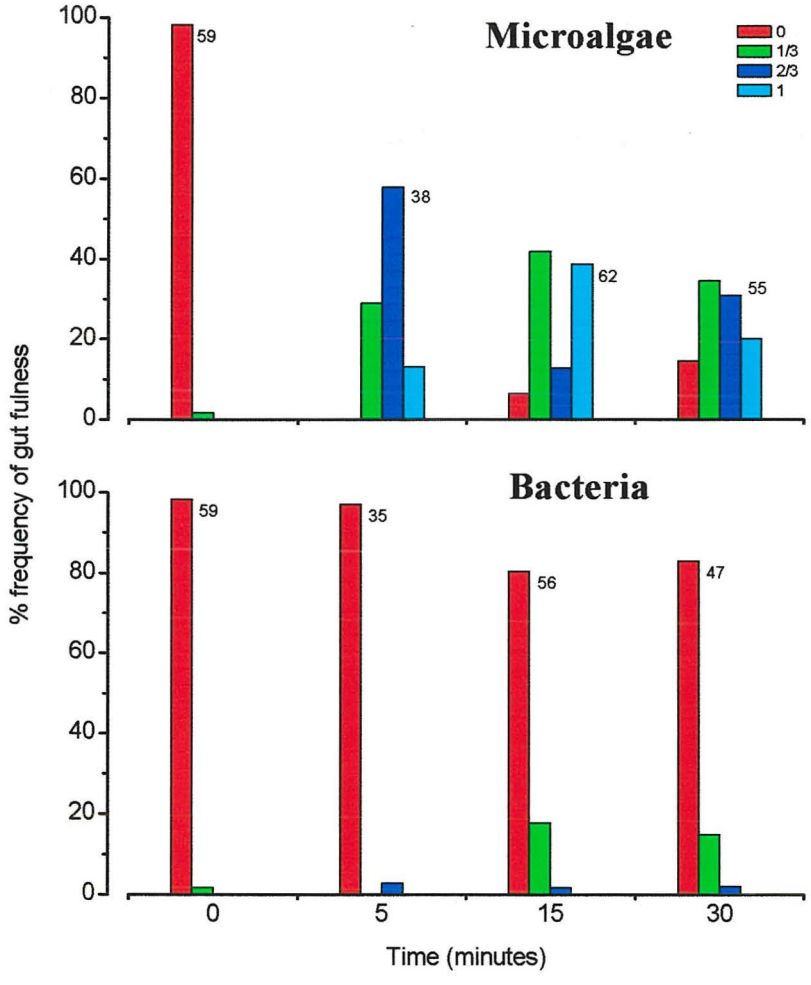

Figure 4.7 - Frequency of gut fullness (%) of recently moulted *Litopenaeus Vannamei* Zoea$_1$ feeding on microalgae (50.000 cells ml^{-1} *Chaetoceros muelleri*) or bacteria marked with malachite green (*Vibrio harveyi*, CAIM79 strain 1.26×10^6 CFU ml^{-1}). Numbers 0, 1/3, 2/3 and 1 represent an ordinal scale ranging from empty gut to complete fullness. Column numbers represent individuals observed at each time. Animals were starved for 2 h in 0.45 µm filtered sterilised seawater with water exchanges every hour prior to feeding.

Bacteria in the digestive tract of penaeid shrimp larvae - FLB observations

Fluorescent labelled bacteria (FLB) were observed along all regions of both $Zoea_1$ and $Zoea_2$ digestive tract irrespective of the bacterial concentration used or the duration of contact of the larvae with FLB (Figs. 4.9, 4.10, 4.11). After long periods (40 min) of exposure to FLB, large background fluorescence green noise was always observed in the midgut anterior and lateral caecae (Fig. 4.9 A, B, C, D; Fig. 4.10 A, B, C) when compared to other regions of the digestive tract (Fig. 4.9 A, B, C, D; Fig. 4.11 A, B, C).

FLB were frequently observed "intact" as individual discrete cells within the peritrophic membrane in the midgut trunk and hindgut region (Fig. 4.9 D; Fig. 4.11 A), whilst FLB in the anterior and lateral midgut caeca regions were difficult to observe because of background fluorescent noise (Fig. 4.10). At higher magnifications, clumps and monodispersed FLB could be seen dispersed in the anterior and lateral midgut caecae (Fig. 4.10 B) and then, progressively, concentrated or "packed" and isolated inside the peritrophic membrane, and transported along the midgut trunk (Fig. 4.11 A, C), following antiperistaltic contractions of the gut walls (Fig. 4.9; Fig. 4.11). These movements were best observed at the posterior end of the larva where faeces are expelled (Fig. 4.11 B, C, D). Faecal strands of larvae fed exclusively with FLB frequently included digested bacterial biomass that did not fluoresce (Fig. 4.9 D; Fig. 4.11 D). This pattern was more evident when larvae were fed FLB together with microalgae (Fig. 4.12 A, B). It was impossible to verify whether bacteria do indeed resist digestion and survive inside the gut. Released and distal ends of trailing faeces (Fig. 4.12 B) were observed to fluoresce more than internal or proximal faecal material (Fig. 4.12 C). Larvae fed exclusively with bacteria showed a significant decrease in the FLB number inside the midgut trunk within 30 min after feeding (χ^2=27.07, 2 df, $p<0.05$ - Figure 4.13).

When larvae were left for more than 12 h in a FLB solution, they become fouled with clumps of bacteria, similar to the debris normally observed under commercial larviculture (Fig. 4.14).

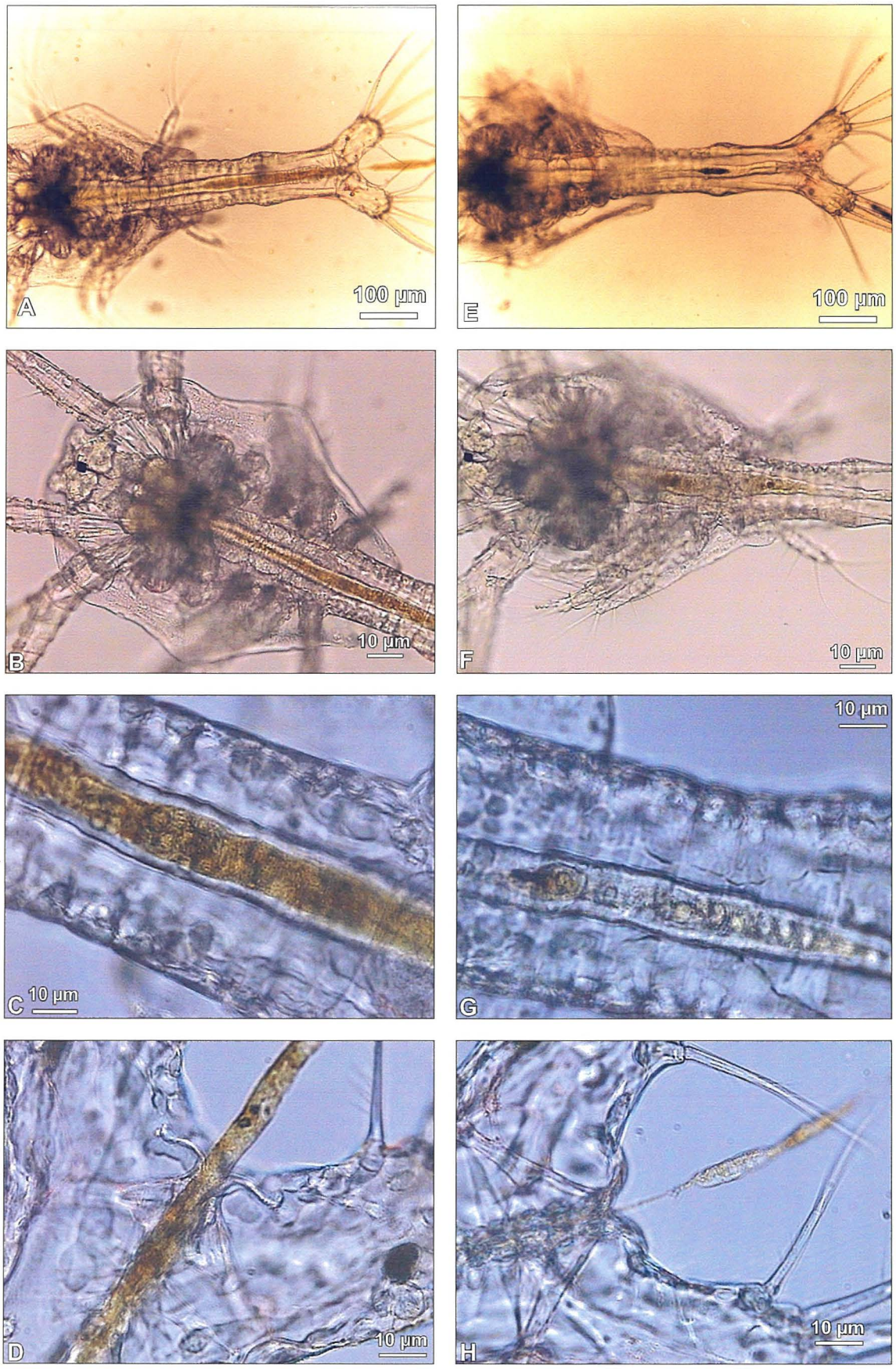

Figure 4.8 – Microphotographs of whole mounts of recently moulted $Zoea_1$ of *Litopenaeus Vannamei* feeding on microalgae (50.000 cells ml^{-1} *Chaetoceros muelleri*) or bacteria marked with malachite green (*Vibrio harveyi*, CAIM79 strain 1.26×10^6 CFU ml^{-1}). Animals were starved for 2 h in 0.45 µm filtered sterilised seawater with water exchanges every hour prior to feeding. Note changes in complete gut fullness, anterior and lateral midgut caeca fullness, midgut extension and faeces thickness.

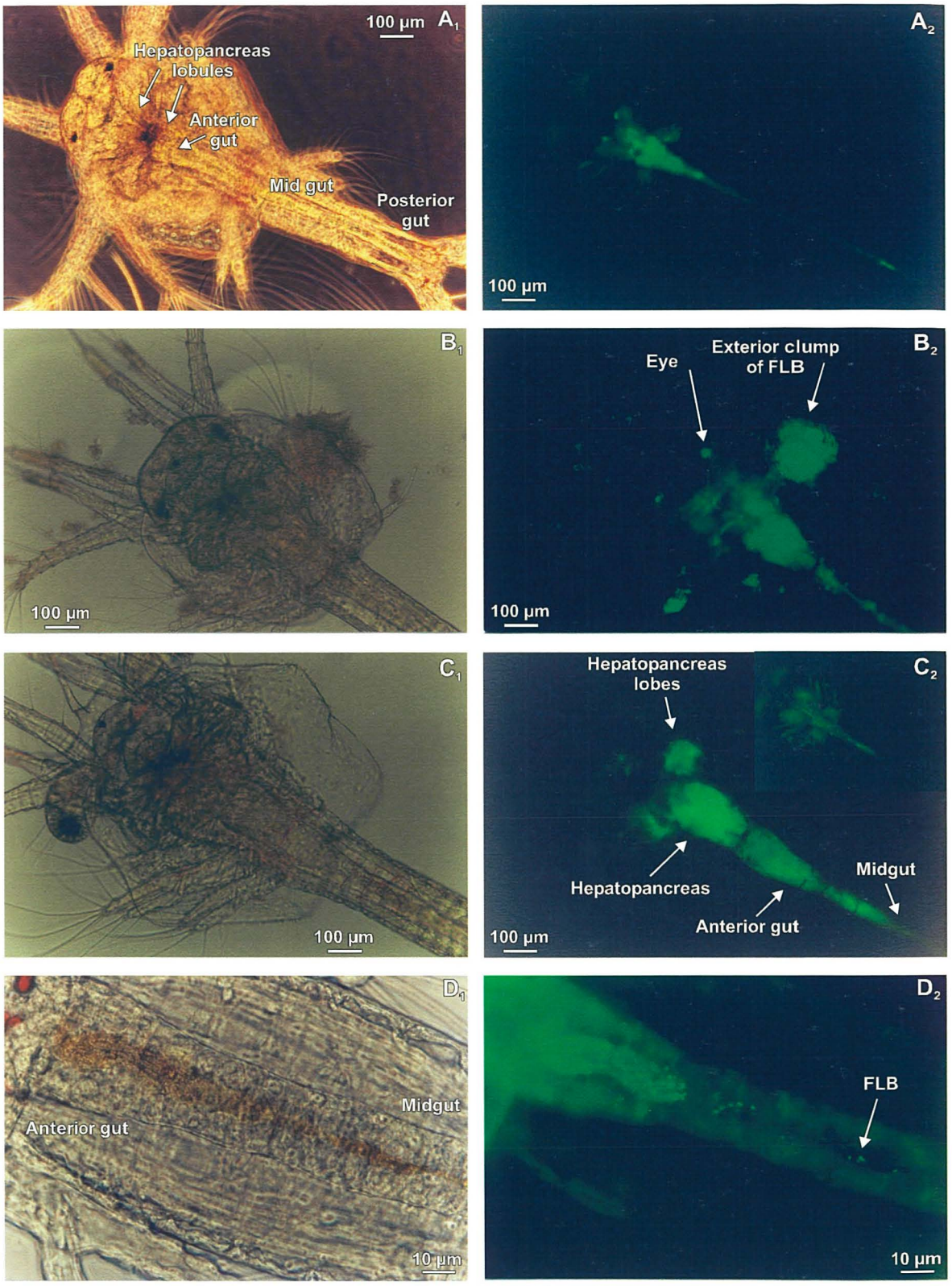

Figure 4.9 - Normal light and identical epi-fluorescence microphotographs of live Zoea$_1$ and Zoea$_2$ of *Litopenaeus vannamei* feeding on fluorescent labelled bacteria alone (*Vibrio harveyi*, CAIM79) or in combination with microalgae (*Chaetoceros muelleri* 150 000 cells mL^{-1}). All photographs represent different individuals unless otherwise specified. **9A** - Dorsal view of FLB on anterior and lateral caeca and in the midgut of a Zoea$_1$ feeding on 6.6×10^6 FLB for 2-3h. **9B** - Dorsal view of FLB on the anterior and lateral caeca and in the midgut of an advanced Zoea$_1$ fed exclusively on 1.12×10^7 FLB for 12 h. Note a clump of FLB bacteria attached to the exterior carapace. **9C** – Dorsal view of anterior and lateral caeca and anterior opening to the midgut trunk of an advanced Zoea$_2$ fed bacteria only (8.85×10^6 CFU mL^{-1}) for 1-2 h. Smaller pictures show equivalent area of a Zoea$_1$. Note the different area of the hepatopancreas from one stage to another. **9D** - Monodispersed and clumps of FLB in anterior and midgut of an advanced Zoea$_1$ feeding on 6.6×10^6 FLB for 15-20 min. Note entrance to the lateral midgut caecum on the top left corner.

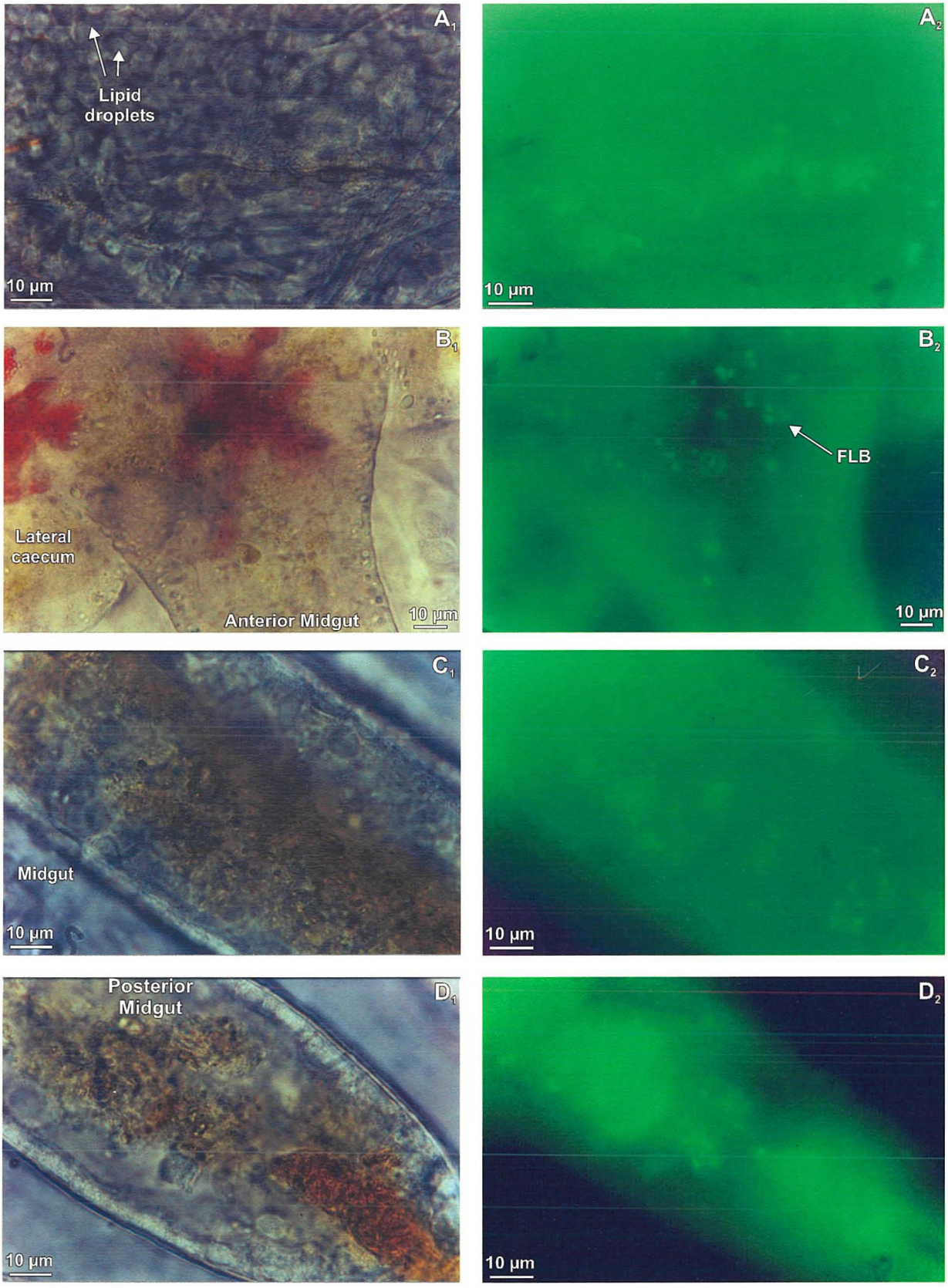

Figure 4.10 - Normal light and identical epi-fluorescence microphotographs of live $Zoea_1$ and $Zoea_2$ of *Litopenaeus vannamei* feeding on fluorescent labelled bacteria alone (*Vibrio harveyi*, CAIM79) or in combination with microalgae (*Chaetoceros muelleri* 50.000 cells ml^{-1}). All photographs represent different individuals unless otherwise specified. **10A** – Dorsal view of anterior and lateral caeca of an early $Zoea_1$ feeding on bacteria (1.52×10^7 CFU ml^{-1}) and microalgae for 40 min. Note lipid droplets. **10B** – Dorsal view of monodispersed FLB in lateral midgut caeca and anterior midgut of an early $Zoae_1$ feeding on bacteria (1.26×10^7 CFU ml^{-1}) and microalgae. **10C** – Midgut trunk of a $Zoea_2$ fed 1.4×10^7 bacteria only for 12 h. **10D** – Posterior midgut of a $Zoea_2$ fed 1.4×10^7 bacteria only for 12 h.

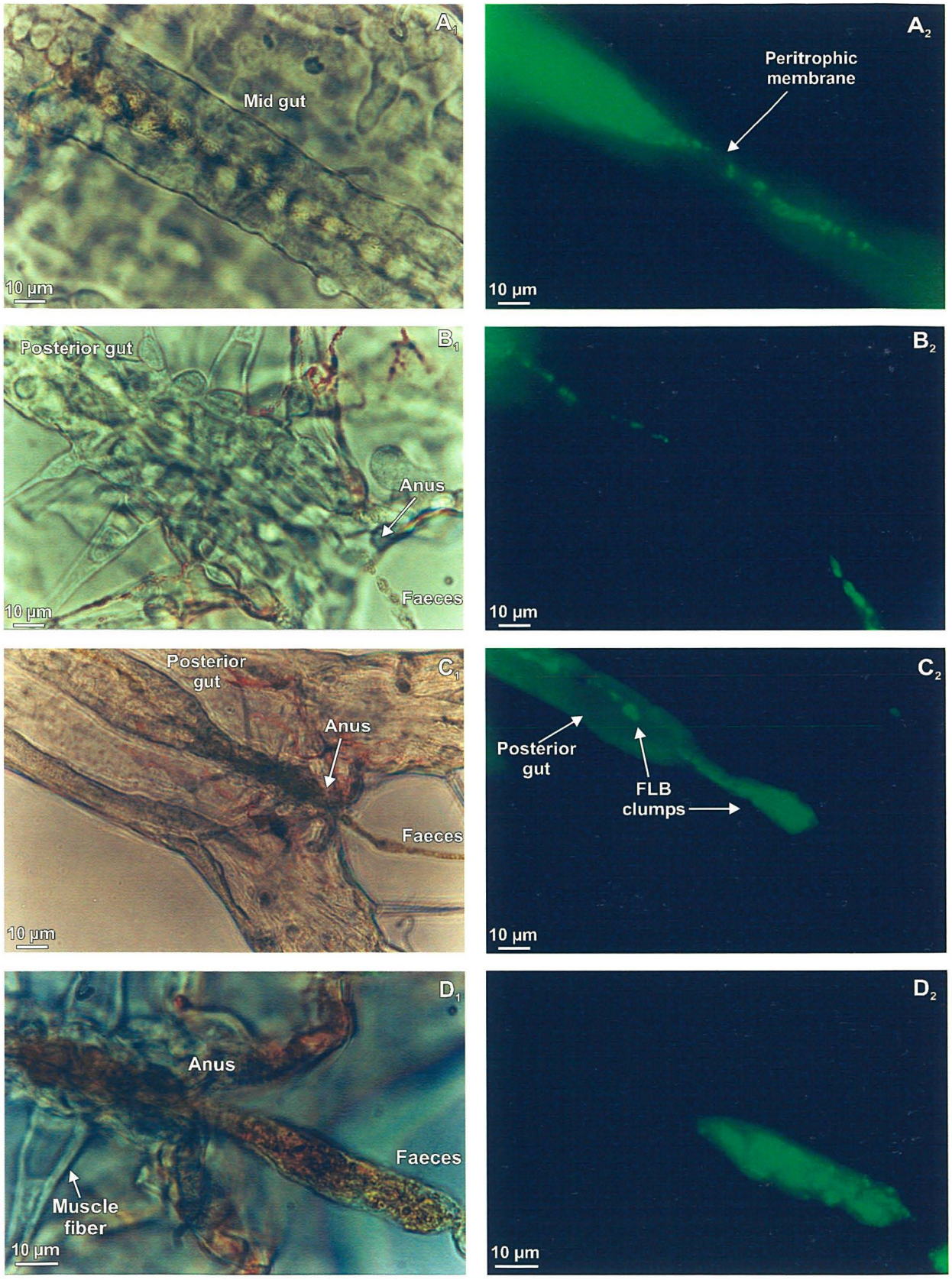

Figure 4.11 - Normal light and identical epi-fluorescence microphotographs of live $Zoea_1$ and $Zoea_2$ of *Litopenaeus vannamei* feeding on fluorescent labelled bacteria alone (*Vibrio harveyi*, CAIM79) or in combination with microalgae (*Chaetoceros muelleri* 50.000 cells ml^{-1}). All photographs represent different individuals unless otherwise specified. **11A** - Monodispersed FLB in midgut of an early $Zoea_1$ feeding on 1.52×10^7 FLB and microalgae for 40 min. **11B** - Monodispersed FLB in posterior gut and faeces of an early $Zoea_1$ feeding on 1.52×10^7 FLB and microalgae for 40 min. **11C** –Posterior gut, anus and faeces of larvae kept under the same conditions as before.. **11D** – Anal region and faeces with a clump of FLB of a $Zoea_2$ fed 1.4×10^7 bacteria only for 1-2 h.

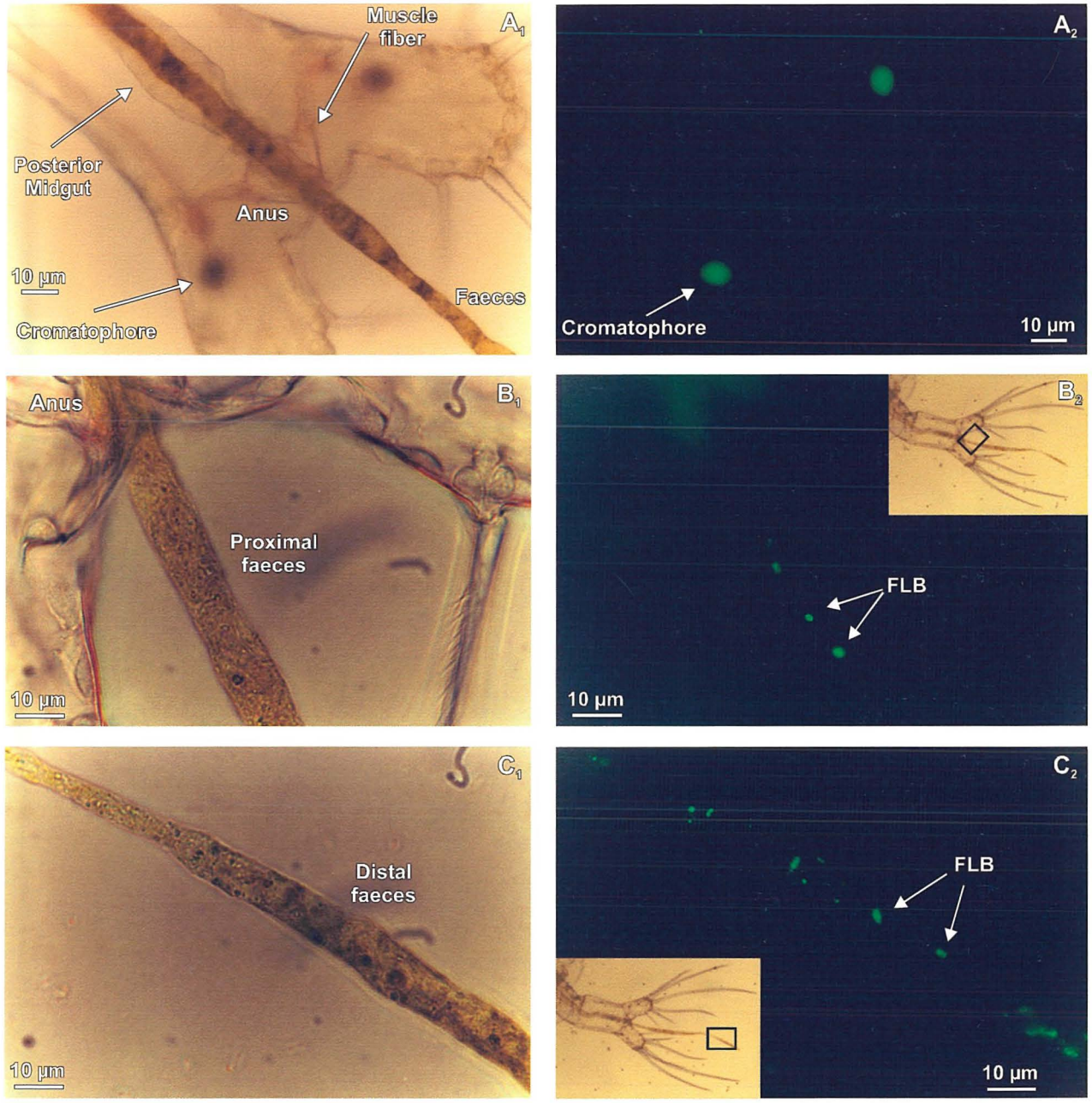

Figure 4.12 - Normal light and identical epi-fluorescence microphotographs of live Zoea$_1$ of *Litopenaeus vannamei* feeding on bacteria (*Vibrio harveyi*, CAIM79 Strain). All photographs represent different individuals unless otherwise specified. **12A** – Posterior midgut and faeces of an early Zoea$_1$ feeding on bacteria (1.26×10^7 CFU ml^{-1}) and microalgae. **12B** – A few FLB in the anal region and proximal faeces of an early Zoea$_1$ feeding on bacteria (1.26×10^7 CFU ml^{-1}) and microalgae. Small picture shows the same individual at a lower magnification showing area of larger magnification picture. **12C** – Several FLB in distal faeces from the same individual as in 12 B.

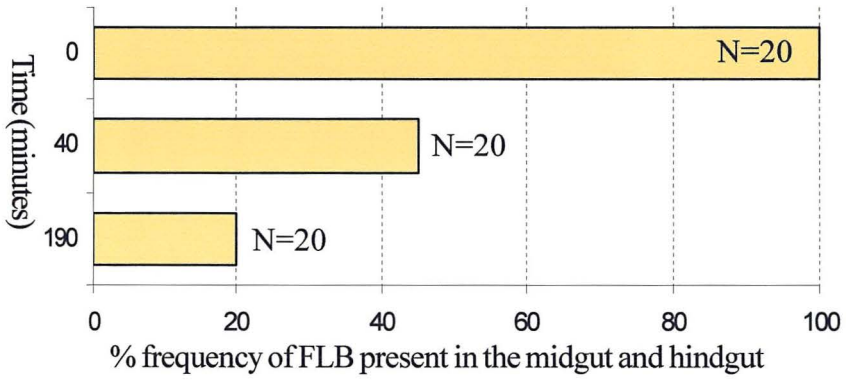

Figure 4.13 – Bacteria evacuation time from the midgut of Zoea$_1$ of *Litopenaeus vannamei*. Presence/absence frequency of fluorescent labelled bacteria (FLB) in the midgut and hindgut of *Litopenaeus vannamei* Zoea$_1$ fed with a bacteria concentration solution of 1.52×10^7 ml^{-1} (*Vibrio harveyi*, CAIM79 strain).

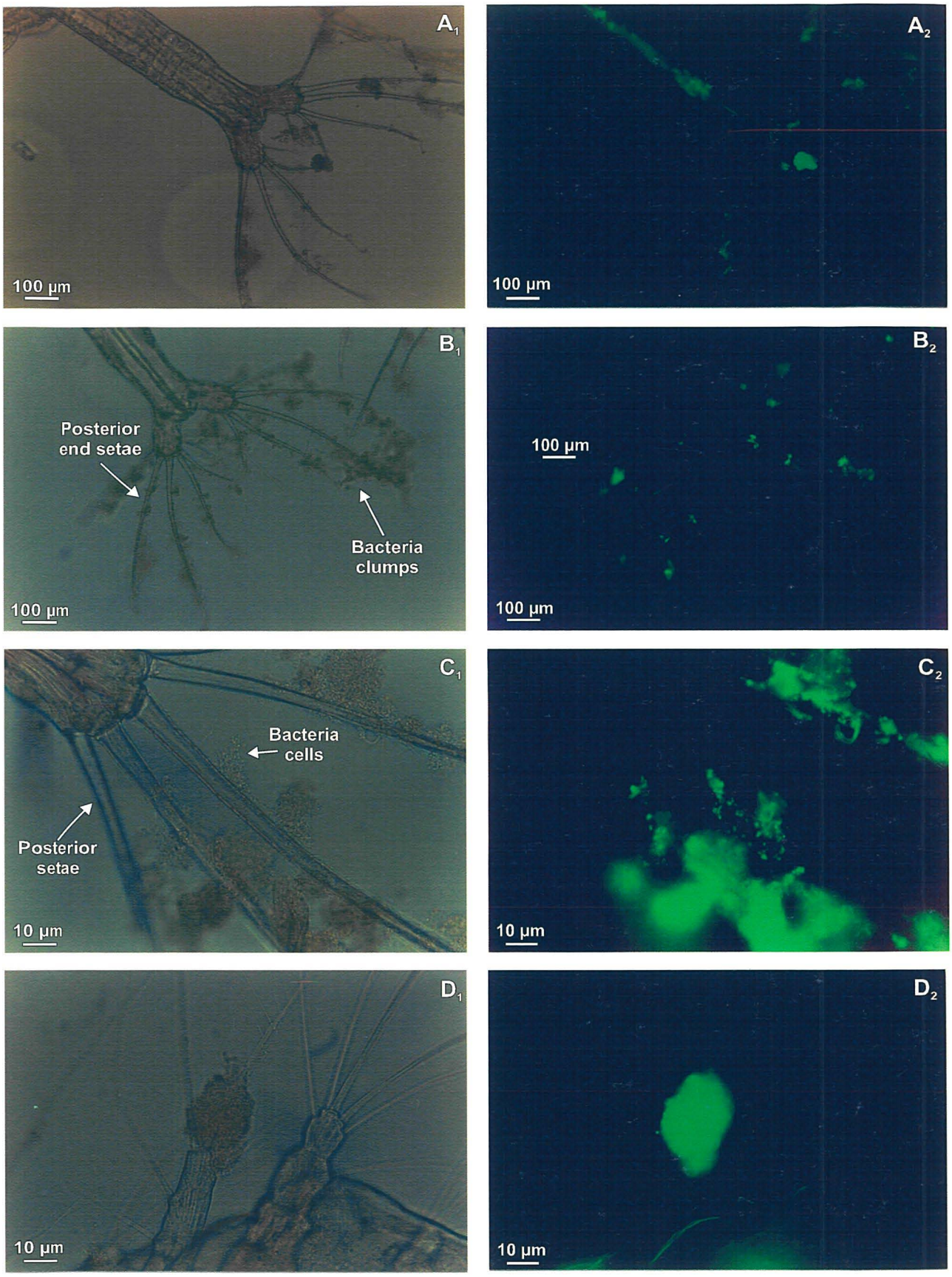

Figure 4.14 - Normal light and identical epi-fluorescence microphotographs of live $Zoea_1$ and $Zoea_2$ of *Litopenaeus vannamei* feeding exclusively on non-pathogenic bacteria (1.12×10^7 CFU ml^{-1} *Vibrio harveyi*, CAIM79 strain) for 12 h. **14A** – Dorsal view of FLB on the midgut and hindgut of an advanced $Zoea_1$. Note the clump of FLB bacteria attached to the posterior setae. **14B** - Bacterial fouling on posterior end setae of an advanced $Zoea_1$ **14C** - Detail of FLB clumps on posterior end setae of an advanced $Zoea_1$. **14D** – Detail of FLB clump attached to setal end of a $Zoea_2$ fed on bacteria for 12 h ($1.4\ 10^7$ CFU ml^{-1}).

DISCUSSION

Nauplius$_5$ morphology

The clearly defined lumen and peristaltic activity in Nauplii5 stages, disagrees with the assertion of Lovett and Felder (1989), that the walls of the foregut are adpressed and that the midgut cannot be clearly distinguished in Nauplii$_5$. Although this might be due to differing development between species or histological sample preparation artefacts, it is reasonable to believe that the nauplii samples used by these authors were not as advanced and close to moulting into Zoea$_1$ stage. The foregut and hindgut of Nauplii$_5$ already show differentiated chitinised cells, which according to Freeman (1993), are of epidermal origin. The yolk vacuoles described by Zilch (1978) in earlier naupliar stages of *Penaeus trisulcatus* were observed inside the primordials of the anterior and lateral midgut caecae.

Lovett and Felder (1989) state that the foregut does not communicate with the midgut until Zoea$_1$, the stage at which the gut becomes complete and the larva commences feeding. We infer that the internal gut environment of advanced stage Nauplius$_5$ may already be exposed to external conditions. The anus is open, even if the mouth is not in Nauplii$_5$. The existence of antiperistalsis (anal drinking) strongly suggests that the interior tissues of the posterior gut may be in contact with the external environment through a similar process, and consequently to the microflora present in the water column, as early as Nauplius$_5$. Antiperistalsis waves in the midgut trunk have been described for the subsequent larval stages (Zoea$_{1-3}$) by Lovett and Felder (1990c). These authors propose that anal drinking and antiperistalsis in shrimp postlarvae may function in the anteriad expansion of hepatopancreas microtubules by means of hydraulic pressure, as well as in aiding defecation. Water that enters the anus can be carried anteriad as far as the anterior end of the midgut. Thus, if the anal pore is open before the mouth, then anal drinking may serve as the initial entrance for water into the primitive alimentary canal, expanding it and providing some hydraulic pressure to the caecae. The ecological implications of these observations, assuming that internal tissues of the eggs and early nauplii are sterile, would be that several hours before normal feeding activity begins at Zoea$_1$ stage, the developing anterior and lateral caecae, the midgut trunk and the hindgut may be inoculated with bacteria and other microbes present in the water at that time.

Zoea$_1$ morphology

Recently moulted Zoea$_1$ are capable of ingesting, processing and evacuating microalgae from a very early stage, a few minutes after moulting. The midgut trunk of *Litopenaeus vannamei* Zoea$_1$ and Zoea$_2$ larvae are similar to the anatomical descriptions made for *Litopenaeus setiferus* by Lovett and Felder (1989), consisting of a simple tube lined with epithelial cells and two pairs of caecae that are similar in both structure and size. The hindgut is well differentiated in comparison to the anal pore of Nauplius$_5$, but in the Zoea$_1$ stage and during all subsequent development, it remains a simple tube with chitinised cells with the junction to the midgut trunk located within abdominal segment 6. The observation of strong and frequent peristaltic movements of the hindgut supported by rectal dilator muscle fibres and short spines ornamenting the anus, assists in pulling faecal strands out, confirming the observations of gut kinematics made by Lovett and Felder (1990c). Pressure exerted on the sides of these spine ridges is thought to help break the faecal strand.

Unlike the intermittent growth and development restricted to ecdysis in the chitin-lined foregut and hindgut, development in the midgut is more gradual and any resident bacterial flora are likely to remain securely attached to the surface of either the midgut trunk or the anterior and lateral caecae. Healthy $Zoea_1$ in an environment with abundant food resources will feed almost continuously with some food particles in the foregut passing directly to the midgut trunk and others being diverted to the anterior and lateral caecae (Lovett & Felder 1990c). Oral drinking is accomplished by peristaltic waves along the oesophagus and occurs regularly, even when food is not being ingested, as has been described for all developmental stages of *Litopenaeus setiferus* (Lovett & Felder 1990c). Both of these mechanisms can contribute to the entry of bacteria into the larval digestive tract. Digested food is packed into a peritrophic membrane by peristaltic and antiperistaltic contraction waves and eventually is expelled to the exterior through the hindgut. While the peritrophic membrane may protect delicate epithelia of the midgut trunk from abrasive material (Forster 1953), it also may play a major role in the separation of chyme and faecal matter from extra-peritrophic water that is taken up by anal drinking and moved anteriorly to inflate the midgut (Lovett & Felder 1990c). Therefore, any bacteria that are present in the chyme and faeces are most likely to be expelled to the surrounding seawater, whilst the midgut trunk absorptive epithelium may be colonised with bacteria taken in by anal drinking.

Ingestion of microalgae vs. ingestion of bacteria

There are few references to bacterial ingestion by small planktonic crustaceans. Azad *et al.* (2002) report moderate survival and growth of *Penaeus monodon* larvae from $Zoea_1$ to $Postlarvae_1$ fed with live microalgae and *Artemia* nauplii and varying proportions of the phototrophic bacteria *Rhodovulum sulfidophilum*. Intriago and Jones (1993) report moderate growth and survival results for *Artemia* feeding on a suspension of bacteria. Peterson *et al.* (1978) have shown that *Daphnia* can feed on the very small, naturally occurring bacteria from an Alaskan lake. Bacteria numbers in water vary drastically, but due to their omnipresence, they are surely part of the diet of all planktonic adult and larval crustaceans. However, the actual contribution of bacteria to the total requirements of the animals is likely to be small (Tezuka 1971). Present study results indicate that at 5, 15 and 30 min, nearly all larvae had 1/3, 2/3 and 3/3 of the gut full when feeding on microalgae, whereas most larvae feeding on bacteria had empty guts at 5, 15 and 30 min, similarly to the initial time 0 starved situation.

It is well known that planktonic, filter-feeding crustaceans have different efficiencies in sieving and ingesting different size particles from the water (Grahame 1983). Decapod larvae employ either filter or raptorial feeding methods depending upon their trophic feeding level (Jones *et al.*, 1997). For exclusive filter feeders, such as shrimp $Zoea_{1-2}$, the encounter rate with a potential food item is a chance encounter and generally, dependent upon the food particle concentration. To feed, these larvae use branched appendages covered with many fine setae. Both mechano- and chemoreception are involved in food selection once contact has been made Jones *et al.* (1997). The difference in size between microalgae and individual bacteria may explain why microalgae are captured more efficiently than bacteria; the bacteria may be too small to be caught in the inter-setular spaces of the feeding mouthparts (Boyd 1976). In addition, certain microalgae, such as the diatom *Chaetoceros muelleri* used in these observations, have long spines and form large

chains of cells attached to each other, which effectively increases their probability of being captured by the larval feeding appendages.

In copepods a very wide size range of particles are typically collected by the various feeding appendages and gathered under the labrum (Yule & Crisp 1983). Smaller particles are accumulated until a bolus of particles is formed at the mouth, bound together by labral gland secretion (Grahame 1983). This bolus is partially pushed into the mouth by the bases of the mandibles and partly sucked in by peristaltic movements of the oesophagus or foregut. Single, larger particles that are small enough to enter the mouth whole, are immediately ingested in the above manner (Yule & Crisp 1983). Aggregates of particles too large to enter the mouth are broken up by the mandibles; although much is ingested, some food is lost. According to Yule and Crisp (1983), particles too large to enter the mouth whole, yet too tough for the animal to break up, are quickly rejected and flicked out from the labrum by the mandibles to be carried away in the current. Although there are differences in the feeding appendages of shrimp larvae and copepods, the overall feeding mechanisms of $Zoea_1$ shrimp larvae, should not be much different from those of copepods. In the present study, clumps of bacteria would be ingested using the first described mechanism, whilst microalgae could have been ingested with the same mechanism or directly without accumulation and bolus formation. It is likely that larval sieving efficiency is increased when high densities of bacteria result in the formation of large clumps.

Food particle size and mechanical filtration limits, however, are not the only determinant factors influencing ingestion rates of planktonic filter-feeding organisms. Scent can play an important role in food particle selection. Working with copepods, Price *et al.* (1983) postulate that if remote detection is achieved by chemoreception, encounter probabilities should be related to the characteristics of the cloud of chemical exudates surrounding each cell. This cloud of chemical exudates has been described for microalgal cells and termed as "phycospheres" by Bell and Mitchell (1972). Because shrimp larvae are small (1-10 mm), their physical world is dominated by viscous forces rather than the inertial forces and therefore, the feeding appendages operate at Reynolds numbers of only 10^{-2} to 10^{-1} meaning that flow is laminar (Koehl & Strickler 1981). A consequence of laminar flow is that when a $Zoea_1$ flaps its feeding appendages to propel water past itself, it will not stir the water and thus will not confuse the direction from which chemical signals, such as "phycospheres" are coming. Intermittent swimming and feeding behaviour, characteristic of many small, planktivorous crustaceans may therefore, be related to resting periods or to accurately sense nearby chemical or mechanical cues in the surrounding environment (Koehl & Strickler 1981; Yule & Crisp 1983; Price *et al.* 1983). The patterns of food detection, feeding mechanisms, and selection of food by *Homarus gammarus* lobster larvae have been described by Kurmaly *et al.* (1990), which demonstrate that these carnivorous larvae eliminate most non-nutritious particulate material. The mouthparts of early and late stage penaeid larvae are capable of selecting between irregular and rounded particles and exhibit chemosensory discrimination (Kurmaly *et al.*, 1989). According to Jones *et al.* (1997), penaeid larvae are capable of differentiating potential food items on the basis of scent and texture. According to these authors, preferred particles include bland or flavourless substances such as carbon, latex beds or chromic oxide, natural prey items and artificial diets with certain biological flavours, whilst rejected particles, which accumulate as a bolus below the mouth, include acidic or alkaline substances, strongly flavoured particles such as haemo-

globin and in particular rancid or oxidised lipids. Therefore, we speculate that differences in the ingestion rate for microalgae and bacteria by $Zoea_1$ in the present study may be due to both a negative "odour" of the particular strain of bacteria used and its smaller size.

Drinking activity is thought to have an osmoregulatory function in early fish larvae (Tytler and Blaxter, 1988; Tytler *et al.*, 1990), and for filter feeders, may act as a "probing for food" activity. Peristalsis movements in the oesophagus have been described in shrimp postlarvae and are evidence of "oral drinking" activity (Lovett & Felder 1990c). Although oral drinking activity has not yet been demonstrated for nauplii and $Zoea_1$ larval stages, it is likely that such activity exists. When microalgae are not present in the water, ingestion of bacteria could be accidental through routine drinking activity. When microalgae are present, ingestion of bacteria may continue to be accidental, especially when bacteria are specifically attached to the algal cells. Bacteria are known to attach to the outside silica carapace of marine diatom cells (Coleman 1980), and other microplankton (Makemson *et al.* 1992).

Bacteria in the digestive tract of penaeid shrimp larvae - FLB observations

After leaving the anterior and lateral midgut caecae, digested material, including bacteria, is encased and compressed in the peritrophic membrane with no opportunity for contact with the intestinal lining (Zachary & Colwell 1979; Lovett & Felder 1990c). Sochard *et al.* (1979) found considerably lower numbers of bacteria in the dissected gut of copepods after elimination of faecal pellets than when faecal material was present, indicating that most bacteria were flushed with faeces. For a bacterium to be a potential colonizer of the shrimp larvae digestive tract, it must attach to the gut walls and reproduce in order to remain in such a "flow-through" environment. As the entire foregut and hindgut epidermal lining is shed at every moult to another ontogenetic stage (Freeman 1993), and the estimated time for gut evacuation is 10-20 min (Jones *et al.* 1997), which is similar to the generation time of the *Vibrio* strain used in the present study observed in experimental conditions, it can be concluded that most bacteria will be flushed out before they manage to reproduce inside the larval gut.

Present study observations of the more intense background fluorescence green noise in the midgut anterior and lateral caecae as compared to other regions of the digestive tract raises the hypothesis that depending on the bacterial strain, some bacteria may traverse the larval gut without prolonged contact with enzymes and therefore resist digestion. Those bacteria that do have a prolonged contact with the larval gut enzymes are digested, partially or completely, and the fluorescent stain is released and attaches to the larval tissues. This hypothesis agrees with Lovett and Felder observations (1990a; 1990b; 1990c), where they describe the back and forth movement of microalgal cells inside the different regions of the foregut subjected to higher enzyme activity. Some cells never enter the anterior and lateral midgut caecae and continue directly from the foregut to the midgut trunk; other microalgal cells do enter these organs and, again with back and forth movements, may stay for short or long periods before being returned to the midgut trunk. In the present study, individual and clumps of fluorescent labelled bacteria could be seen dispersed in the anterior and lateral midgut caecae, and then, progressively, concentrated or "packed" and isolated inside the peritrophic membrane in the midgut trunk and hindgut region. Furthermore, faecal strands of larvae fed exclusively with FLB frequently included digested bacterial biomass that did not fluoresce, a pattern that was more evident

when larvae were fed FLB together with microalgae, since digested microalgae biomass was more abundant. Clearly, bacteria can be ingested inadvertently when the larva is feeding on microalgae. It was not possible to verify whether bacteria do indeed resist digestion and survive inside the gut.

The peritrophic membrane of arthropods, which is composed of chitin and protein, is thought to act as a barrier to mechanical abrasion, as well as microbial infection of the gut epithelium (Forster 1953; Mercer & Day 1959; Zimmerman *et al.* 1975; Brandt & Adang 1978). In the present study no bacteria were observed in the extraperitrophic space of the midgut trunk either free or attached to the gut lining. Indeed, the peritrophic membrane acts as a "barrier" to colonisation of the midgut trunk epithelium by bacteria ingested through the mouth. However, ingested bacteria could potentially establish in the foregut, the anterior and lateral midgut caecae and anterior part of the midgut trunk where the peritrophic membrane is produced. Due to the leaching of the fluorescent stain these areas were difficult to observe because of background noise, so that more specific stains are required. Colonisation of the midgut trunk may be only achieved by bacteria gaining access to the extra-peritrophic space through the observed antiperistaltic waves or "anal drinking".

Larvae fed exclusively with bacteria showed a significant decrease in FLB number inside the midgut trunk within 30 min after feeding, even when larvae were not fed microalgae which stimulates peristalsis and therefore the speed of food transition time (Lovett & Felder 1990c; Jones *et al.* 1997). Thus, it appears that the retention time of these bacteria in the gut is quite short, suggesting that this particular strain of bacteria would not readily colonize the mid and posterior regions of the midgut trunk of $Zoea_1$. In preliminary observations larvae that were subjected to either 2 or 48 h immersion in water with FLB and then transferred to clean, sterile seawater, showed predominance of fluorescence in the foregut and midgut caecae regions. These data, together with the observation that FLB are packed inside the peritrophic membrane soon after ingestion, would indicate that the bacteria are merely transient components in the $Zoea_1$ gut.

Released and distal ends of trailing faeces were observed to fluoresce more intensely than internal or proximal faecal material. This either indicates different periods and efficiencies in prior feeding, or suggests colonisation of exposed faeces by bacteria present in the water. External bacteria can attach and reproduce on the released faecal strands, Luminescent strains of *Vibrio harveyi* have been reported to proliferate in fish faecal material (Makemson & Hermosa 1999), and they have also been reported to be the most abundant bacteria in *Penaeus monodon* faeces (Lavilla-Pitogo *et al.* 1992). This process may be delayed by the peritrophic membrane which still covers egested faecal strands (Lautenschlager *et al.* 1978). If bacteria survive and are viable after the period inside the larval digestive tract, they may also grow and reproduce on the faecal strands. Sochard *et al.* (1979) found that the release of copepod gut bacteria via faecal pellets provides a mechanism for the wide distribution of these bacteria. In the highly intensive shrimp larval rearing systems, faecal strands will eventually be reduced to a small enough size to be re-ingested through degradation activity by endo- and epiphytic bacteria, and the highly turbulent flow of water due to strong aeration. With high stocking densities this process may represent an important additional mechanism for bacterial proliferation and dispersion in the system and provide increased chances of colonization of digestive tracts of other individuals.

Most studies on the bacterial flora of the digestive tracts of both shrimp larvae and postlarvae demonstrate that *Vibrio* and *Pseudomonas* are the dominant genera (Table 1.1). Dempsey *et al.* (1989), working with *Litopenaeus setiferus* and *L. aztecus* adults, found as many bacteria on the hindgut lining as its contents and suggest large numbers of bacteria adhere to the gut lining following epifluorescence stained bacteria observations. Dempsey and Kitting (1987) hypothesise that the gut flora of penaeid shrimps assists the digestion of starch and cellulose in algal detritus ingested by the shrimp, and that these bacteria resist various phenolic compounds leached from seagrass detritus that could interfere with bacterial metabolism. They found large numbers of quickly growing bacteria strains including the genera *Flavobacterium* Bergey *et al.* 1923, *Cytophaga* Winogradsky 1929, *Photobacterium* Beijerinck 1889, *Alcaligenes* Castellani and Chalmers 1919, *Pseudomonas, Caulobacter* Henrici and Johnson 1935, *Xanthamonas* Dowson 1939, *Altermonas* Baumann *et al.* 1972, *Chromobacterium* Bergonzini 1880, *Aeromonas* Stanier 1943 and *Vibrio*, and investigated their cellulose, lipase, amylase, gelatinase and chitinase enzyme activity in the digestive tract of *L. aztecus*. They conclude that the ability of the intestinal flora to withstand natural levels of phenolic compounds which may appear in the shrimp diet, as well as to degrade various components of the shrimp diet, may prove beneficial to the shrimp digestion process. Yasuda and Kitao (1980) studied the bacterial flora in the gut of *Marsupenaeus japonicus* larvae and found *Vibrio* to be the dominant genus in $Zoea_1$ stages and *Pseudomonas* in adults.

Tanasomwang and Ruangpan (1995) studied the aerobic bacterial flora of *Penaeus monodon* from the $Nauplius_5$ to the $Postlarva_2$ stages in relation to the microflora of rearing water and feed and also found *Vibrio* and *Pseudomonas* to be the dominant genera. Hameed (1993) studied the aerobic heterotrophic bacterial flora of eggs, larvae and postlarvae of *Fenneropenaeus indicus* and larval rearing tank water. Again, *Vibrio* was found to be the dominant genera followed by *Pseudomonas* in eggs, larvae and postlarvae. Suzuki et al (1990) found that *Vibrio* and *Pseudomonas* predominate both in external and internal bacterial flora of the crab *Portunus trituberculatus* larval culture, and bacterial counts increased as developmental stages progressed. *Vibrio cholera* has been observed to attach preferentially to the hindgut and not to midgut of crabs (Huq *et al.* 1986). Two Gram positive genera, *Clostridium* Prazmowski 1880 and *Peptostreptococcus* Kluyver and van Niel 1936, and two Gram negative genera of obligate anaerobic bacteria, *Bacteroides* Castellani and Chalmers 1919 and *Fusobacterium* Knorr 1922 were identified from the intestine of adult *L. vannamei* (Dixon *et al.* 2001). However, most isolates could not be identified beyond the genus level indicating that new, previously undescribed species may be present. Enzyme analyses of these anaerobic isolates were found to produce esterase, alpha-galactosidase, alpha-fucosidase, trypsin, lipase, and alpha- and beta-glucuronidase. However, these obligate anaerobic bacterial strains are not likely to be found in the digestive tract of first feeding shrimp larvae due to the short length of the midgut trunk, and the high rate of oral and anal drinking which continuously pumps water rich in oxygen into the digestive tract.

CONCLUSIONS:

1) Advanced shrimp Nauplius$_5$ possess a hindgut, an anal pore and exhibit antiperistaltic movements in a well developed midgut trunk (anal drinking) which may enable colonization of the digestive tract by bacteria present in the water column before the mouth is opened and the start of normal feeding activity.
2) Zoea$_1$ can filter bacteria present in the culture water, although they preferentially select and are more efficient at filtering microalgae.
3) When fed on microalgae, Zoea$_1$ ingest bacteria present in the culture water or attached to the microalgal cells.
4) Depending on the bacterial strain, some bacteria may travel through the larval gut without prolonged contact with enzymes and resist digestion remaining viable when egested in the faeces. Others have a prolonged contact with larval gut enzymes and are digested, partially or completely.
5) Bacteria ingested through the mouth are more likely to establish in the epithelia of the foregut, the anterior and lateral midgut caecae and anterior part of the midgut trunk than bacteria taken in through the anus.
6) Bacterial colonisation of the midgut trunk and hindgut may be preferentially achieved by bacteria gaining access to the extra-peritrophic space by antiperistaltic waves or "anal drinking".
7) Most bacteria may be flushed out before they reproduce inside the larval gut.
8) More specific fluorescent stains and staining techniques are required to observe bacterial presence in the anterior parts of the larval digestive tract, as well as the use of transmission electron microscopy to evaluate bacterial attachment to the larval gut epithelium.

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CHAPTER 5

Accumulation of ammonia and nitrite in intensive shrimp postlarvae (*Litopenaeus vannamei*) and crab juvenile (*Callinectes sapidus*) culture systems: zero exchange continuous recirculation versus batch water exchange

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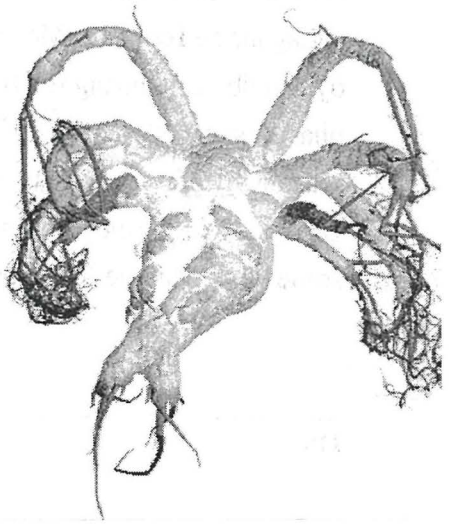

INTRODUCTION

Recirculation systems, not only diminish the volume of water discharged from aquaculture sites, avoiding release of potentially hazardous substances such as antibiotics, hormones, fertilizers and other feed inputs, but also help maintain constant important variables that affect the growth and development of cultured species, such as temperature, salinity, pH and dissolved oxygen (Huguenin & Colt 1992; Timmons & Losordo 1994; Reinersten & Haaland 1995). Recently, low or no-exchange production systems are considered to assist in preventing the spread of bacterial and especially virus diseases, increasing the biosecurity of farms (Pantoja *et al.* 1999; Lee *et al.* 1998). These systems also expand the area where marine and freshwater aquaculture can be accomplished, reducing the coastal zone impact of such activity (Lygren & Blanquet 1997; Davis *et al.* 2002).

The shrimp industry is increasingly under scrutiny from environmental protection agencies, largely because of natural habitat destruction to build ponds, but also for the enormous volumes of water returned to the natural environment, often without the adequate pre-treatment (Wickins & Lee 2002). Although economic use of recirculation for large scale grow-out ponds is still to be achieved, larval rearing and nursery raceway ponds, where early postlarvae are grown to juvenile sizes prior to pond stocking, require smaller volumes of water and therefore are strong candidate units where recirculation systems could be implemented (Mallasen & Valenti 1998; Davis & Arnold 1998). Maturation units, using considerable less volumes of water are other candidates for recirculation (Menasveta *et al.* 1991). Furthermore, the shrimp industry has yet to perfect the high assimilation efficient artificial feed products. Present feeds are known to leach proteins and other nutrients, which impact both the water and the immediate environment.

Although not as common and widespread as shrimp, the culture of mud crab (*Scylla* spp.) in South-East Asia and the mitten or river crab (*Eriocheir sinensis*) in China and Korea is now widespread (Wickins & Lee 2002). The majority of farmed crabs come from extensive ongrowing and fattening of wild-caught juveniles or polyculture operations (Castanos 1997; Overton & MacIntosh 1997; Wickins & Lee 2002), while some are raised in semi-intensive monoculture (Chen 1990; Trino *et al.* 1999; Wickins & Lee 2002; Williams *et al.* 1999). In recent years advances have been made in crab maturation (Millamena & Quinitio 2000), hatchery and nursery (Blackmon & Eggleston 2001; Brick 1974; Hamaski & Hatai 1993; Heasman & Fielder 1983) and feed technology (Millamena & Quinitio 2000; Millikin *et al.* 1980) while prospects for restocking natural populations are being actively investigated in Japan (*Portunus trituberculatus, P. pelagicus* and *Scylla* spp.)(Imamura 1999), Indonesia and Vietnam (*Scylla* spp.; Dr. Le Vay pers. com.). Some attempts have been also made to culture *Callinectes sapidus* juveniles for re-stocking over-fished populations in Laguna de Terminos, Mexico (Biol. Mauricio Orellana, pers. com.). Despite considerable mortality caused by cannibalism during the first stages, other questions remain to be solved in the hatchery and nursery phases, such as poor water quality due to the natural food added (frequently, minced fish muscle) and seasonality of water quality in tropical coastal areas (drastic differences in salinity and temperature). In the USA and Mexico most soft-shell crab farms rely on animals from the wild and utilize recirculating systems of some kind (Malone & Burden 1988; Manthe *et al.* 1983; Webster 1998).

For an efficient implementation of closed water recirculation systems for commercial scale crustacean culture, new water management protocols that permit productions similar to those of the traditional protocols, are required. According to Wickins and Lee (2002), for all aquaculture operations, but specially those where the water is treated, it is desirable to know the maximum and minimum acceptable levels of changes that occur in water chemistry so that effort is saved in achieving unnecessary goals. Unfortunately there is a shortage of data based on long-term growth studies with crustaceans and much reliance is placed on values extrapolated from short-term acute tests and from studies with fish (Wickins & Lee 2002). Unlike the crustaceans used in traditional laboratory tolerance tests, farmed crustaceans are exposed to mixtures of metabolites, minerals or toxins in the water that may act synergistically or antagonistically. For example, toxicity of ammonia is exacerbated by high pH (Armstrong *et al.* 1978), whilst nitrite effects can be reduced by the presence of chloride ions (Meade & Watts 1995). Although recirculation technology is capable of processing the excess nutrients released into the water from decaying feed, faeces and excretion, it cannot do so instantly and animals have to acclimate to higher-than-normal ammonia, and nitrite concentrations before biofilter units start operating at full capacity.

The present work compares the patterns of ammonia, nitrite, nitrate and total proteins accumulation using either traditional batch water exchange or continuous closed recirculation. Each water management protocol was replicated over four daily percentage water exchanges (0, 30, 60 and 90%). The patterns of nutrient accumulation were correlated to shrimp and crab postlarvae growth and survival.

MATERIAL AND METHODS

Animals

First instar *Callinectes sapidus* crabs (0.93 ± 0.16 mm carapace width and 10.43 ± 4.51 mg wet weight) were obtained from a local hatchery, Campeche, Mexico. These crab postlarvae resulted from a single spawn of a mature gravid female from the wild. Larvae were fed a mixture of microalgae, rotifers, *Artemia* and minced fish muscle. *Litopenaeus vannamei* postlarva5 (1.07 ± 0.48 mg wet weight) from a single female spawn and grown following standard shrimp larviculture procedures were obtained from Industrias Pecis, Yucatan, Mexico. Stocking densities of 180 and 360 animals m^{-2} (25 and 50 individuals per tank) for crabs and shrimp, respectively were used.

Experimental culture system

The system consisted of 21 experimental plastic tanks (41L×34W×30H cm) containing approximately 20 L and constant aeration (**Fig.** 5.1). For crabs, plastic refuges made of PVC tubing and patches of nylon mesh

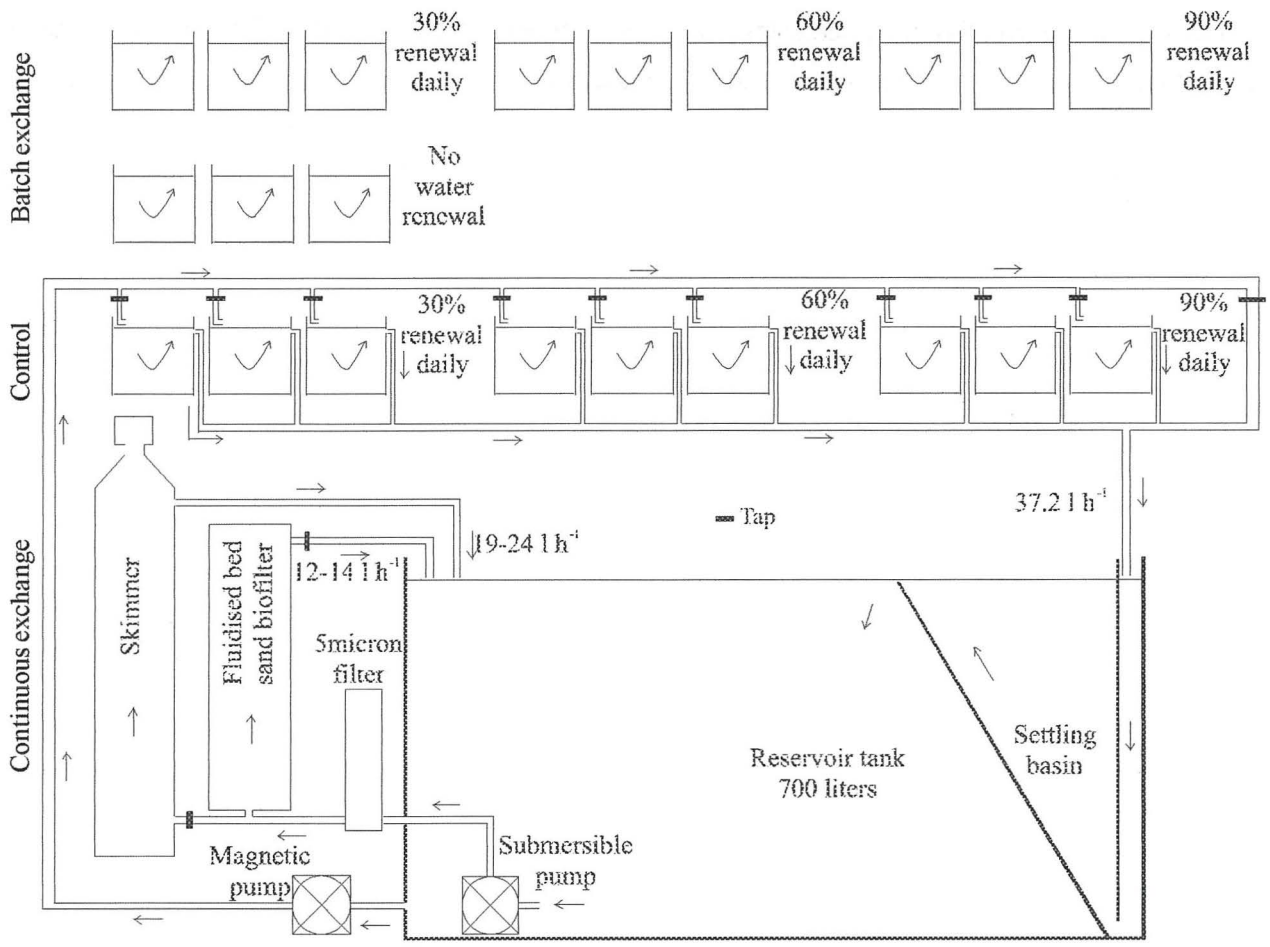

Figure 5.1 – Diagram of the experimental system used. Drawings are approximate, but not to scale

were added to each tank in similar numbers and areas. In order to calculate the rates required to achieve 30, 60 and 90% water volume exchange of each tank per day, an exponential decay equation was used:

$$f = V_0 \times \left(1 - e^{Ln\left(\frac{1-x}{t}\right)} \right) \quad (5.1),$$

where, f is flow (ml min^{-1}), Where V_0 is the total available water volume (L) in each tank, x is the percentage water exchange expressed as a proportion and t is time (min). Please see Appendix 1 for derivation of the equation. Individual tank flows were re-calibrated twice every day and allowed to vary within ± 5% of the average rate of 44.9 ± 2.4 ml min^{-1}. In experimental conditions, flow rates reproduced the calculated values with small deviations. The average flow rates for 30, 60 and 90% exchange per day treatments were 4.5±0.7, 13.2±0.7, 36.8±2.9, and 5.0±0.8, 12.7±1.1, 35.9±4.0 ml min^{-1} for crabs and shrimps, respectively (**Fig.** 5.1, 5.3 A_{1-2}). The two trials were completed in sequence, first with shrimp and then with crabs. One third of the water from the first trial was used in the second trial, and was kept recirculating without animals during the 55 days between the two trials. The system was neither conditioned or innoculated prior to any trial.

Diet

Both species were fed with an artificial diet (53% protein content – **Table** 5.1) given at 120-60% of the total biomass of each tank, 2-3 times a day. Pellets were presented with sizes between 100 and 250 mm for the first 10 days, and thereafter between 340-500 and 340-800 for crabs and shrimps, respectively.

Table 5.1 – Percentage inclusion of different ingredients in the experimental artificial diet used in trials with *Litopenaeus vannamei* and *Callinectes sapidus*. CP indicates crude protein, CL indicates crude lipid and cbh indicates carbohydrates; all expressed as % what was fed.

Ingredients	% as fed	CP	CL	CBH
Anchovy flour	31	21	3	
Soy flour	20	12		
Shrimp flour	13	6		
Squid flour	14.5	10		
Soy Lecithin	1.5		1.5	
Fish liver oil	1.5		1.5	
Cholesterol	0.5		0.5	
Wheat starch	8.5			9
Mixture of vitamins	1.7			
Mixture of minerals	0.8			
Rovimix Stay-C	2			
Carboximethyl Cellulose (CMC)	5			
Totals		**49**	**7**	**9**
Total digestible energy (kJ g^{-1})	15.2			

Experimental design

Two methods of water exchange were used: **Batch** - Water was siphoned out of the tank with a 1cm diameter pipe with a nylon mesh covering the end. Remaining food and faeces were siphoned with gentle movements to avoid stressing the animals. New water was sand filtered, sedimented for 2 days, 1µm cartridge filtered and salinity adjusted with drinking water filtered through a carbon filter. This new water was added gently with a graduated plastic container to the sides of the tanks. **Continuous** - Water from a recirculation system was added continuously to the tanks through plastic taps calibrated 3 times a day and drained back to a 700 L capacity reservoir where the water was constantly recirculated through a parallel system that included a 5µm cartridge filter, a fluidised sand bed biofilter and a protein skimmer (**Fig.** 5.1). The total volume of the system including tanks added to 1300 L. Water renewal rates of 30, 60 and 90% per day were used for both methods of exchange with a common control of no exchange, and therefore, 0% water renewal. Three replicates were used per treatment (**Table** 5.2). Only part of the faeces and uneaten food was siphoned out of the system through a sieve. Siphoned water was returned to the system.

Table 5.2 – Experimental design used in both *Litopenaeus vannamei* and *Callinectes sapidus* trials. Numbers between brackets represent number of replicates.

	Method of water exchange			**totals**
	Batch	**Nil**	**Contin.**	
Water renewal rate	0% (3)			3
	30% (3)		30% (3)	6
	60% (3)		60% (3)	6
	90% (3)		90% (3)	6
totals	9	3	9	21

Table 5.3 – List of comparisons made using Schefe's contrast analysis (with hanging control group) on relative mortality rate (number of dead animals · day $^{-1}$), relative growth in weight (mg · day $^{-1}$) and length (mm · day $^{-1}$), final weight (mg) and final length (mm) of juvenile *Callinectes sapidus* and *Litopenaeus vannamei* postlarvae. CONT – continuous recirculation water exchange; STAT – batch or batch water exchange; CONTROL –treatment that served as a control condition for both factors.

Between exchange methods	CONT (pooled) vs BATCH (pooled)
Exchange methods vs no exchange	CONT (pooled) + BATCH (pooled) vs CONTROL
	CONT (pooled) vs CONTROL
	STAT (pooled) vs CONTROL

Water quality variables

Total ammonia was measured using a phenol-nitroprusside reaction, and nitrite using a sulfanilamide reaction following Parsons *et al.* (1984) techniques adapted to small microplate volumes. Samples were collected every three days during the morning (9-11 A.M.) and immediately analyzed. Non ionized ammonia fraction was calculated following the average of four slightly different determination models (Whitfield 1974; Khoo *et al.* 1977; Johansson & Wedborg 1980; Spotte & Adams 1983) using values of pH, temperature and salinity for each tank on the day and time of total ammonia determination. The pH electrode was calibrated weekly. Proteins were measured with a similar periodicity using a Pierce BCA total protein assay kit based on the binchinchinoic acid reaction. Nitrates were analysed every 10 days or longer using the standardized Hach kit techniques. Ortophosphates were also analysed using a Hach kit technique at the beginning and end of the experiment. Due to logistic problems it was not possible to follow phosphate concentration in the shrimp trial. Oxygen was measured with a YSI electrode, calibrated daily.

Biological variables

Change in weight (g) was evaluated by sampling three random animals from each tank every 10 days to re-calculate each tank biomass. Initial weight was estimated from 50 shrimp postlarvae and 30 first instar crabs. At the end of the experiment all survivors were counted, measured and weighed. The relative mean mortality rate was calculated according to equation (2.1). Assuming that growth of shrimp postlarvae follows an exponential increase, the average relative growth rate (weight gained per unit of time and per unit of weight) was calculated with equation:

$$\text{Relative Growth Rate G} = \frac{Ln(W_{t_1}) - Ln(W_{t_0})}{t_1 - t_0} \qquad (5.2)$$

Where G is the average relative growth rate between time t_0 (initial) and t_1 (final), and W_t is the weight of the population in each replicate at a given time t.

Statistical analysis

Shapiro-Wilk's normality and Bartlett's or Levene's homogeneity of variance tests were used prior to analysis of variance. Scheffe's contrast analysis with hanging control group was used to detect differences due to water exchange methods and percentage of water exchange on the following response variables: relative mortality rate (intrinsic or specific mortality - number of dead animals · day^{-1}), relative growth in weight (intrinsic or specific growth - mg · day^{-1}) and final weight (mg) of both crabs and shrimp postlarvae (**Table** 5.3).

RESULTS

Physico-chemical variables

Conditions were kept nearly constant for each treatment throughout the experiments with both species

Temperature:

Temperature in the reservoir was significantly higher than in the experimental tanks ($p<0.001$, crabs; $p<0.001$, shrimp) due to the submersible pump (**Fig.** 5.2 A_{1-2}), however this did not affect the tanks where the temperature was kept reasonably constant and considerably lower throughout the experimental period.

Salinity:

Salinity decreased proportionally to the increase in rate of exchange, although the absolute values were quite different with lower salinities used to grow crab juveniles. The higher average values throughout the experimental periods were observed in the reservoir water, and continuous exchange treatments, but reached a maximum in the 0% exchange treatment in both experiments (**Fig.** 5.2 $B_{1,2}$). Observed differences to the batch water exchange treatments are highly significant ($p<0.001$, crabs; $p<0.001$, shrimp)

pH:

The higher values of pH in the water in the reservoir and 90% exchange continuous tanks for *Callinectes sapidus* experiment were significantly above all other treatments, but not different between them ($p<0.001$) (**Figure** 2 – C_1, C_2). Values for pH were higher and more variable in the shrimp postlarvae trial. In this trial, both 30% exchange treatments (batch and continuous exchange) showed unexpectedly lower values than all other treatments, although not significantly different ($p=0.867$).

Dissolved oxygen:

Oxygen levels kept fairly constant throughout the experimental period with a non-significant tendency for higher concentration in the reservoir water ($p=0.761$, crabs; $p=0.233$, shrimp) (**Fig.** 5.2 D_{1-2}).

Flow rate:

In both experiments, lower continuous exchange flow rates (30 and 60%), were maintained linearly constant and within the desired values (**Fig.** 5.3 A_{1-2}), whilst for 90% continuous exchange condition, variation increased enormously due to the difficulty of maintaining such an exact high flow with the taps used. Under conditions of 60% continuous exchange for shrimp and 90% continuous exchange for crabs, significant differences between the average flows of the replicate tanks occurred ($p = 0.001$ and $p < 0.001$, respectively), although these differences were of 1.0 and 0.6 ml min^{-1} and are not considered in further analysis. Corresponding average flow rates were very similar but significantly different between experiments ($T_{30\%} = -7.84$, $p = 0.000$, $DF = 322$; $T_{60\%} = 5.22$, $p = 0.000$, $DF = 249$; $T_{90\%} = 2.75$, $p = 0.006$, $DF = 282$).

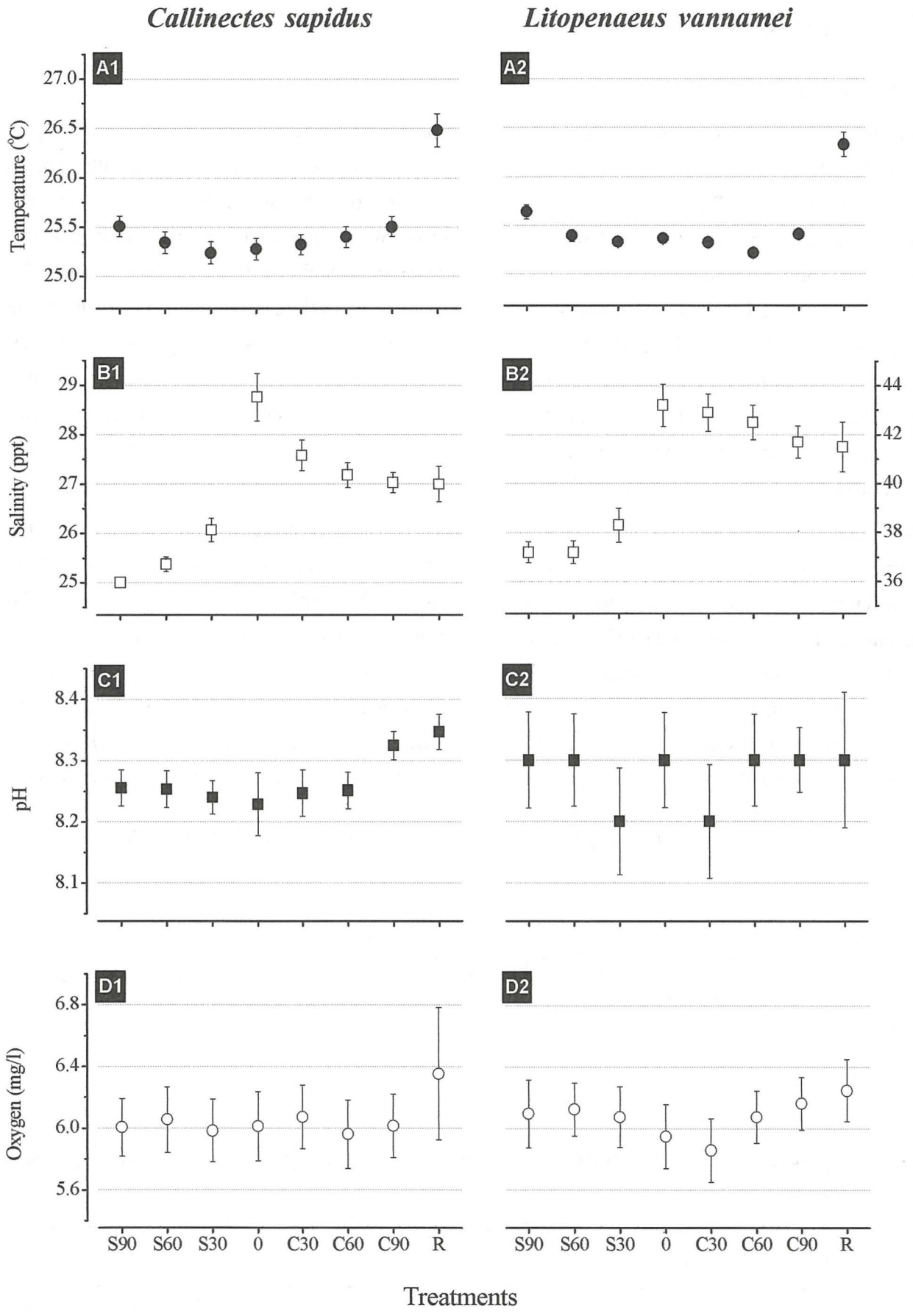

Figure 5.2 – Mean temperature, salinity, pH and dissolved oxygen (± SE) during experiments with *Callinectes sapidus* (42 days) and *Litopenaeus vannamei* (30 days) in static or batch water exchange (S) and continuous recirculation (C), and 3 daily exchange rates (30, 60 and 90%). "E0" indicates the control with no water exchange and "R" for reservoir.

Water quality chemical variables

Callinectes sapidus

For the crab trial, the pattern of un-ionised ammonia accumulation throughout the experimental period closely followed the pattern shown by total ammonia concentration, due to a nearly constant average pH (**Fig.** 5.4, A_{1-2}, B_{1-2}). The highest un-ionised ammonia concentration was observed on day 15 in the 0% water exchange control treatment. During the first 15 days of the experiment, the gradual increase in total ammonia concentration was directly related to the percentage of exchange (**Fig.** 5.4, B_{1-2}). At the 18th day, within a period of 3 days, the ammonia concentration decreased to values similar to the initial ones for both treatments (batch and continuous). Maximum concentration (3.52 mg L^{-1}) was reached with 0% exchange on day 15. Continuous exchange gave generally lower concentrations of ammonia which peaked and decreased earlier than for batch exchange (**Fig.** 5.4, B_{1-2}). Since the ammonia concentration in the seawater used in the batch water exchange was very low (always below 0.1 mg L^{-1}), there was an accumulation of this chemical despite water exchange, at this particular stocking density and feeding regime.

The sudden decrease in ammonia concentration coincides in all treatments with a sharp increase in nitrite concentration (**Fig.** 5.4, B_{1-2} C_{1-2}) between days 10 and 12. Around 25 days after the start of this trial, nitrite concentrations reached their maximum levels with considerable variation (4.4 mg L^{-1}). Despite variability, accumulation of nitrite in the batch water exchange treatments was sometimes double the concentration reached in the continuous exchange tanks. From day 16 onwards, the animals in the batch water exchange tanks were permanently exposed to nitrite concentrations higher than 1mg L^{-1}. The direct relationship between percentage exchange and the ammonia concentration was not as clear for nitrite, due to the high associated variability. Nevertheless, with continuous exchange, the 90% treatment has a lower concentra-

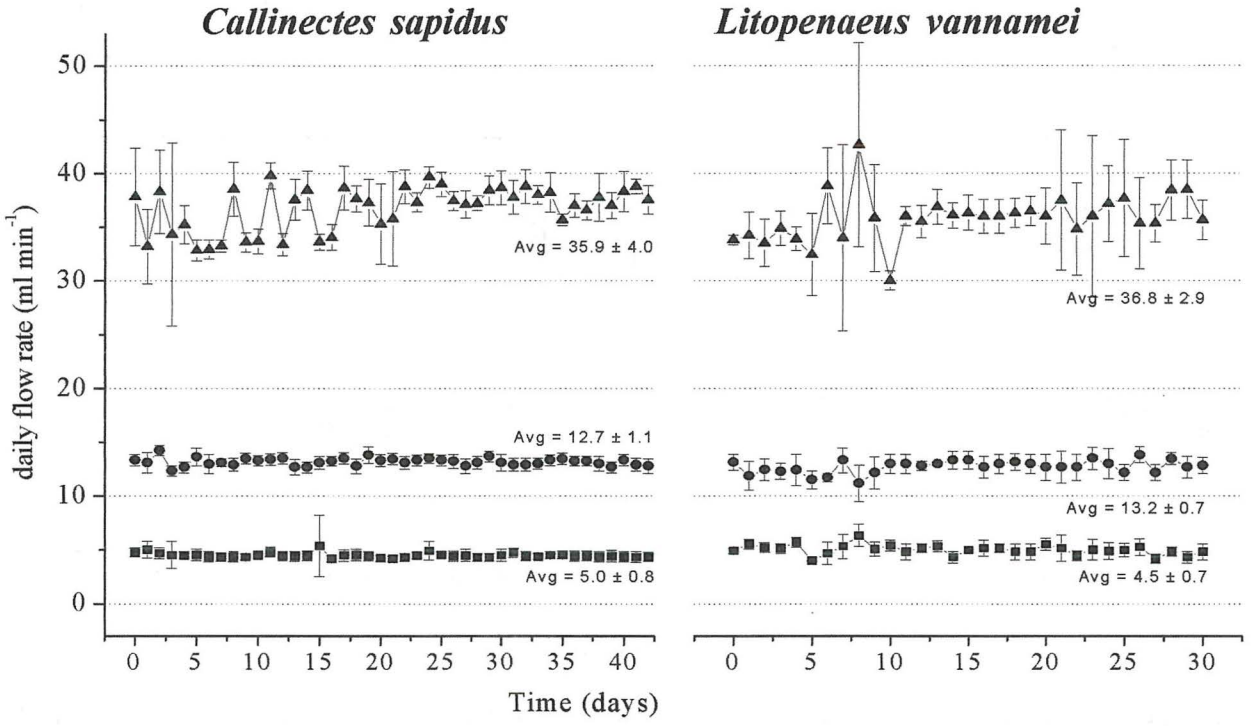

Figure 5.3 – Daily average flow rate (± SE) of tanks with three different percentage of water renewal: ■ 30%; ● 60%; ▲ 90%.

tion of nitrite than 0, 30 and 60% treatments. Again, the seawater used for the batch water exchange and the reservoir water had very low concentrations of nitrite throughout the experiment.

Nitrate concentration in the water rose sharply towards the end of the experiment (**Fig.** 5.4, D_{1-2}). The 0% exchange treatment registered the highest concentration (70 mg L^{-1}), whilst, in the reservoir, nitrates were lowest. For batch water exchange treatments, an inverse relation was observed between the percentage of exchange and nitrate concentration. In continuous water exchange all treatments had values around 28 mg L^{-1}. Reservoir water showed comparatively low concentrations (8 mg L^{-1}).

Initial total orthophosphate concentration in the crab trial was considerably high, and a maximum of 89 mg L^{-1} was attained in the reservoir (**Fig.** 5.6). However, tanks from the recirculation system showed significantly lower concentrations,. Batch water exchange tanks gave lower concentrations of this ion when compared with continuous recirculation, but were nevertheless still high, at around 14 mg L^{-1}. The 0% exchange control treatment gave higher concentrations of orthophosphate than all other tanks (**Fig.** 5.6).

Total protein concentration was variable at the beginning of the experiment, after which a sudden decrease and stability were observed (**Fig.** 5.4, E_{1-2}). A trend can be observed where the higher the percentage of exchange treatments, the lower the protein level (**Fig.** 5.4, E_{1-2}). Extremely high protein concentrations were found in the drain water/foam from the protein skimmer (maximum of 344 mg L^{-1}).

Litopenaeus vannamei

The pattern of un-ionised and total ammonia concentration throughout the experimental period are nearly identical, except from day 21 onwards, when a sudden drop in pH reduced the fraction of un-ionised ammonia in the 30 and 60% batch and continuous exchange treatments (**Fig.** 5.5, A_{1-2}) whilst total ammonia values, remained high (**Fig.** 5.5, B_{1-2}). Total ammonia and nitrite concentrations were generally higher in the continuous recirculation treatments as opposed to batch water exchange. Increase in the concentration of these substances was proportional to the rate of exchange (**Fig.** 5.5, B_{1-2}, C_{1-2}). Concentration of ammonia and nitrite in the continuous recirculation system reservoir were very low. Nevertheless, there was an accumulation of ammonia and nitrite in the continuous recirculation treatments, especially in the tanks with lower exchange rates (**Fig.** 5.5, B_{1-2}, C_{1-2}).

Ammonia concentration rose to peak around day 20, with a later decrease more accentuated in batch exchange treatments (**Fig.** 5.5, B_{1-2}). The 0% water exchange control treatment showed the highest ammonia concentration peaking earlier than all other treatments. At day 30, the treatment's ammonia concentration ranking distribution is exactly inverse to that of the first 17 days, having the lowest ammonia concentrations. On a broader scale, it seems that after 17 days, ammonia concentration in tanks using batch water exchange stabilizes and oscillates between 0.2 and 1.5 mg L^{-1}. This situation was not observed in continuous recirculation treatments, where, the 90% water exchange did not rise continuously but, instead, maintained ammonia concentrations below 0.8 mg L^{-1}. The 30 and 60% continuous exchange treatment tanks, on the other hand, accumulated much higher ammonia concentrations, than the parallel batch water exchange treatments (**Fig.** 5.5, B_{1-2}).

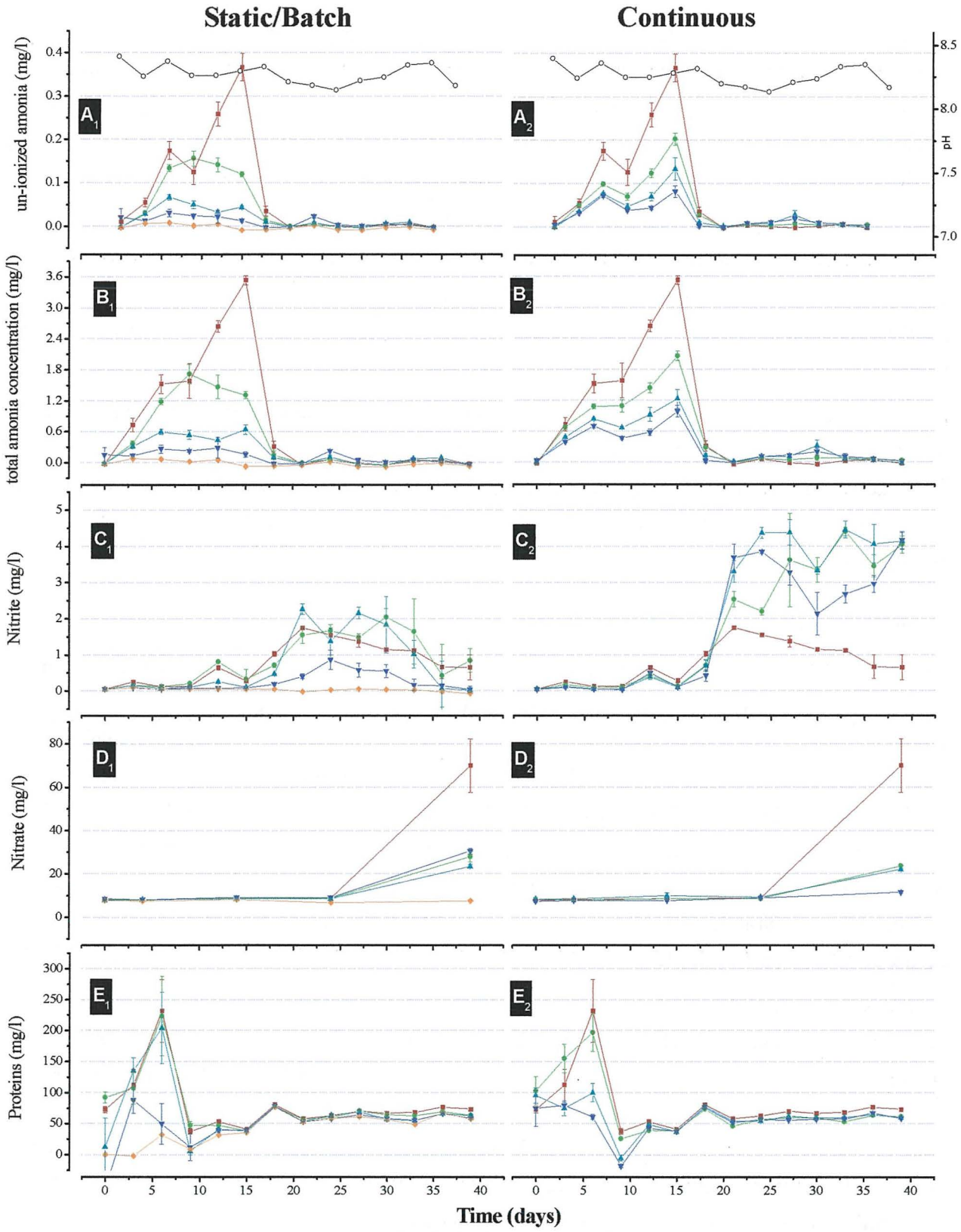

Figure 5.4 – Changes in the mean concentration of un-ionised ammonia, total ammonia, nitrite, nitrates and proteins (mg L^{-1} ± SE) in tanks with juvenile *Callinectes sapidus* throughout the experimental period in static or batch water ex-change (S) and continuous recirculation (C) with 3 daily exchange rates (30, 60 and 90%). "E0" indicates the control with no water exchange. Values are means of 3 replicate tanks (mg L^{-1} ± SE). For graphs A_1 and A_2, the top line with open circles corresponds to the right axis which denotes progress in overall (all experimental tanks) pH variation. ■ 0%; ● 30%; ▲ 60%; ▼ 90%; ◆ reservoir.

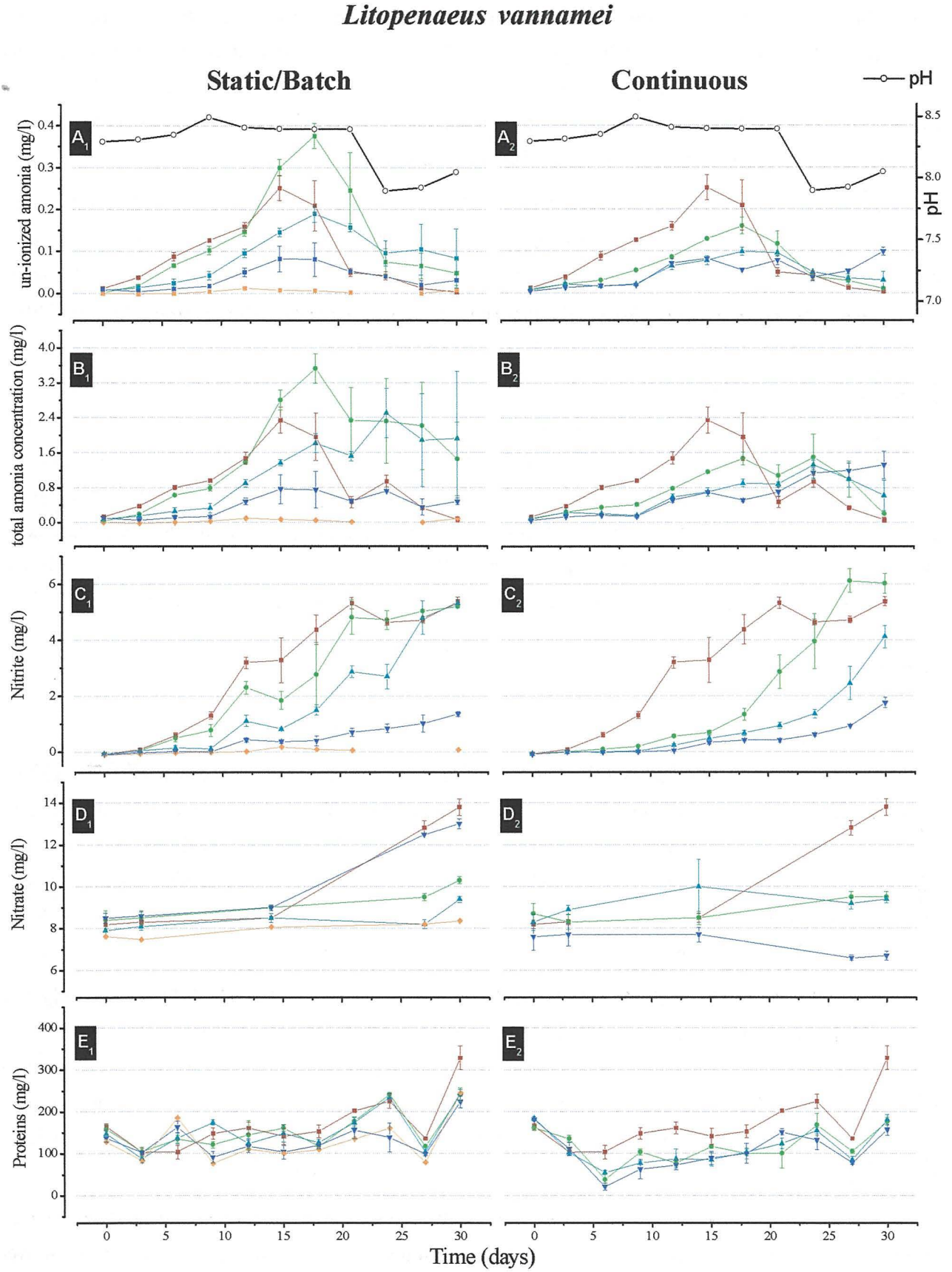

Figure 5.5 – Changes in the mean concentration of un-ionised ammonia, total ammonia, nitrite, nitrates and proteins (mg L^{-1} ± SE) in tanks with juvenile *Litopenaeus vannamei* throughout the experimental period in static or batch water exchange (S) and continuous recirculation (C) with 3 daily exchange rates (30, 60 and 90%). "E0" indicates the control with no water exchange. Values are means of 3 replicate tanks. For graphs A_1 and A_2, the top line with open circles corresponds to the right axis which denotes progress in overall (all experimental tanks) pH variation. ■ 0%; ● 30%; ▲ 60%; ▼ 90%; ◆ reservoir.

In both batch and continuous water exchange, nitrite concentration increased with the rate of exchange, reaching similar levels at end of the 30 days (**Fig.** 5.5, C_{1-2}). However, the rise of nitrite concentration was delayed in the batch water exchange treatments, showing a "power function" type increase, whilst for continuous recirculation the increase was approximately linear from the first days. The control (no water exchange) was again the first treatment to accumulate nitrite following an early increase in ammonia levels.

Nitrate concentration present on the seawater used to fill the system was high from the first day of the experiment and later fluctuated between 7 and 10 mg L^{-1} in all treatments, except for the 90% continuous recirculation and the no exchange control (**Fig.** 5.5, D_{1-2}). The increment in nitrate concentration in the experimental tanks occurred after day 14, at a time when the treatments with highest concentrations of nitrite were stabilising their nitrite accumulation (**Fig.** 5.5, C_{1-2}, D_{1-2}).

Total protein concentration showed a decrease during the first days, especially in the batch water exchange tanks (**Fig.** 5.5, E_{1-2}). Later, it started to accumulate linearly, especially in the batch water exchange treatments (**Fig.** 5.5, E_2). There was a sharp decrease on day 27, followed by another increase. In the continuous recirculation tanks, protein concentrations were not much different from the 0% water exchange control and slightly higher than batch water exchange. Total protein concentration in the drain water from the protein skimmer accumulated gradually to reach just under 1400 mg L^{-1} after 30 days. This dataset was not plotted to conserve scale comparisons between batch and continuous water exchange treatments.

Biological variables

Callinectes sapidus

Survival was high during the first 15 days (**Fig.** 5.8 A_{1-2}), followed by a gradual reduction to 45-60%, with no significant differences between batch and continuous water exchange (**Table** 5.4). The control group showed a steeper decline in survival during the period between days 15 and 35, although it did not differ from other treatments at the end (**Table** 5.4 – **Fig.** 5.7 A). Variability in survival increased towards the end of the experiment. Rate of death or relative mortality, was not different between batch and continuous water exchange pooled (**Table** 5.4 – **Fig.** 5.8 A) and there were no differences to the control treatment (**Table** 5.4).

After 42 days, juvenile crabs reached a carapace width between 11–14 mm and weighed 100-200 mg (**Fig.** 5.8, B_{1-2}; C_{1-2}), corresponding to an overall average absolute growth rate of 0.27 mm and 3.70 mg per day. There was a significant effect of batch and continuous water exchange treatments on the final carapace width and final wet weight (**Table** 5.4). Both final carapace width and final wet weight of animals under batch and continuous exchange conditions were significantly different from the control animals (0% exchange) either separately or pooled together (**Table** 5.4).

In terms of rate of growth, both in weight and carapace width, there was a trend of proportionality to percentage of water exchange (**Fig.** 5.7, B, C). Animals exposed to zero water exchange (control) grew significantly slower than animals from both continuous and batch water exchange treatments (whether data are taken separately or pooled together – **Table** 5.4). Overall, animals from static or batch water exchange treatments grew faster than animals submitted to continuous water exchange (**Table** 5.4).

Table 5.4 – Probability values of Scheffe's contrast analysis of variance (with hanging control group) on differences due to water exchange methods and percentage of water exchanged on % survival, relative mortality rate (number of dead animals · day^{-1} · animals alive^{-1}), relative growth in weight (mg · day^{-1} · mg^{-1}) and length (mm · day^{-1}), final weight (mg) and carapace width/length (mm) juvenile *Callinectes sapidus* and *Litopenaeus vannamei* postlarvae. ("Cont." = continuous)

	Callinectes sapidus				*Litopenaeus vannamei*			
	Cont. vs Static/Batch	**Cont. + Static/Batch vs Control**	**Cont. vs Control**	**Static/Batch vs Control**	**Cont. vs Static/Batch**	**Cont. + Static/Batch vs Control**	**Cont. vs Control**	**Static/Batch vs Control**
% survival	0.373	0.344	0.235	0.564	0.007**	0.056	0.414	0.009**
Relative mortality rate	0.350	0.425	0.286	0.676	0.006**	0.058	0.440	0.009**
Final carapace width/length (mm)	0.019*	0.000**	0.002**	0.000**	0.938	0.097	0.113	0.124
Relative growth rate (mm day^{-1})	0.018*	0.000**	0.002**	0.000**	0.993	0.116	0.140	0.139
Final wet weight (mg)	0.028*	0.002**	0.016*	0.001**	0.833	0.014*	0.017*	0.023*
Relative growth rate (mg day^{-1})	0.015*	0.000**	0.005**	0.000**	0.836	0.010**	0.012*	0.016*

* and ** denotes significant differences at ± = 0.05 and 0.001, respectively

Litopenaeus vannamei

Survival was significantly higher for animals held in continuous exchange conditions, than for those held in static or batch exchange conditions (**Fig.** 5.9 A_{1-2}, **Table** 5.4). Lowest survival occurred in the 90% batch water exchange (76.7%), whilst, the highest was observed in the 0% exchange (99.3%). Overall survival in static or batch water exchange treatments was significantly lower than in the control (**Table** 5.4). In terms of rate of death or relative mortality, animals submitted to static or batch water exchange died significantly faster than those from continuous exchange and control treatments (**Table** 5.4 – **Fig.** 5.7 D).

After 30 days, shrimp postlarvae reached a carapace length between 2.52±0.05 – 2.95±0.04 mm and weighed 143.1±5.8 – 206.4±7.9 mg, having started with 0.7 ± 0.02 mm and 1.07 ± 0.07 mg, respectively (**Fig.** 5.9, B_{1-2}; C_{1-2}). This corresponds to an overall average absolute growth rate of 0.07 mm and 6.00 mg per day. The pattern of weight gain throughout the experimental period was similar for both continuous and batch 30 and 60% exchange. Slowest weight gain was observed in the 90% batch exchange, whilst the fastest occurred in the 90% continuous recirculation (**Fig.** 5.9 B_{1-2}). There was no significant effect of batch and continuous water exchange treatments on the final carapace length of shrimp postlarvae, neither was the final length of animals from these treatments different to that of the control animals (**Table** 5.4). The final wet weight of animals submitted to batch and continuous exchange conditions was not significantly different in either treatments, but was significantly different from control animals (0% exchange), either separately, or pooled together (**Table** 5.4). Although not as evident in crabs, a trend of proportionality between relative growth rate (both in length and weight) and percentage water exchange is observed (**Fig.** 5.7, E F). However, no significant differences in relative rate of growth in length could be found (**Table** 5.4; **Fig.** 5.7, F). Overall, animals from static or batch water exchange treatments gain weight at the same rate as animals submitted to continuous water exchange, but animals submitted to zero water exchange (control) gain weight at a significantly lower rate compared to animals from both continuous and static or batch water exchange treatments (separated and combined – **Table** 5.4; **Fig.** 5.7 E).

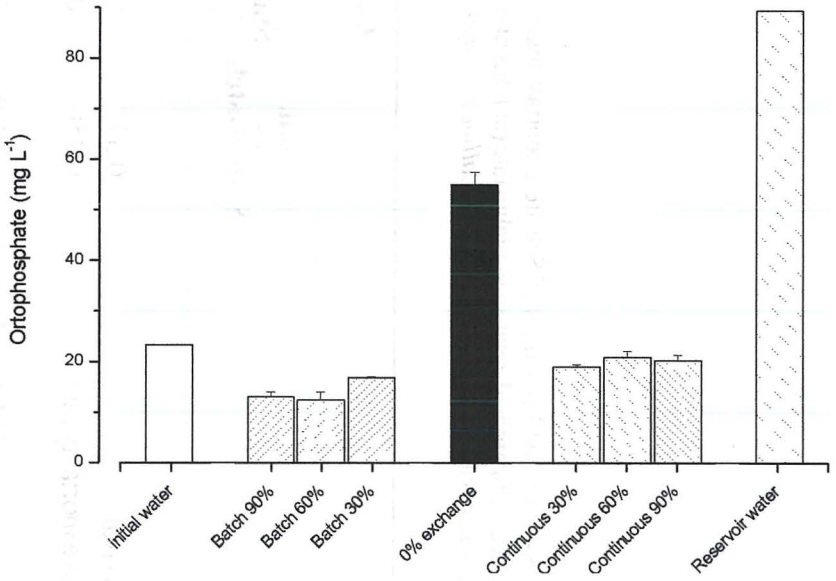

Figure 5.6 – *Callinectes sapidus* postlarvae experiment - Concentration of orthophosphate ions (mg L^{-1} ± SE) present in the initial water and at the end of the experimental period in static or batch water exchange and continuous recirculation with 3 daily exchange rates (30, 60 and 90%). "0% exchange" indicates the control with no water exchange. Values are means of 3 replicate tanks.

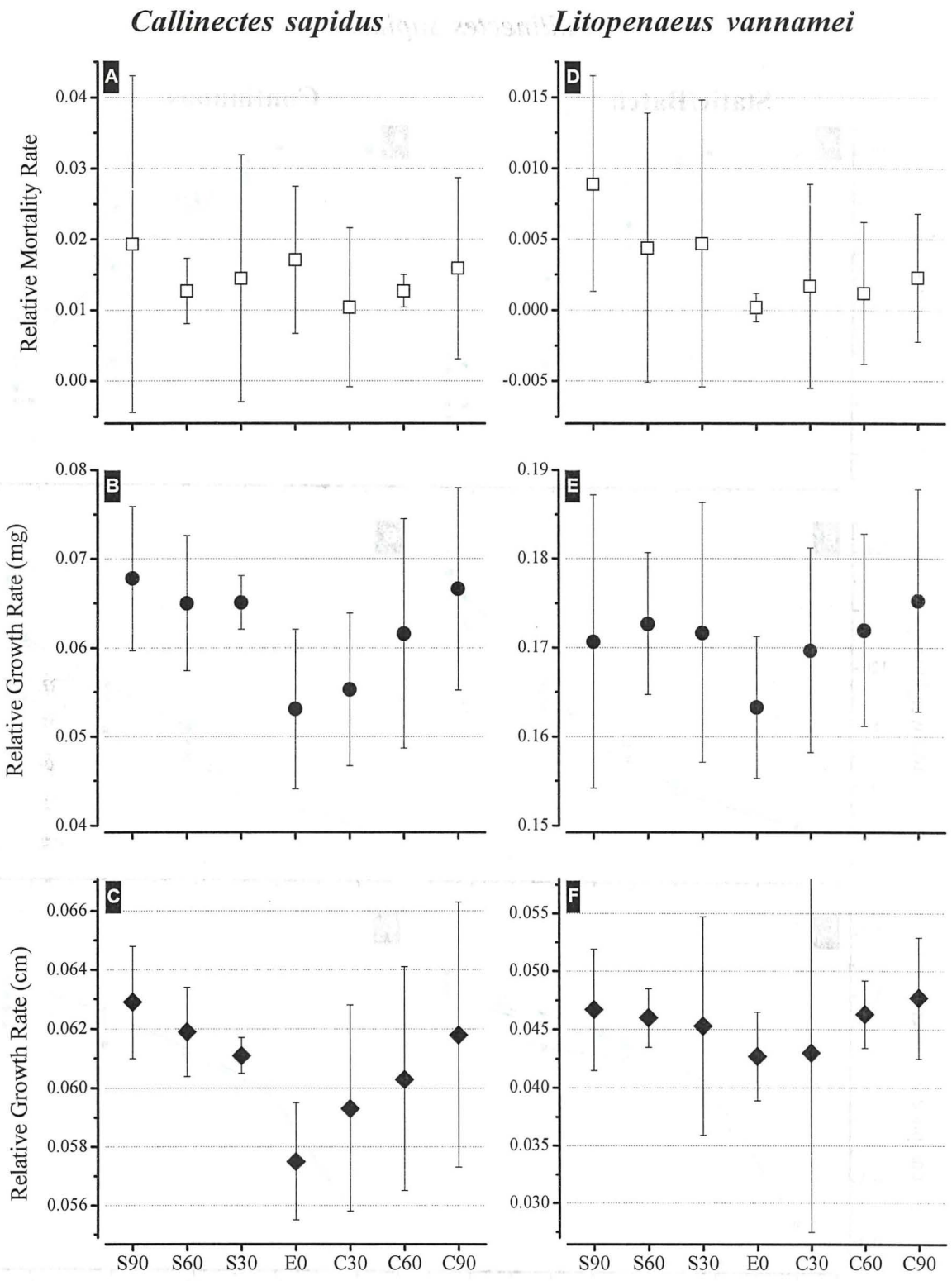

Figure 5.7 – Mean relative rates of mortality (number of dead animals · day $^{-1}$ · animals alive^{-1}) and growth in weight and length (mg · day $^{-1}$ · mg^{-1}; cm · day $^{-1}$ · cm^{-1}, ± SE respectively) of *Callinectes sapidus* (42 days) and *Litopenaeus vannamei* (30 days) in static or batch water ex-change (S) and continuous recirculation (C) with 3 daily exchange rates (30, 60 and 90%). "E0" indicates the control with no water exchange.

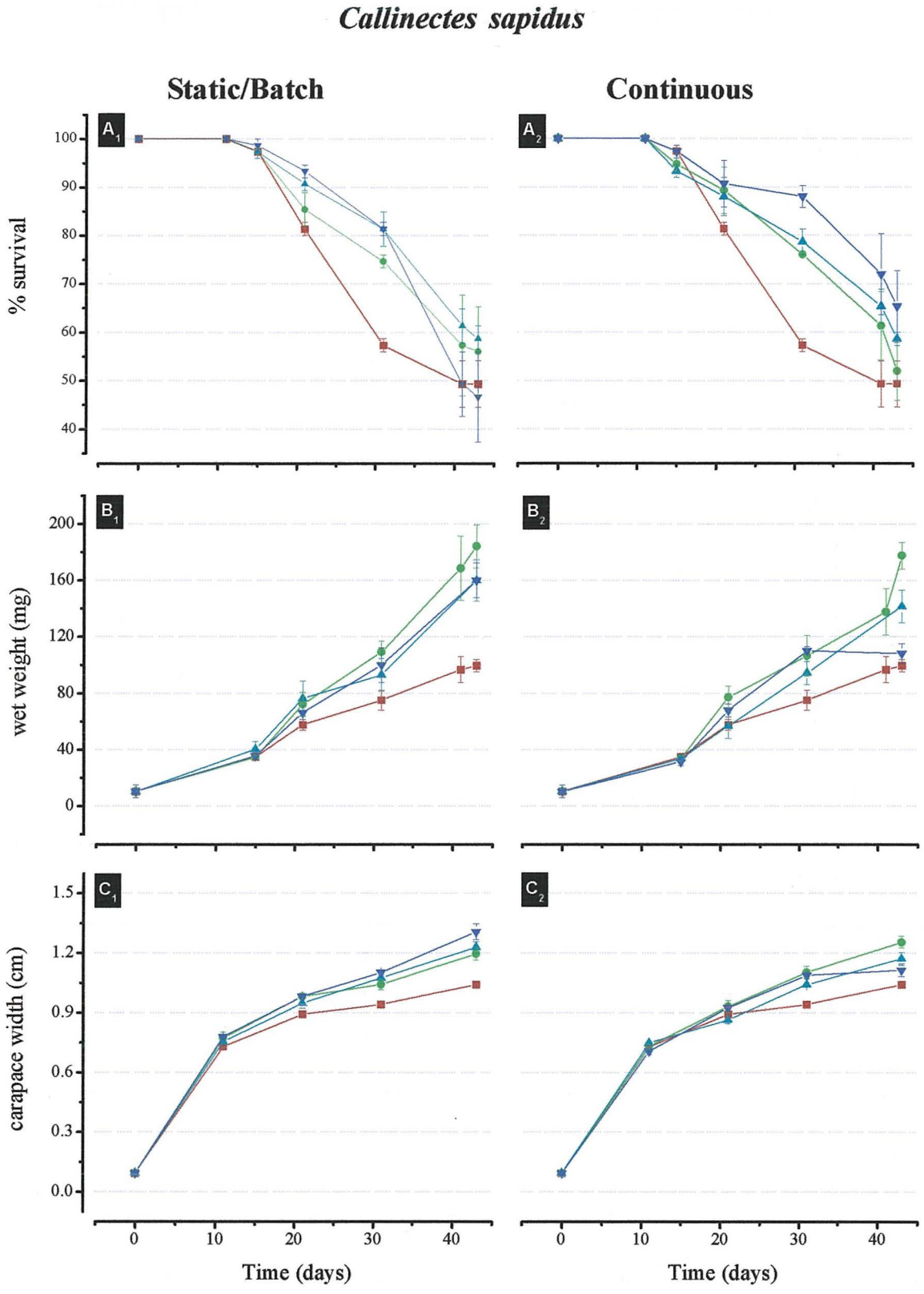

Figure 5.8 – Percent survival (% ± SE) based on an initial stocking density of 25 juvenile *Callinectes sapidus* (180 animals m^{-2}). For wet weight and carapace width/length, the initial values are based on measurements of 30 animals. Final and intermediate values include all survivors. ■ 0%; ● 30%; ▲ 60%; ▼ 90%.

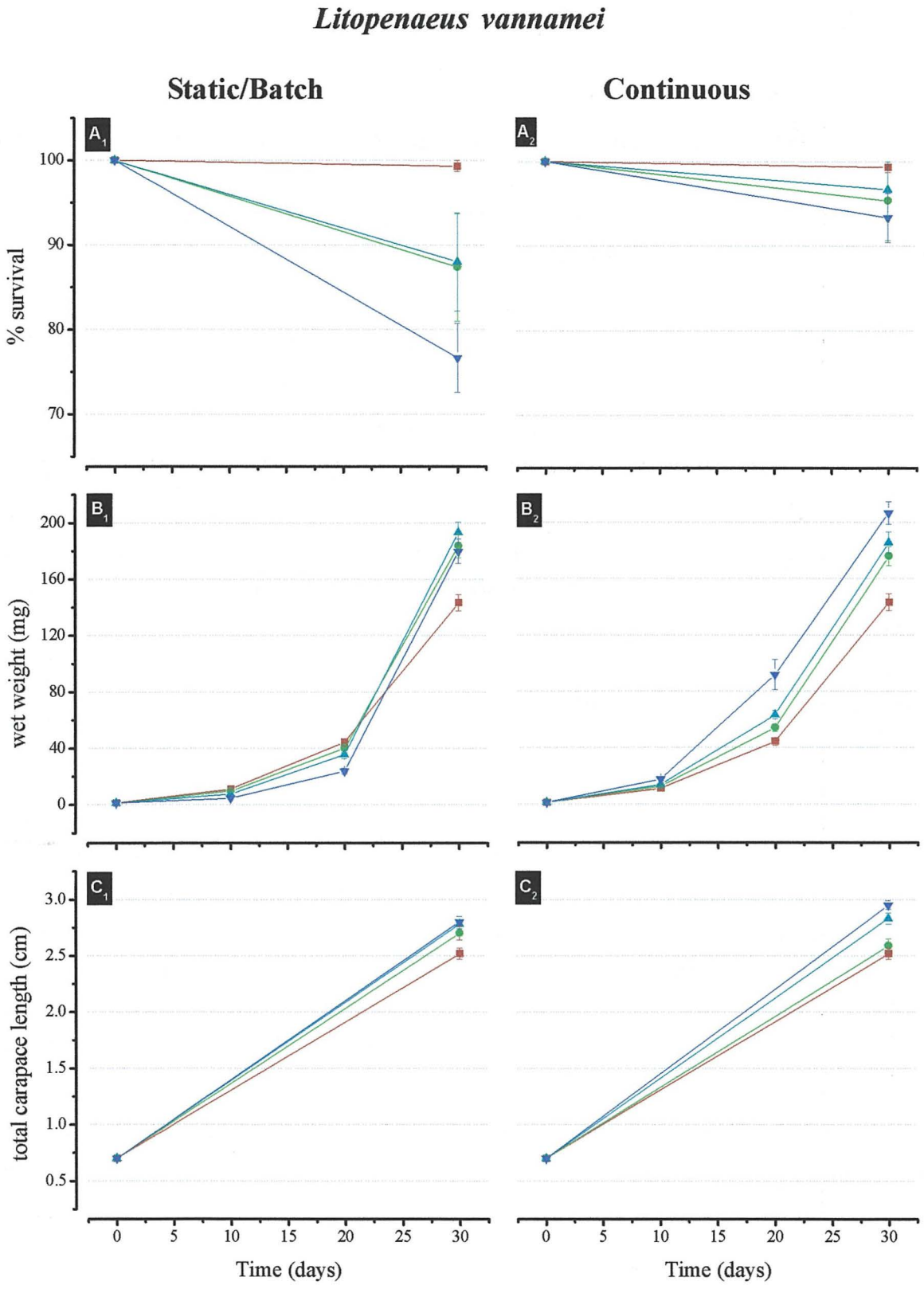

Figure 5.9 – Percent survival (% ± SE) based on an initial stocking density of 50 juvenile *Litopenaeus vannamei* (360 animals m^{-2}). For wet weight and carapace width/length, the initial values are based on measurements of 30 animals. Intermediate values represent averages from 3 randomly selected individuals. ■ 0%; ● 30%; ▲ 60%; ▼ 90%.

DISCUSSION

Physico-chemical variables

The concave trend in the temperature values for the treatments in the first experiment with *Callinectes sapidus*, despite insignificant differences between them (0.3 °C of maximum difference), reflects the placement of the tanks inside the experimental room with a stratified atmosphere. The use of a stronger ventilator avoided this pattern in the second experiment with *Litopenaeus vannamei* postlarvae.

Significant differences between the control with no exchange and batch water exchange treatments in both experiments were due to evaporation with no freshwater compensation. This problem can be easily rectified with the addition of a seawater drip, plus occasional compensation with freshwater.

Oscillations in pH greatly influenced the concentration of the non-ionized ammonia fraction.

The higher oxygen concentration in the reservoir water is due to experiments being conducted in a continuous closed circuit (high water turnover rate) between the reservoir with two aeration lines and a protein skimmer where water was constantly aerated close to saturation levels. The slight decrease in the continuous recirculation with 30% water exchange treatment of shrimp is probably due to un-aerated tank(s) for periods of longer than a day.

Water quality chemical variables

Callinectes sapidus

The highest un-ionised ammonia concentration observed on day 15 in the 0% water exchange control treatment was below the 96 h LC_{50} reported for *Callinectes sapidus* (1.87 and 2.72 for pre and intermoult, respectively - Lakshmi *et al.* 1984). The expected direct relation between the rate of continuous exchange and the concentration of ammonia indicates a sub-optimal water quality situation, even at the highest exchange rate tested (90%), when compared to the insignificant concentration of ammonia in the reservoir. Caution should be taken when interpreting these results, since 90% water exchange of a 20 L tank, does not mean that there is an 18 L day^{-1} exchange volume. In reality, using the equation 1, a 90% water exchange/day represents a changed volume of 44 L day^{-1}.

Around 25 days after the start of this trial, nitrite concentrations reached their maximum levels with considerable variation (4.4 mg L^{-1}). Manthe *et al.* (1983), report more than 95% successful moulting in a commercial closed recirculation shedding system for production of soft shell *Callinectes sapidus* at nitrite and ammonia concentrations below 1 mg L^{-1}. Increased moulting mortality was observed when concentrations of nitrite approached 1.6 mg L^{-1}. This low lethal concentration value for nitrite may be related to the moulting process, as it has been demonstrated that crabs and shrimp increase their susceptibility to ammonia and specially to nitrite toxicity during this period (Mawatari & Hirayama 1975). However, Ary and Poirrier (1989) report 96 h LC_{50} concentrations of 58-88 and 72–121 mg L^{-1} (pre- and inter-moult periods, respectively) and Lakshmi *et al.* (1984) 25-29 mg L^{-1}, which are much higher values, even for pre-moult periods. Such variance in the reported toxic nitrite levels makes interpretation of present results difficult in terms of

potential toxicity for the juvenile crabs. The continuous recirculation performed better in extracting nitrite from the water, as indicated by the difference between batch and continuous water exchange nitrite accumulation patterns. It was expected that these results would be reflected in growth and survival of the animals but, due to variation no significant differences were seen. The direct relationship between percentage exchange and the ammonia concentration was not as clear for nitrite, due to the high associated variability. Nevertheless, with continuous exchange, the 90% treatment has a lower concentration of nitrite than 0, 30 and 60% treatments. Low nitrite concentrations in the seawater used for the batch water exchange and the reservoir water throughout the experiment indicates that there was an excessive accumulation in experimental tanks due to insufficient water exchange which led to the establishment of a nitrifying bacterial community in the tanks themselves, producing the fluctuations in ammonia and nitrite concentrations.

Nitrate concentration showed a sharp rise towards the end of the experiment, suggesting a transformation from nitrite. Values around 28 mg L^{-1} in all treatments of continuous water exchange indicates an insufficiency of the recirculation system to extract or degrade nitrates. Reasonably low concentrations in the reservoir (8 mg L^{-1}), however, raise some questions as to the interpretation of these results. Values of nitrate concentration exceeding 350 mg L^{-1} were observed in a *Callinectes sapidus* soft-shell production closed recirculating system with no apparent effects to the moulting adult crabs (Manthe *et al.* 1983).

High initial total orthophosphate concentration in the crab trial was probably due to accumulation in the water from a previous trial. However, tanks from the recirculation system showed significantly lower concentrations, suggesting that orthophosphate was being used up in these tanks.

The observed sudden decrease and stability in total protein concentration at the beginning of the experiment coincided with the forced use of another technique to measure it. Results from day 12 onwards are therefore untrustworthy. Nevertheless, the first days of the experiment indicate a lower concentration of proteins in the reservoir, which may be explained by extremely high concentrations found in the drain water/foam from the protein skimmer.

Litopenaeus vannamei

Although low there was an accumulation of ammonia and nitrite concentrations in the continuous recirculation treatments, especially in the tanks with lower exchange rates. Batch water exchange treatments also showed an accumulation of these substances, demonstrating that with these stocking densities and feeding regimes, all percentage exchange treatments were insufficient to promote levels of these chemicals close to zero. However, survival and growth results suggest that tolerance to the concentration of ammonia and nitrite existed without deleterious consequences. The un-ionised form of ammonia (NH_3-N) is considered to be toxic to most aquatic life (Armstrong *et al.* 1978; Chin & Chen 1987) and accumulation of ammonia or its intermediate product, nitrite, in culture systems with unconditioned biological filters, or little water exchange, causes mortality and affects growth of cultured animals (Colt & Armstrong 1979; Spotte & Adams 1983). The ionised form has also been reported to be toxic at high concentrations, but under non-experimental conditions this is unlikely to occur as un-ionised ammonia would have already caused a stress situation (Armstrong 1979). The values attained by the un-ionized ammonia fraction in the present study (**Fig.** 5.5, $A_{1\text{-}2}$) are above the reported safe levels of 0.16 mg L^{-1}, but are below the 96 h LC_{50} concentration (1.60 mg

L^{-1}) for *Litopenaeus vannamei* postlarvae and juveniles at 35‰ (Lin & Chen 2001). Desirable ranges and levels of ammonia and nitrite concentration reported for penaeid shrimp in Wickins and Lee (2002) are 0.09-0.11 mg L^{-1} for un-ionised ammonia and 0.1-0.25 mg L^{-1} for nitrite (Brock & Main 1994; Olivares & Yule 2000). Cavalli *et al.* (1998) reported a lower range for un-ionised ammonia (<0.02-0.07 mg L^{-1}) when in presence of nitrite (1.5 mg L^{-1}) for adult *Farfantepenaeus paulensis*. Animals in the present study were submitted to concentrations as high as 0.37 mg L^{-1} momentarily (30% continuous recirculation treatment), and concentrations above 0.10 mg L^{-1} for as long as 16 days (0 and 30% continuous recirculation treatment – **Fig.** 5.5, A_{1-2}). Despite these potentially adverse conditions, animals survived and grew reasonably well (**Fig.** 5.9, A_{1-2}, B_{1-2}, C_{1-2}), indicating that they were capable of acclimating to high concentrations of un/ionised ammonia.

The ammonia concentration peak around day 20, with a later decrease more accentuated in batch exchange treatments, suggests a "trigger" concentration that further stimulates the growth of bacteria in the tanks that transform ammonia into nitrite. The 0% water exchange control treatment showed the highest ammonia concentration peaking earlier than all other treatments. This can be understood assuming that the increased rate of ammonia accumulation stimulates the growth of nitrifying bacteria earlier than in other treatments. Such an accumulation pattern was particularly evident in the batch water exchange treatments. Although reaching lowest final ammonia concentrations, lower percentage exchange treatments provided less time for acclimatisation to a maximum concentration earlier on and, perhaps, a more prolonged exposure to overall higher ammonia concentrations. The 30 and 60% continuous exchange treatment tanks, accumulated higher ammonia concentrations, than the parallel batch water exchange treatments. Since the ammonia input from the continuous recirculation system reservoir water is negligible, it is not clear why ammonia reached such high concentrations in these treatments without triggering the activity of a nitrifying bacterial community in the tanks earlier, as occurred in the batch water exchange treatment tanks. Total ammonia values in the present study were always below the reported safe levels of 3.95 mg L^{-1}, and below the 96 h LC_{50} concentration (39.54 mg L^{-1}) for *Litopenaeus vannamei* postlarvae and juveniles (Lin & Chen 2001).

The rise in nitrite concentration was delayed in the batch water exchange treatments. This indicates that the nitrifying process started earlier in the continuous recirculation, but since all the ammonia generated in these tanks is extracted by the biofilter alone, the rate of ammonia production exceeded that of ammonia conversion into nitrite during the early days. In the batch water exchange, the accumulation of ammonia resulted from that remaining which was not extracted with the discarded siphoned water. Consequently, ammonia was taken out from the system without the need for bacteria and, therefore, it was only between day 15 and 20 that ammonia reached a high enough concentration in batch water exchange tanks to start the nitrifying process, as indicated by the rise in nitrite at that time (**Fig.** 5.5, C_{1-2}). In continuous recirculation, this process started before day 12, by which time a significant rise in nitrite concentration had occurred, presumably as a product of ammonia conversion which was already high.

The total ammonia and nitrite results confirm that under these particular experimental conditions, the nitrifying bacterial community required 15-20 days to establish the first step in the nitrifying process ($NH_4 \rightarrow NO_2$). The following step ($NO_2 \rightarrow NO_3$) did not occur before 25 days into the culture cycle. However, after a full culture cycle, the second run should have shown slowest acclimation times providing biofilter units are not

left to dry or stagnate. Due to the experimental conditions used in the present study, there was not enough natural light to stimulate and induce an algal bloom to consume ammonia and contribute to feed. This may explain the reduced growth rate of animals held in the present system, as compared to growth rates of animals grown in outdoor large culture tanks.

The unusually high nitrate concentration observed in natural seawater may be related to the seawater pumping station, which is close to a urbanized shore and within a tropical estuarine environment. The increment in nitrate concentration in the experimental tanks occurred after day 14, at a time when the treatments with highest concentrations of nitrite were stabilising their nitrite accumulation, indicating that the bacterial community was already involved in transforming nitrite into nitrate. Although the accumulation of nitrates in the 0% water exchange control can be explained by the early development of the nitrifying process as compared to the rest of the treatments, the reason for the 90% continuous recirculation showing a similar rise in nitrate concentration is unexplained. This treatment showed low concentrations of both ammonia (0.8 mg L^{-1}) and nitrite (1.4 mg L^{-1}) and the 5 mg L^{-1} increase above the average initial nitrate concentration could not result solely from the conversion of nitrite. Accumulation of nitrates, in this case, must be due to other sources. Nitrates are much less toxic than ammonia and nitrite, however, in excess, they are reported to reduce growth and survival of cultured crustaceans (Wickins 1976; Muir *et al.* 1991).

The total protein concentration present in the culture water was intended to serve as an indicator of the leaching of organic matter from the feed pellets used during the experiment, as well as to indicate the protein skimmer capacity to remove them from the water of the tanks submitted to continuous recirculation. The decrease in total protein concentration during the first days, especially in the batch water exchange tanks, was probably due to the initial leaching of the feeds and concomitant growth of bacteria that colonised the tanks. Later, since protein intake from feed was continuous and tended to increase as shrimp grew larger, it probably exceeded the bacterial community turnover capacity and started to accumulate. The sharp decrease on day 27, followed by another increase is unlikely to be due to a sudden extra performance by the skimmer, and more likely is due to the quality of reagents used for those particular determinations. In the continuous recirculation tanks, protein concentrations were not much different from the 0% water exchange control and slightly higher than batch water exchange. This indicates that physical batch water exchange is a better method to remove excess proteins in the water, but it also suggests that a concentration of between 160 and 220 mg L^{-1} represents the minimal concentration below which the process of protein skimming is not efficient. However, total protein concentration in the drain water from the protein skimmer indicates that the system was working despite irregularity in the process of protein skimming which depends on the amount and period between feeding, and the stage of clogging of the cartridge filter, that influences flow though the skimmer. From these results it appears that both methods of water exchange have similar efficiencies and that a recirculation system with an efficient protein skimmer or foam fractioner is capable of keeping total protein concentration within safe levels.

Biological variables

Callinectes sapidus

The increase in % survival variability towards the end of the experiment was principally due to observed cannibalism by the first animals which moulted to a large enough size to predate others, despite the abundant presence of PVC tube shelters and nylon mesh in each tank. Significant differences in growth rate between exchange treatments and the 0 % exchange control indicate that the growth of crab juveniles in the later condition was impaired by high ammonia and nitrite concentrations. Present results confirm that crabs can be fed with artificial diet pellets and can grow well in recirculation conditions.

Litopenaeus vannamei

Although highest survival was observed in the 0% water exchange control, suggesting an unexpected postlarval resistance or tolerance of major changes in water quality, the animals in these tanks were the slowest growers, reflecting the sub-optimal conditions induced by the accumulation and long exposure to high concentrations of ammonia and nitrite. Although not conclusive, results suggest that animals grow slightly better in continuous recirculation, probably due to increased food intake, since batch water exchange tanks were siphoned prior to water exchange, eliminating uneaten food and faeces. While this procedure eliminates solid organic matter that stimulates further heterotrophic bacterial growth, it also eliminates potential food not yet eaten by the omnivorous and detritivorous feeding behaviour of penaeid shrimp. Furthermore, although incoming seawater was brought to the experimental salinity and left acclimating to attain the approximate temperature required, instantaneous changes in these and other (chemical) variables produce an ever-changing environment requiring constant adaptation from the cultured animals, and hence an energy cost. Additionally, the effect of the mechanical stress induced by batch water exchange induced many consecutive tail flip escape responses from shrimp which most probably represented an additional energy expense. It is clear that continuous recirculation provided a more stable, slow changing environment, eventually more suitable for the growth of juvenile crustaceans.

General discussion

Both commercial or experimental pellets and frozen fresh food are known to leach nutrients into the water which stimulates bacterial blooms in the culture water augmenting the BOD, and, together with excreta, raises ammonia and nitrite concentration in the water. The operational conditions of a simple totally closed recirculation system, evaluated in this study, confirm that such system is capable of mitigating these problems to levels similar to batch water exchange open systems and provides an acceptable environment for crustacean growth.

Provided that most decapod crustaceans adapt well to captivity conditions (Wickins 1976), the use of recirculation systems reduces the environmental impact of the farms, and decreases the risks associated with the potential release of toxic substances normally used in the industry, as well as diminishing the risk of disease spread. Furthermore, since the residence time of culture water is increased, a better control of variables such as temperature and salinity, is possible (Huguenin & Colt 1992; Timmons & Losordo 1994).

Most studies have been dedicated to shrimp juveniles or adults. The present study uses postlarvae of both shrimp and crabs in an ontogenic phase where they are susceptible to disease and to even low concentrations of toxic substances.

Present results support the use of recirculation systems for these development stages particularly in nursery systems. Shrimp nursing culture systems are increasingly popular since they provide farmers with hardy juveniles that have been fully acclimatised to the environment which they are likely to encounter during ongrowing. Nursed juveniles are usually transferred to ongrowing ponds when they have reached 0.1-2 g, by which time they already have completed the development of their digestive system (Lovett & Felder 1989; Lovett & Felder 1990b; Lovett & Felder 1990a) and as most weak animals will have died, a realistic estimate can be made of the number stocked (Wickins & Lee 2002). This greatly improves control over stocking densities and greatly assists in the calculation of feeding regimes. According to Wickins and Lee (2002), another advantage of nursery ponds is the relative ease with which predators can be eliminated, and that they can be used for "stockpiling" reserves of juveniles for periods as long as 6 months. More intensive nursery systems using raceways, sometimes within greenhouse structures, have been developed as a means of saving space, improving biosecurity, rearing bait shrimp, and producing juveniles ready for early stocking in a limited ongrowing season (Samocha *et al.* 1993). The present study results confirm that closed continuous recirculation systems can be readily applicable to commercial nursery growing units.

Present survival and growth results using continuous recirculation were similar to results obtained with traditional batch water exchange, favouring the use of recirculation as an environmentally sound method. Although recirculation systems represent an initial higher capital investment and may require high energy supply, technology is rapidly developing creating units that are more efficient, less expensive and with higher volume processing capacity.

The 0% exchange control treatment produced good survival and growth results, considering that no water was changed for 30 and 42 days for shrimp and crab postlarvae, respectively. This treatment simulated zero exchange aerobic, heterotrophic systems. Recently, these systems have been applied to large commercial scale operations in order to reduce the risk of pathogens gaining entrance to the system, to improve the efficiency of nutrient assimilation, and to eliminate effluent discharges with the consequent advantages in terms of sustainable aquaculture and environmental protection (Hopkins *et al.* 1995; McIntosh 1999; McIntosh 2000; McNeil 2000). In reduced and zero water exchange systems feeding strategies have also undergone a fundamental reappraisal, placing emphasis on promoting a pond ecosystem that minimizes water quality fluctuations and maximizes the efficiency of nutrient assimilation by the crop (Wickins & Lee 2002). Such increase in nutrient assimilation efficiency is achieved by conserving uneaten feed and faecal waste, ensuring that it is suspended in the culture tanks to maintain aerobic decomposition. In this way shrimp may reprocess feed and nitrogen assimilation efficiency can be improved. As a result the level of protein in feeds can be reduced, perhaps to as little as 20%, to greatly reduce production costs (Chamberlain 1998).

CONCLUSIONS

Present results confirm that a simple and cheap continuous closed recirculation system is a biologically viable alternative to standard batch exchange procedures in the commercial production of decapod crustacean postlarvae. Water quality was maintained within tolerable levels by both systems, assuming that the animals acclimate to a gradual increase in potentially toxic substances such as ammonia and nitrite. Both survival and growth using continuous recirculation were close to results obtained with traditional batch water exchange. Overall, animals submitted to the highest percentage continuous water exchange treatments, provided lowest accumulation of total ammonia and nitrite, highest growth rates and lowest mortality, although 60 and 30% also gave acceptable results. The use of continuous recirculation with a % exchange between 60 and 90% is recommended.

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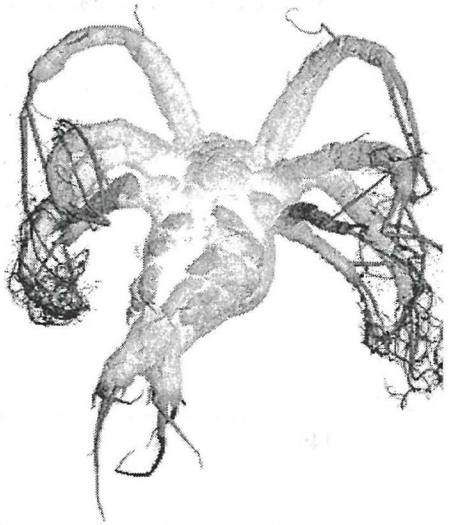
CHAPTER 6
Stocking density of early shrimp postlarvae (*Litopenaeus vannamei* and *Farfantepenaeus brasiliensis*) under zero exchange recirculation conditions: changes in water quality, survival and growth

Parts of this chapter were presented at Larvi2001, 3rd Fish and shellfish larviculture symposium, Ghent University, Ghent, Belgium, 3-6 September 2001.

Simões, N.; Hidalgo, L.E. and Jones, D.A. (2001). Optimal stocking density of *Litopenaeus vannamei* early postlarvae under closed recirculation. Poster and "mini-paper" presented at Larvi2001, 3rd Fish and shellfish larviculture symposium, Ghent University, Ghent, Belgium, 3-6 September 2001.



INTRODUCTION

Recirculation systems reduce water discharges from aquaculture sites, control levels of toxic substances such as ammonia and nitrite, and assist stabilisation of temperature, salinity, pH, and dissolved oxygen in culture water (Huguenin & Colt 1992; Timmons & Losordo 1994). Recently, recirculation systems have also been used to reduce water intake as a further control measure to prevent disease outbreaks, and increase biosecurity, especially in shrimp culture (Wickins & Lee 2002). Nowadays, recirculation technology for crustacean culture can be both feasible and cost effective in maturation, hatchery and nursery production units that require small water volumes. However, this technology is expensive, and therefore research efforts should be directed to increase the efficiency of the process.

Whilst most technical aspects for the implementation of these systems have been studied (Timmons & Losordo 1994), many biological aspects, such as the stocking density, have not yet been completely examined. Recent work using moderate stocking densities and a range of recirculation rates under completely closed conditions has provided encouraging results in terms of growth and survival of early *Litopenaeus vannamei* postlarvae (Martínez *et al.* 2000). In recent years, much research has been directed to reduce the water exchange for intensive shrimp farming in an effort to reduce environmental impacts on estuarine and marine ecosystems (Reid & Arnold 1992; Hopkins *et al.* 1993; Hopkins *et al.* 1995a; Hopkins *et al.* 1995b; Hopkins *et al.* 1996). This effort has focused particularly on the nursery phase of the production cycle. The nursery system has been used to cultivate postlarvae during 3 to 4 weeks prior to stocking in larger grow-out tanks to maximise survival and growth during one of the most critical periods of the shrimp life cycle. Early postlarvae undergo complex morphological changes in their digestive system as far as $postlarvae_{45}$ (Lovett & Felder 1989). These ontogenetic changes are accompanied by frequent moulting with the consequent physiological stress (Jayasankar & Muthu 1983; Lin *et al.* 1993). Whilst increasing stocking density would mitigate the high cost of using recirculation systems, water quality in nursery grow-out units must be sufficient to reach standard values of postlarvae production variables, such as growth, development, survival, feed conversion ratio and stress resistance.

Farfantepenaeus brasiliensis is a Caribbean Sea penaeid species with commercial scale production potential as an alternative to the introduction of exotic penaeid species in the region such as *Litopenaeus vannamei*, and many aspects of its performance under culture conditions are still unknown.

This study describes and quantifies changes in water quality, survival and growth of *Litopenaeus vannamei* and *Farfantepenaeus brasiliensis* early postlarvae subject to a wide range of initial stocking densities under closed recirculation conditions.

MATERIALS AND METHODS

Growth, survival and stress resistance

Litopenaeus vannamei postlarvae (PL_{8-10}) originated from captive broodstock (3rd generation in captivity) were acquired from a local hatchery (Industrias Pecis, Sisal, Yucatan, Mexico) and acclimated to laboratory water conditions for 8 h. *Farfantepenaeus brasiliensis* larvae were obtained from a single spawning of naturally inseminated females from the wild, captured at 42 to 67 m depth off Isla Contoy, Quintana Roo, Mexico. Larvae were grown to PL_{10} following standard penaeid shrimp larvae procedures (Gaxiola *et al.* 2002). Initial stocking densities (ISD) ranging from 0 (control tank) to 1420 animals m^{-2} (Table 6.1) were tested with zero exchange continuous recirculation and 95% tank water renewal. Shrimp were maintained during 28 and 33 days (*L. vannamei* and *F. brasiliensis*, respectively) in 40-litre white polyurethane tanks (41 L × 34 W × 30 H cm) containing ca. 20 L of water with no sediment and constant aeration. A 13 light –11 dark h photoperiod was used with common neon lamps.

Initial postlarval weight was obtained from a sample of 50 (2.14 ± 0.78 mg; 6.39 ± 0.64 mm) and 30 (2.17 ± 0.66 mg; 7.05 ± 0.54 mm) animals for *Litopenaeus vannamei* and *Farfantepenaeus brasiliensis*, respectively. Growth in wet weight (mg) was evaluated by random sampling of three animals from each tank every 7 days, after which animals were returned to their respective tanks. Mortality was estimated every 7

Table 6.1 – Experimental design used in the *Litopenaeus vannamei* and *Farfantepenaeus brasiliensis* postlarvae stocking density experiments. * - Control tank with no animals and no feeding.

Tank N	Water vol. (L)	PL m^{-2}	Increment	Initial PL/tank
1 *	22.1	0	0	0
2	21.6	20	20	3
3	22.9	40	20	6
4	22.8	60	20	9
5	21.5	100	40	14
6	23.4	140	40	20
7	22.1	180	40	25
8	21.9	220	40	31
9	23.2	260	40	37
10	22.1	320	60	45
11	22.5	380	60	53
12	21.7	440	60	62
13	20.2	500	60	70
14	20.0	580	80	81
15	20.2	660	80	93
16	20.2	740	80	104
17	20.6	820	80	115
18	19.7	920	100	129
19	21.5	1020	100	143
20	21.0	1220	200	171
21	22.8	1420	200	199

days using visual counts of survivors in quiet, non-aerated conditions. All survivors were counted, measured and weighed after 28 or 33 days . Due to continuous movement, small size and camouflage of certain animals amongst debris, some counts were slightly lower than the final survival number. In these cases intermediate survival estimates were corrected with the next "higher-than-final" survivor count, or with the final survival numbers. *F. brasiliensis* postlarvae were more active than *L. vannamei* and there was a heavy loss of animals during the first 7 days due to "jump-out" mortality until they acclimated to the experimental conditions. During those first 7 days all dead animals, dry on the sides of the tanks, were replaced by postlarvae of approximately the same size . Animals that died due to "jump-out" mortality were monitored daily in both experiments. Unfortunately, the data for *L. vannamei* were lost. The mean relative mortality rate and mean relative growth rate were calculated according to equations (2.1) and (5.2), respectively.

At the end of the *Litopenaeus vannamei* experiment, 12 randomly collected animals from each treatment (except the first 3 with only 3, 5 and 8 survivors) were submitted to a physiological stress test adapted from (Samocha *et al.* 1998b). For *Farfantepenaeus brasiliensis* the animals from the first 4 treatments (20-100 postlarvae m^{-2}) were pooled. Animals were introduced in 1-litre glass containers filled with drinking water (<1 ‰) and no aeration for 1 and 5:30 h (*L. vannamei* and *F. brasiliensis*, respectively) after which survival was assessed. Animals were considered dead when they did not respond to touching with a glass rod. Results were represented as proportion of initial number of animals alive.

Water quality

The total ammonia and nitrite concentration was measured every 4 to 5 days using standard phenol-nitroprusside and sulphanilamide reactions techniques, respectively (Parsons *et al.* 1984). The non-ionised ammonia fraction was calculated as the average of 4 models (Whitfield 1974; Khoo *et al.* 1977; Johansson & Wedborg 1980; Spotte & Adams 1983) using values of pH, temperature and salinity specific of each tank on the day and time of total ammonia determination. Nitrates and phosphates were analysed approximately every 10 days using the standardized Hach™kit techniques. Temperature inside the experimental room was maintained at 27.5 ± 0.5 °C with a thermostatically controlled air-conditioner and a ventilator to ensure complete mixing. Temperature, oxygen and water flow for each tank was monitored daily, and adjusted accordingly, with the exception of temperature that was adjusted for the entire system. Salinity was also adjusted by addition of fresh water. Suspended solids were collected by filtering 1 litre from each tank through Whatman GF-C filters (1.4 mm) at the end of the experiment.

Recirculation system

The recirculation system (Fig. 6.1) consisted of one 700 L reservoir connected to a 50-mm cartridge filter, a fluidised sand biofilter and a foam fractioner in closed circuit with a submersible pump. The two experiments were completed in sequence, first with *Litopenaeus vannamei* and then with *Farfantepenaeus brasiliensis* postlarvae with 2 days in between. 80 litres of recirculating water from a previous trial were used as a bacterial community start-up of the *L. vannamei* experiment recirculation system. In addition it was filled with settled natural seawater filtered to 1 mm 5 days prior to the beginning of the experiment. A magnetic pump distributed water to the 21 tanks through plastic taps. The total volume of the system added

to approximately 1300 L. Similarly, one third of the water from the first experiment was used in the second one. Flow was calculated according to equation (5.1).

Diet

Animals were fed 3 times per day with high protein pellets (Table 5.1) prepared according to Rosas *et al.* (2001). Feeding regime started at 120% of the total biomass of each tank with a 20% decrease per week, based on survival and weight estimates of each experimental tank Daily quotas were dispensed in three doses: early morning (25%), midday (25%) and late afternoon (50%). Pellets were presented with sizes between 100 and 250 mm for the first 10 days, and between 500 and 800 thereafter. Feed conversion ratio (FCR) per tank was calculated using the ratio between total mg of feed given and final shrimp postlarvae biomass (mg). Food leftovers and faeces on the bottom of tanks were not siphoned to simulate conditions in large nursery raceways where daily siphoning is impractical.

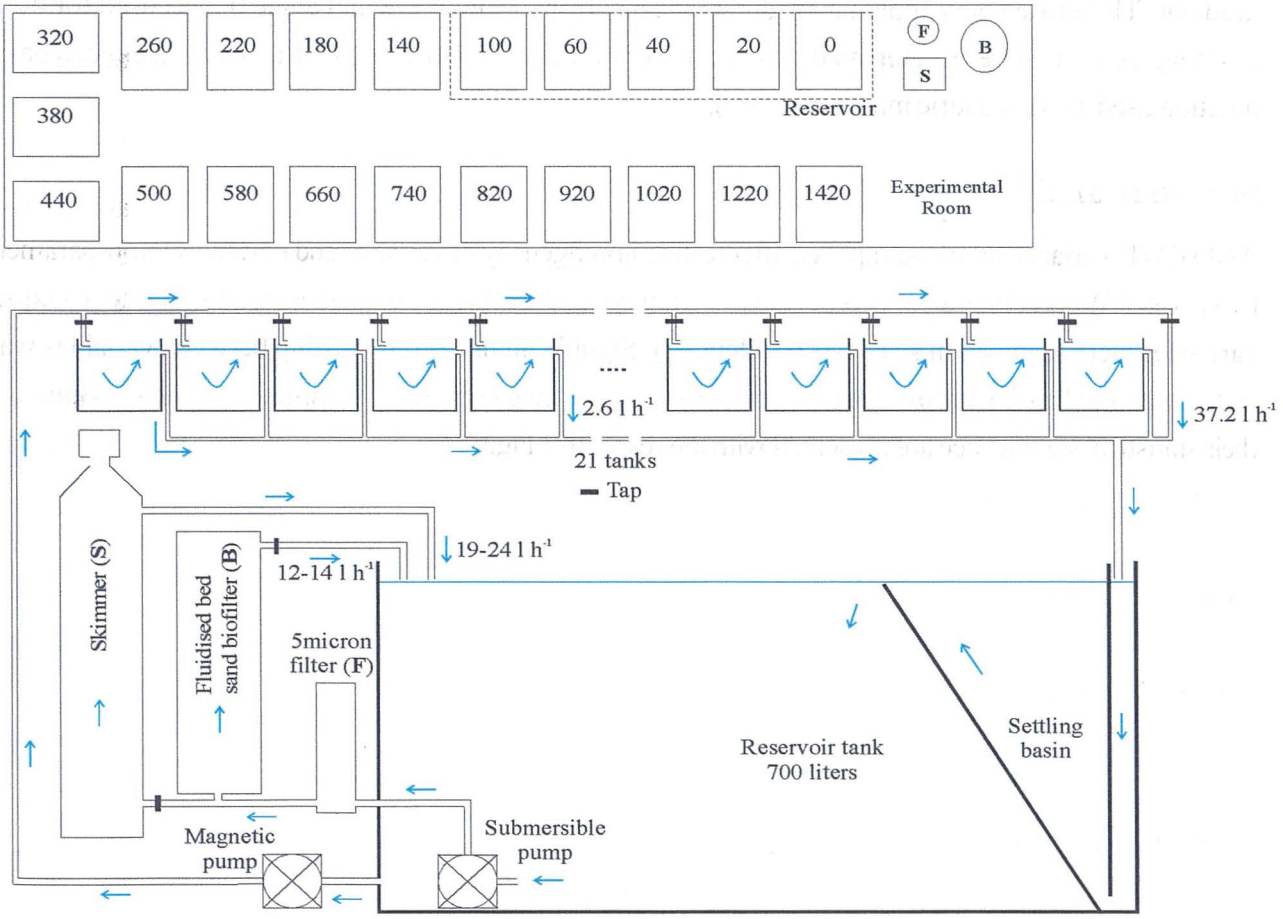


Figure 6.1 – Diagram with details of the recirculation system used and the general disposition of tanks inside the experimental room. Drawings are approximate, but not to scale.

Interpolations

Initial stocking density data had to be expressed as number of postlarvae per dm^2, instead of per m^2, to achieve a balance between the density and time scales (28 days and 14.2 ISD, maximums). Non-ionised ammonia fraction (NH_3), total ammonia ($NH_3 + NH_4^+$) and nitrite (NO_2) data had a matrix structure of 21×9 observations. Percentage mortality, weight gain and biomass per tank data had a matrix structure of 21×5 and 21×6 observations for *Litopenaeus vannamei* and *Farfantepenaeus brasiliensis*, respectively. These data sets were interpolated using the "point krigging" gridding method (Cressie 1991 - no drift, and no nugget effect) with a grid density of 21 lines (densities/tanks) and 28 or 33 columns (days – *L. vannamei* and *F. brasiliensis*, respectively) to ensure representation of the original structure of data. Nitrates (NO_3) and phosphates (PO_4) had an observation matrix structure of 21×4 observations, and therefore a "radial basis function" gridding method was more adequate (Carlson & Foley 1991), maintaining a grid density of 21 lines and 28 or 33 columns. The concentration of all molecules was assumed to change more rapidly per unit of time than per unit of initial stocking density, which is the same as to say that when interpolating at a point, an observation 10 units away in a direction parallel to the ISD gradient is more likely to be similar to the value at the interpolation point than is an equidistant observation in a direction perpendicular to the ISD gradient. Therefore when applying krigging, an anisotropy ratio of 0.8 and angle 0, was used for both gridding methods. The krigging method used a linear variogram with a slope of 1, whilst, the radial basis function used a multiquadric model ($r^2 = 469$).

Statistial analysis

ANOVA (F, variance ratio) was applied after testing homogeneity of variance and normality. Non-parametric Kruskal-Walis (H statistic) was performed for oxygen data due to heterogeneity of variance. Unequal variances were assumed in all *t*-tests (student's *t*). Significant linear relationship between variables was tested with the Pearson's correlation coefficient. Curve fitting equations, parameters, *r* and c^2 values and their statistical significance are presented with the respective Figures.

RESULTS

Water quality

Temperature

Water temperature in the tanks averaged 27.3 ± 0.3 and 27.54 ± 0.22 °C (*Litopenaeus vannamei* and *Farfantepenaeus brasiliensis*, respectively) throughout the experimental period with daily oscillations (Fig. 6.2, A, B). Temperature in the reservoir (28.1 ± 0.4; 28.4 ± 0.2 °C *L. vannamei* and *F. brasiliensis*, respectively) was significantly higher than overall average tank temperature ($p < 0.001$) due to the heat generated by the submersible pump. This local increase in temperature was partially transmitted to the first experimental tanks in the distribution line (Fig. 6.3, A, B). Despite efforts to mix the air layers inside the experimental room with a strong ventilator, a symmetric "U" shaped pattern of significant differences in temperature among tanks ($p < 0.001$) resulted from the way these were laid out within the room (Fig. 6.1 and Fig. 6.3, A, B). For *L. vannamei* these differences, although small, were significantly correlated to postlarvae weight gain, relative growth rate, final % mortality and to relative mortality rate (Table 6.2), whilst, for *F. brasiliensis* they were significantly correlated only to postlarvae weight gain and relative growth rate (Table 6.2).

Dissolved oxygen

Oxygen concentrations averaged 6.07 ± 0.03 and 6.05 ± 0.04 mg L^{-1} at the beginning and steadily decreased to 5.40 ± 0.30 and 5.19 ± 0.31 mg L^{-1} towards the end of the experiment for *Litopenaeus vannamei* and *Farfantepenaeus brasiliensis*, respectively (Fig. 6.2, C, D). Upward oscillations in the overall gently decreasing pattern of dissolved oxygen availability in both experiments relate to an increase in aeration rates (days, 12, 17, 20 and 26 for *L. vannamei* and 13, 27 and 29 for *F. brasiliensis*). Twenty-eight days average oxygen concentration was significantly lower in tanks with higher ISD (Table 6.2; Fig. 6.3, C, D) and correlated significantly to *F. brasiliensis* postlarvae weight gain, relative growth rate, final % mortality and to relative mortality rate (Table 6.2).

Salinity

Salinity increased approximately 5 ‰ throughout the experimental period in both experiments (Fig. 6.2, E, F). For *Litopenaeus vannamei*, initial water was diluted to 34 ‰, the salinity at which the postlarvae arrived at the laboratory, and then rose steadily to reach 38.6 ± 0.5 ‰ at day 27, despite occasional addition of fresh water to compensate high evaporation (days 12 and 21; Fig. 6.2 E). For *Farfantepenaeus brasiliensis*, since broodstock, larvae and postlarvae, had been raised in water at 38 ‰, that salinity was the one used at the beginning of the experiment, although it then rose until it reached 42.6 ± 0.5 ‰ by the end of the experiment (Fig. 6.2 F). Once more, the addition of freshwater on days 19 and 28 was not enough to compensate strong evaporation. Chenges in salinity were significantly correlated to *L. vannamei* postlarval ISD (Table 6.2).

Table 6.2 – Tabulated Pearson correlation coefficients and respective probability values (N = 21) between water quality (daily average and maximum non-ionised ammonia fraction (NH_3), total ammonia (NH_4) and nitrite (NO_2) concentrations (mg L^{-1}), total suspended solids, temperature, dissolved oxygen, salinity and pH) and zootecnical variables (final % mortality, relative mortality rate, final average wet weight, relative growth rate, feed conversion ratio and osmotic stress resistance) for *Litopenaeus vannamei* and *Farfantepenaeus brasiliensis* early postlarvae in 21 initial stocking densities (ISD, Table 6.1) Experiments with *L. vannamei* and *F. brasiliensis* were run for 28 and 34 days, respectively.

	Litopenaeus vannamei							***Farfantepenaeus brasiliensis***						
	Relative growth rate	**Final wet weight (g)**	**Osmotic stress resistance**	**Relative mortality rate**	**Final % mortality**	**Initial stocking density**	**Feed conversion ratio**	**Relative growth rate**	**Final wet weight (g)**	**Osmotic stress resistance**	**Relative mortality rate**	**Final % mortality**	**Initial stocking density**
Initial stocking density	–	0.150 (0.529)	0.118 (0.619)	**–0.828** (0.000)	–0.074 (0.755)	–0.037 (0.877)	–0.070 (0.770)	–	–0.408 (0.074)	–0.423 (0.063)	–0.430 (0.085)	**–0.554** (0.011)	**–0.659** (0.002)	–0.427 (0.060)
Suspended solids	**0.797** (0.000)	–0.157 (0.509)	–0.181 (0.446)	**–0.822** (0.000)	0.212 (0.371)	0.331 (0.154)	0.240 (0.308)	**0.868** (0.000)	–0.277 (0.237)	–0.281 (0.230)	–0.116 (0.657)	–0.377 (0.102)	–0.430 (0.058)	–0.236 (0.304)
Daily NH_3	**0.635** (0.002)	0.037 (0.878)	0.015 (0.951)	**–0.468** (0.037)	–0.075 (0.754)	–0.039 (0.870)	–0.113 (0.634)	**0.978** (0.000)	**–0.456** (0.044)	**–0.456** (0.043)	–0.375 (0.138)	**–0.529** (0.017)	**–0.643** (0.002)	–0.322 (0.154)
Daily NH_4	**0.947** (0.000)	0.087 (0.715)	0.053 (0.825)	**–0.743** (0.000)	–0.086 (0.720)	–0.056 (0.815)	–0.093 (0.697)	**0.989** (0.000)	–0.426 (0.061)	–0.431 (0.058)	–0.386 (0.126)	**–0.498** (0.025)	**–0.606** (0.005)	–0.298 (0.189)
Daily NO_2	–0.037 (0.872)	0.152 (0.523)	0.102 (0.668)	0.151 (0.524)	–0.417 (0.067)	–0.278 (0.235)	–0.417 (0.067)	**0.946** (0.000)	**–0.465** (0.039)	**–0.449** (0.047)	–0.355 (0.162)	**–0.527** (0.017)	**–0.625** (0.003)	–0.315 (0.165)
Maximum NH_3	0.337 (0.135)	–0.100 (0.676)	–0.115 (0.630)	–0.361 (0.118)	0.139 (0.558)	0.146 (0.540)	0.104 (0.663)	**0.918** (0.000)	–0.407 (0.075)	–0.418 (0.067)	–0.354 (0.163)	**–0.488** (0.029)	**–0.606** (0.005)	–0.309 (0.173)
Maximum NH_4	**0.932** (0.000)	0.183 (0.440)	0.155 (0.515)	**–0.681** (0.001)	–0.175 (0.461)	–0.167 (0.481)	–0.182 (0.442)	**0.979** (0.000)	–0.392 (0.087)	–0.410 (0.073)	–0.366 (0.149)	**–0.449** (0.047)	**–0.559** (0.010)	–0.253 (0.268)
Maximum NO_2	**–0.530** (0.013)	–0.086 (0.719)	–0.091 (0.701)	0.399 (0.082)	–0.008 (0.974)	0.121 (0.611)	0.048 (0.840)	**0.904** (0.000)	–0.402 (0.079)	–0.399 (0.082)	–0.293 (0.254)	**–0.465** (0.039)	**–0.561** (0.010)	–0.296 (0.193)
Temperature	–0.050 (0.829)	**–0.529** (0.017)	**–0.490** (0.028)	–0.313 (0.179)	**0.852** (0.000)	**0.751** (0.000)	**0.833** (0.000)	–0.142 (0.540)	0.409 (0.073)	0.346 (0.136)	–0.072 (0.784)	**0.682** (0.001)	**0.645** (0.002)	0.227 (0.323)
Disolved oxygen	**–0.954** (0.000)	–0.097 (0.685)	–0.068 (0.777)	**0.773** (0.000)	0.035 (0.883)	0.000 (1.000)	0.027 (0.910)	**–0.978** (0.000)	**0.456** (0.044)	**0.464** (0.039)	**0.493** (0.044)	**0.566** (0.009)	**0.682** (0.001)	0.291 (0.201)
salinity	**0.563** (0.008)	0.112 (0.639)	0.072 (0.762)	**–0.768** (0.000)	0.036 (0.882)	–0.014 (0.953)	0.025 (0.917)	0.019 (0.936)	–0.144 (0.545)	–0.153 (0.519)	–0.362 (0.153)	–0.212 (0.370)	–0.159 (0.502)	–0.232 (0.311)
pH	**–0.845** (0.000)	–0.124 (0.601)	–0.106 (0.655)	**0.672** (0.001)	–0.018 (0.940)	–0.034 (0.886)	–0.018 (0.941)	**–0.913** (0.000)	0.435 (0.055)	**0.457** (0.043)	**0.593** (0.012)	**0.539** (0.014)	**0.631** (0.003)	0.217 (0.344)

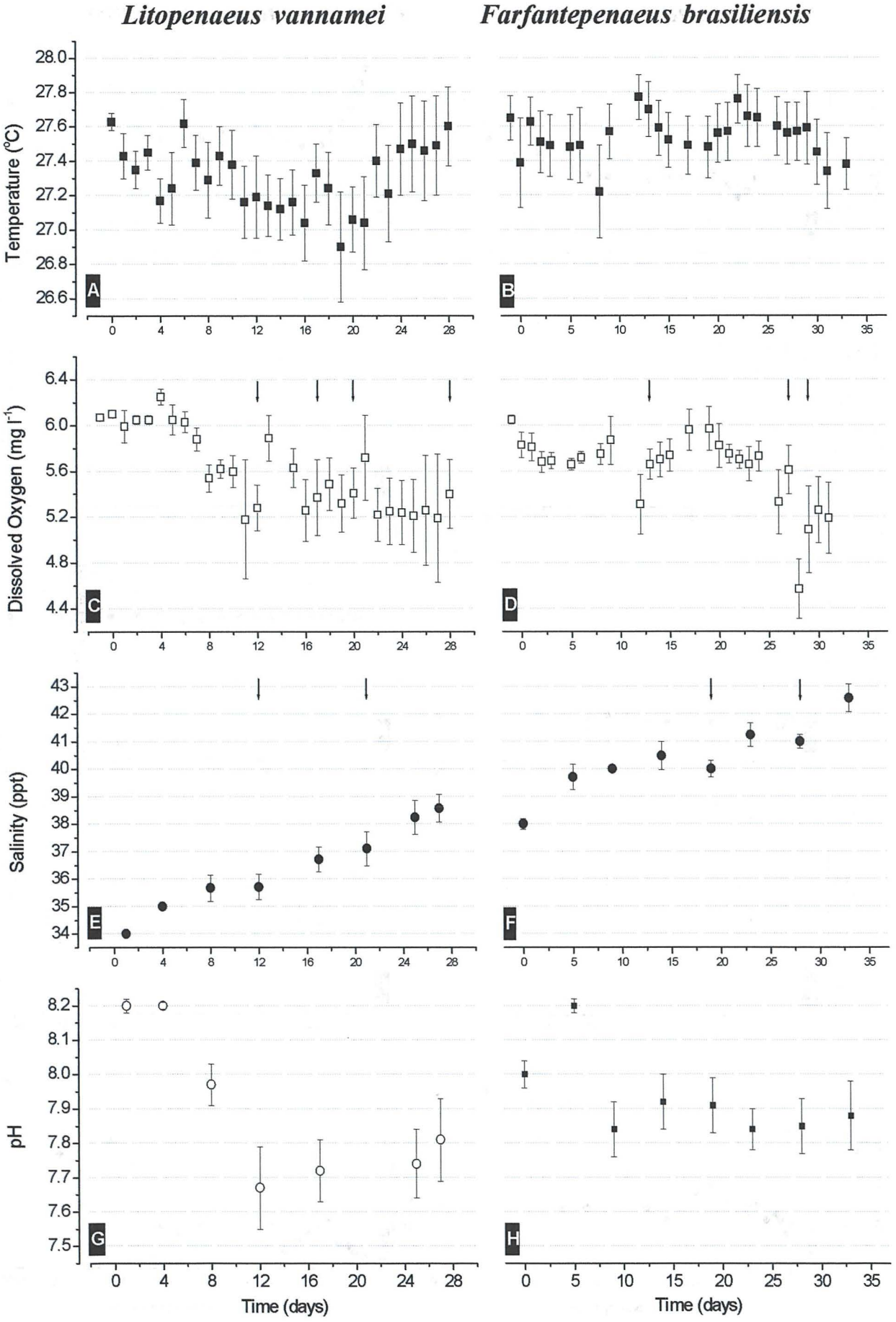


Figure 6.2 – Overall mean daily water temperature, dissolved oxygen, salinity and pH of tanks (± SD). Arrows in Figures **C-D** and **E-F** indicate increase in aeration and addition of fresh water, respectively.

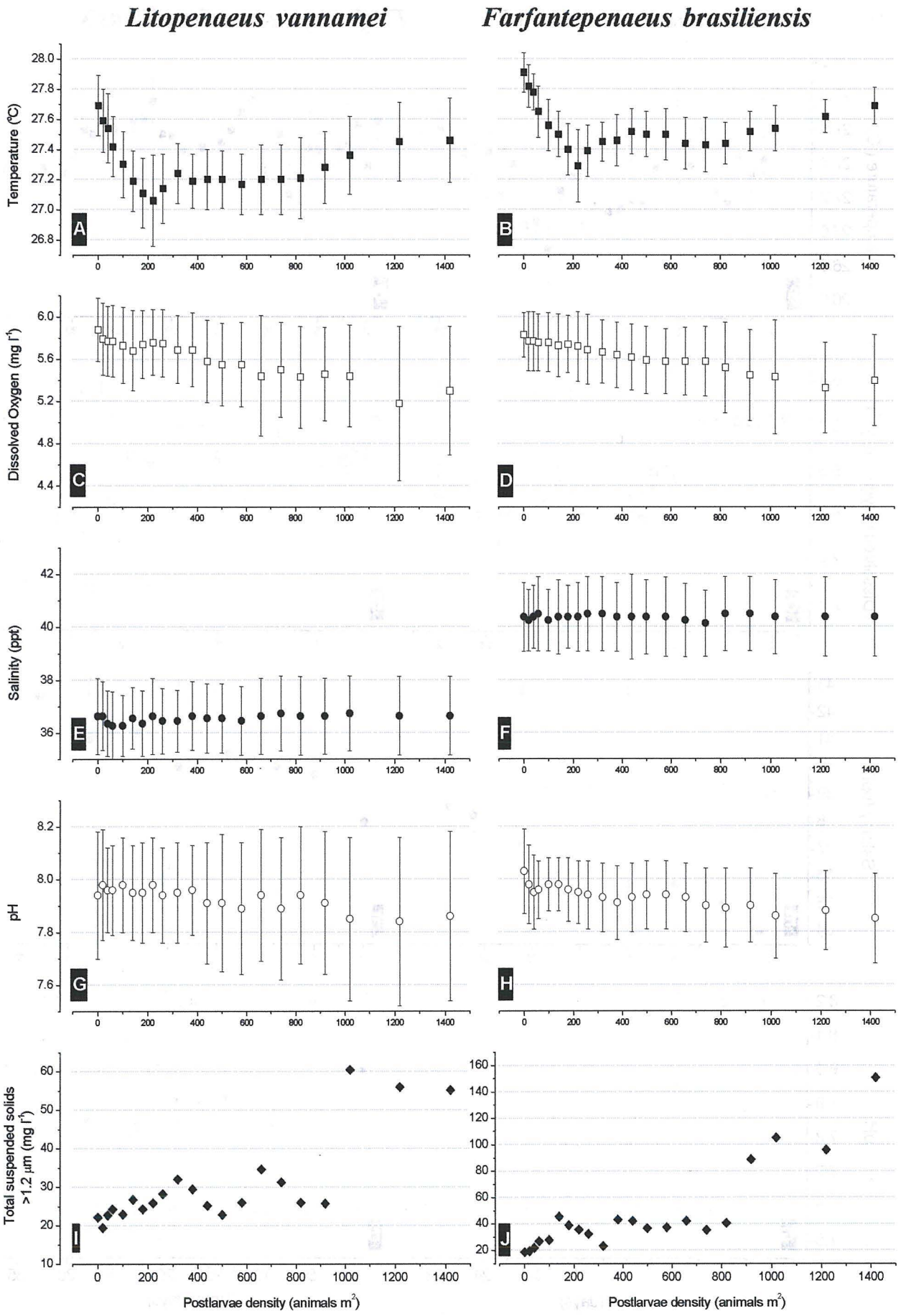


Figure 6.3 – Mean water temperature, dissolved oxygen, salinity and pH (± SD) throughout the experimental period (28 and 34 days, respectively). Results of suspended solids are final values per tank.

pH

For *Litopenaeus vannamei*, values of pH averaged 8.20 ± 0.02 at the beginning and steadily decreased to 7.81 ± 0.12 towards the end of the experiment (Fig. 6.2G), whilst for *Farfantepenaeus brasiliensis*, pH values started at 8.00 ± 0.01, raised to 8.2 ± 0.01 and then decreased and stabilise around 7.7 (Fig. 6.2 H). There was a highly significant and strong correlation between the decrease in pH and increase in shrimp postlarvae ISD (Table 6.2; Fig. 6.3, G, H).

Suspended solids

At the end of the experiment the suspended solids increased proportionally with increasing ISD (Table 6.2 and Fig. 6.3, I, J), although in the experiment with *Farfantepenaeus brasiliensis* postlarvae, values almost doubled those of *Litopenaeus vannamei* experiment. White mats of bacteria, fungi and possibly heterotrophic microalgae grew on accumulated debris made of uneaten food and faeces on the bottom of the tanks (as these were not siphoned out). The extension and thickness of such mats increased with ISD as a consequence of the feed quantity added daily, from scattered crumbs to homogeneous "carpets" covering almost completely the tank bottoms. Increased aeration and movement of animals towards the end of the experiment in tanks with over 660 postlarvae m^{-2}, disrupted these mats and re-suspended most of the debris in the water column. Heavy debris particles that settled down were no longer organised in mats, but instead in little "hills" which were preferred places for shrimp postlarvae to bury. Strongly significant negative correlations were found between final total suspended solids and both average and final dissolved oxygen concentrations ($r = -0.732$, $p < 0.001$; $r = -0.602$, $p = 0.005$, respectively for *L. vannamei*, and $r = -0.827$, $p < 0.001$; $r = -0.780$, $p < 0.001$, for *F. brasiliensis*).

Non-ionised ammonia, total ammonia and nitrite in control tanks

Since control tanks had no postlarvae, it can be considered that their Figures represent water quality as it entered the experimental tanks. For the *Litopenaeus vannamei* experiment, non-ionised ammonia fraction in the control tank without animals (ISD = 0) started at 0.27 mg L^{-1} at day 1, reached 0.94 mg L^{-1} at day 4, and by day 12 concentration dropped to 0.24 mg L^{-1} (Fig. 6.4 A). Total ammonia concentration attained 11 mg L^{-1} on day 10-14, but decreased to as low as 3-4 mg L^{-1} from day 21 onwards (Fig. 6.4 B). Nitrite concentration rose steadily to reach a maximum of 8.9 mg L^{-1} on day 14, after which it decreased to 2.5 mg L^{-1} at the end of the experiment (Fig. 6.4 C). For the *Farfantepenaeus brasiliensis* experiment values of non-ionised ammonia, total ammonia and nitrite in the control tanks were below those of the *L. vannamei* trial. Non-ionised ammonia started at 0.14 mg L^{-1} at day 1, and fluctuated between a minimum of 0.058 mg L^{-1} at day 9 and a maximum of 0.27 mg L^{-1} at day 14, throughout the experimental period (Fig. 6.5 A). Total ammonia concentration attained 4.8 mg L^{-1} on day 14, and oscillated between 2.7 to 3.9 mg L^{-1} from then onwards (Fig. 6.5 B) in an overall pattern much like the one of non-ionised ammonia fraction. Nitrite concentration started at 0.01 mg L^{-1} at day 1 and then rose steadily, but never went above 0.42 mg L^{-1} (Fig. 6.5 C). In other tanks with animals, the rise of total ammonia was followed by the increase of nitrite, which in turn was followed by an accumulation of nitrates in both experiments (Figs. 6.4, 6.5). This evolution reflects

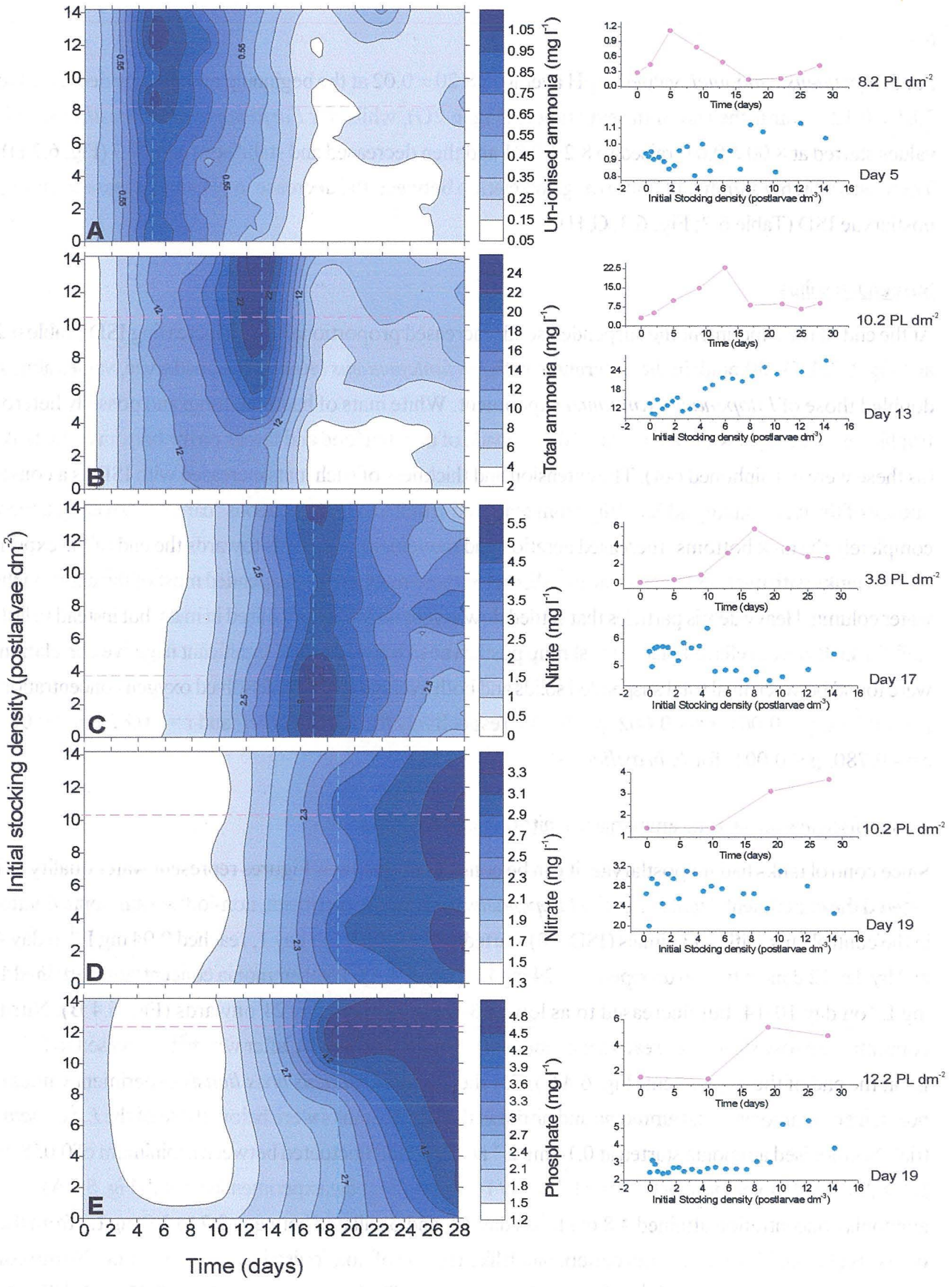


Figure 6.4 – Non-ionised ammonia fraction (**A**), total ammonia (**B**), nitrite (**C**), nitrate (**D**) and phosphate (**E**) contour plots based on 21 initial stocking densities (ISD) of early *Litopenaeus vannamei* postlarvae (Table 1) throughout the experimental period (28 days). Values of postlarvae density in dm^{-2} apply only to Figures; text uses the equivalent postlarvae m^{-2} (postlarvae $dm^{-2} \times 100$). Graphs on the right present real data points along respective transect lines of contour plots on the left. Refer to text for details on the interpolation procedures used.

the succession of nitrifying microbe populations that have been previously described in seawater environments (Wheaton *et al.* 1994).

Total ammonia

In the *Litopenaeus vannamei* trial, total ammonia values increased steadily to reach a maximum of 27 mg L^{-1} in the tank with the highest ISD at days 10-12 (Fig. 6.4 B). The extreme concentrations around these days declined shortly after (day 14), and within 4 days nearly all tanks were below 8 mg L^{-1}. From day 16 onwards, concentrations of total ammonia stabilised at levels as low as the control tank levels, and returned to their initial concentrations in tanks with 400 or less postlarvae m^{-2}. In the *Farfantepenaeus brasiliensis* trial, total ammonia values increased and reached a maximum of 17.8 mg L^{-1} in the tank with the second highest ISD at day 14 (Fig. 6.5 B). Such maximum concentration was considerably lower than that of the *L. vannamei* trial and although it also declined shortly after, total ammonia did not return to the initial values. In fact, tanks with 580 or more postlarvae m^{-2} presented total ammonia concentrations above 7 mg L^{-1} throughout the rest of the experimental period (Fig. 6.5 B). Furthermore, the increase in total ammonia concentration was not as homogeneous throughout the ISD range as in the *L. vannamei* trial, and an effect of ISD can be clearly seen with a rise in total ammonia occurring earlier in tanks with more animals. In both trials, the daily average and the maximum concentration of total ammonia was clearly correlated to ISD (Table 6.2). These variables were also significantly correlated to the final wet weight and relative growth rate of *F. brasiliensis* postlarvae (Table 6.2).

Non-ionised ammonia fraction

For the *Litopenaeus vannamei* experiment, peak concentrations of total ammonia were preceded in time by peaks of the un-ionised ammonia fraction, and rose sharply from day 0, peaked at day 5 and then steadily decreased to concentrations lower than the initial values (Fig. 6.4, A, B). It should be taken into account that these observations are closely related to the high pH levels at the beginning of the trial (Fig. 6.2 G). Concentrations of these two water quality variables increased with increasing ISD. A total ammonia concentration threshold was observed at approximately 0.04 postlarvae m^{-2}, below which all tanks had similar concentrations (< 16 mg L^{-1} Fig. 6.4 B). A similar non-ionised ammonia fraction concentration threshold was observed at approximately 700 postlarvae m^{-2}, although it occurred earlier in the experimental period (0.95 mg L^{-1} Fig. 6.4 A). Such a pattern was not as evident in the *Farfantapenaeus brasiliensis* experiment where both the un-ionised ammonia fraction, and total ammonia shared an almost identical concentration distribution and had lower overall concentration values (Fig. 6.5, A, B). The only exception was an early secondary peak of un-ionised ammonia fraction around day 5 in tanks with higher ISD that coincided with the maximum pH registered during the experimental period (Fig. 6.2 H).

Nitrite

In the *Litopenaeus vannamei* trial, nitrite concentration was below 1 mg L^{-1} until day 9, when it started to rise steadily to peak at days 16-18 (6.4 mg L^{-1} at 440 postlarvae m^{-2}, Fig. 6.4 C), whereupon it decreased to levels as low as 0.22 mg L^{-1} even in tanks with the highest ISD. Nitrite concentration started to increase

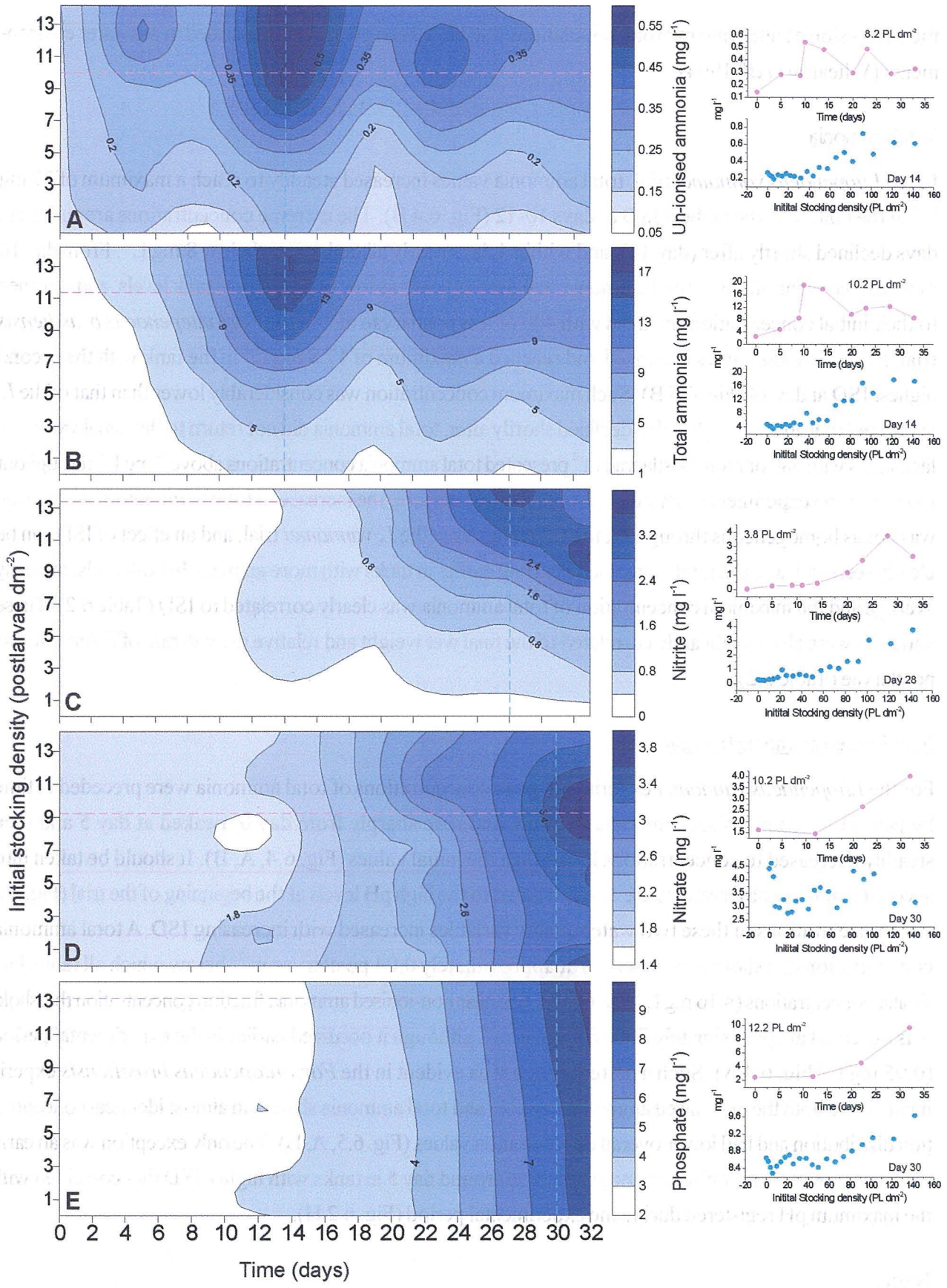


Figure 6.5 – Non-ionised ammonia fraction (**A**), total ammonia (**B**), nitrite (**C**), nitrate (**D**) and phosphate (**E**) contour plots based on 21 initial stocking densities (ISD) of early *Farfantepenaeus brasiliensis* postlarvae (Table 6.1) throughout the experimental period (32 days). Values of postlarvae density in dm^{-2} apply only to Figures; text uses the equivalent postlarvae m^{-2} (postlarvae $dm^{-2} \times 100$). Graphs on the right present real data points along respective transect lines of contour plots on the left. Refer to text for details on the interpolation procedures used.

when total ammonia values were above 12 mg L^{-1} and peaked approximately 4 days after the total ammonia concentration reached its maximum. The nitrite peak, however, presented the highest nitrite concentrations in tanks with low ISD. A completely different pattern resulted from the *Farfantepenaeus brasiliensis* trial observations where overall nitrite accumulation reached a much lower concentration, the increase started only around day 12 and there was a strong and highly significant strong relation to the ISD gradient at day 28 when the maximums were observed (Table 6.2; Fig. 6.5 C).

Nitrate

Nitrate concentration started to increase around day 10 in both experiments and reached maximums of 3.6 and 4.0 mg L^{-1} towards the end of the experimental period for *Litopenaeus vannamei* and *Farfantepenaeus brasiliensis*, respectively (Figs. 6.4, D and 6.5, D). There was no clear positive relationship between NO_3^- and ISD.

Phosphate

Phosphates began to accumulate at the same time as nitrates, and showed a trend to increase with ISD in the *Litopenaeus vannamei* trial with a clear peak of >5 mg L^{-1} around day 19 (Fig. 6.4 E), although final values were not significantly correlated to ISD ($r = 0.369$, $p = 0.100$). In the *Farfantepenaeus brasiliensis* trial, initial phosphate concentration was higher and reached much greater values at the end of the experimental period with a strong and highly significant relation to ISD ($r = 0.835$, $p < 0.001$), although at day 28 phosphates values were only around 1 mg L^{-1} above the equivalent date values of the *L. vannamei* trial.

Poslarvae mortality

Litopenaeus vannamei postlarvae mortality began on days 3-4 and affected tanks with ISD of 150 to 600 postlarvae m^{-2} (Fig. 6.6 A). It was within this range of ISD that percentage mortality continued to increase steadily reaching maximum values at the end of the experimental period. Animals at approximately 900 postlarvae m^{-2} were also affected, although by day 22, percentage mortality was only 30%, much less than the percentage mortalities (above 50%) observed since day 14 at ISD 150 to 600 postlarvae m^{-2}. The highest mortality (59 %) was observed at 580 postlarvae m^{-2} during the last days of the experimental period. The only two tanks maintaining initial stocking density throughout the experiment were those with 20 and 100 postlarvae m^{-2}. Surprisingly, the tanks with the highest ISD gave relatively low mortality (10-20%). For the *Farfantepenaeus brasiliensis* trial, the postlarvae maintained at densities between 0 and 180 postlarvae m^{-2} were the most affected, reaching a maximum of 78.6% mortality at 100 postlarvae m^{-2}. However, such results may be a consequence of calculation artefacts since few dead animals would cause exceptionally elevated % mortalities, due the low number of animals per tank at the low initial stocking densities (Table 6.1). For the rest of the tanks, all mortality occurred between day 10 and 15, after which population numbers remained unaltered for the rest of the experimental period (Fig. 6.6 B). Mortality was gradual and constant in the *L. vannamei* experiment, suggesting a continuous lethal/negative pressure, whilst for *F. brasiliensis* it was concentrated in high mortality between day 10 and 15.

If the unusually low mortalities at the three highest ISD in the *Litopenaeus vannamei* experiment are excluded, the relationship between the number of dead postlarvae and ISD is linear (Fig. 6.7 A, B), indicating that the effect of ISD is of an additive nature: the number of dead postlarvae increases proportionally with increasing ISD. Relative mortality rate showed no significant correlation with ISD (Table 6.2; Fig. 6.7 C, D). Such observations suggest that the speed at which mortality occurred can not be explained by the ISD gradient alone, inferring that other factors were responsible for observed relative mortality rates in both species. However, if the three highest ISD treatments from *L. vannamei* are excluded from calculations, there is a significant positive correlation with ISD ($r = 0.551$, $p = 0.022$), which raises some confusion in the interpretation of these results since the low mortalities from the three highest ISD treatments were unexpected. Potential explanations for these three observations are suggested and debated separately in the discussion section.

Many postlarvae died stuck on the dry sides of tanks at the highest density treatments during the first days of the experiment, especially in the *Farfantepenaeus brasiliensis* experiment where postlarvae had to be replaced during the first 7 days to maintain ISD (Fig. 6.8 A). This behaviour was also observed for *Litopenaeus vannamei* although to a much lesser extent. As animals grew, overcrowding in the higher ISD tanks induced a general chain reaction of escape responses that eventually caused mortality by shrimp jumping out of the tanks. Although death due to this behaviour rose linearly with increasing ISD, the proportion of dead postlarvae in each tank showed a bell shape suggesting that at higher ISD animals acclimate to overcrowding conditions and reduce the intensity of the chain-reaction escape response behaviour.

Postlarvae stress resistance

For *Litopenaeus vannamei*, results from the physiological stress test show that the animals' resistance to a drastic salinity change increases with ISD, as the proportion of dead animals decreased along the ISD gradient (Fig. 6.7 E). The animals' stress resistance shows a strong negative linear relation to ISD (Table 6.2), although a second order polynomial fits the data better (Fig. 6.7 E). It was also negatively correlated to the maximum observed total ammonia concentration and to the average (28 days) total and non-ionised ammonia fraction concentrations (Table 6.2). Although not significant at a probability level of 0.05, the animals' stress resistance was positively correlated to the observed maximum nitrite concentration (Table 6.2), reflecting an inverse relation to the change in this variable. *Farfantepenaeus brasiliensis* postlarvae, on the other hand, showed a stronger resistance to the osmotic stress test with a much lower proportion of dead animals and with a non-significant relation between resistance and ISD (Table 6.2; Fig. 6.7 F).

Postlarvae growth

The overall increase in weight, averaging sampled animals from all tanks every 7 days, is represented in Figure 6.8 A, B. Although *Litopenaeus vannamei* postlarvae grew in average larger than *Farfantepenaeus brasiliensis* and in a shorter period of time (7 days difference), this later species grew more steadily, whilst *L. vannamei* more than doubled in weight in the last 7 days of the experiment (Fig. 6.9 A, B). The evolution of such species' differential weight gain through time can be followed by ISD in Figures 6.6 C and D. Both species grew faster in lower ISD treatments. This difference in other treatments starts to be evident from day

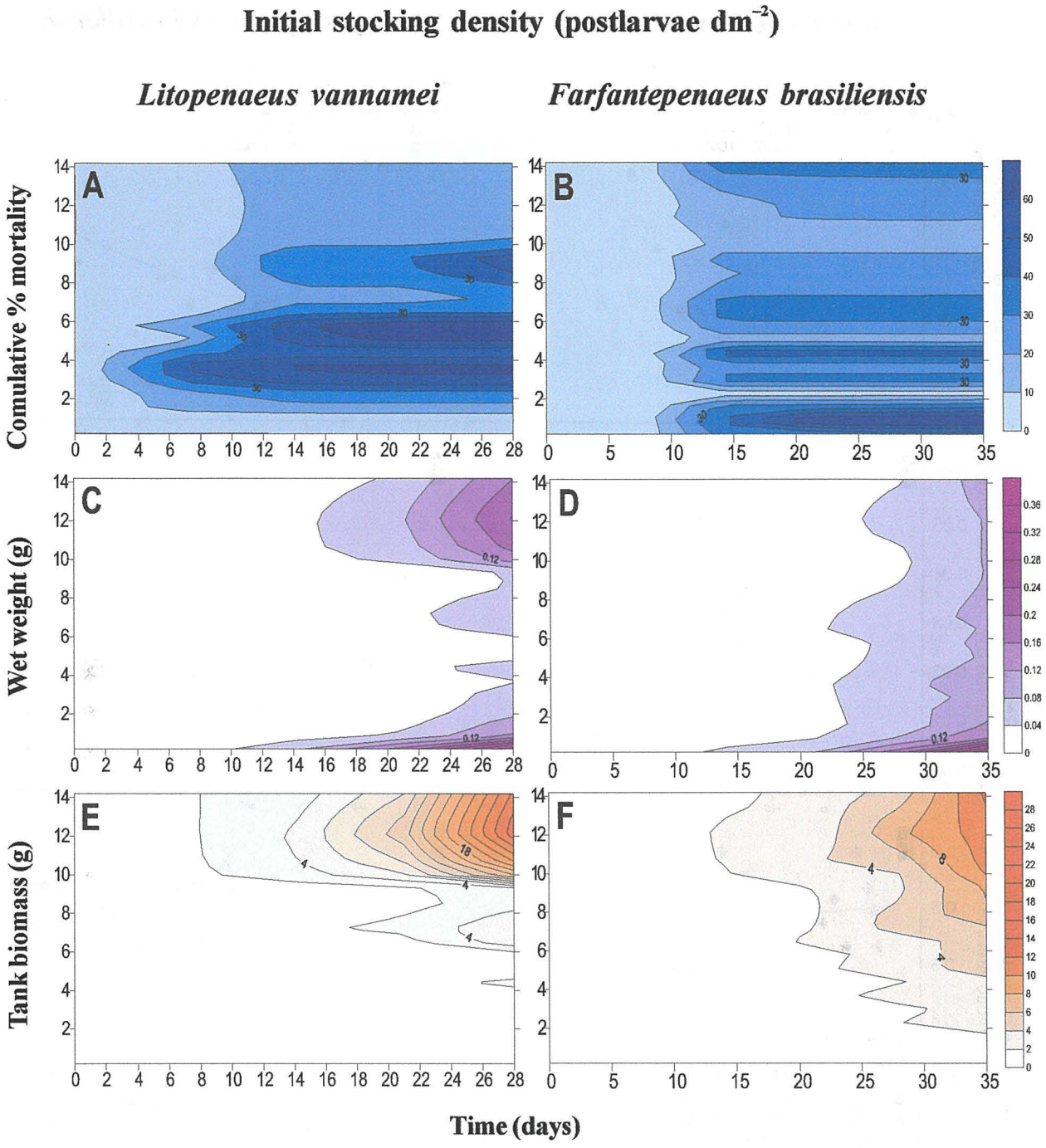


Figure 6.6 – Evolution of percentage mortality, mean individual wet weight and total biomass per tank in contour plots based on 21 initial stocking densities (ISD) of early *Litopenaeus vannamei* **(A, C, E)** and *Farfantepenaeus brasiliensis* **(B, D, F)** postlarvae dm^{-2} throughout the experimental period (28 and 32 days, respectively).}

14 onwards and culminates at the end of each experimental period with high wet weight peaks at the inferior right corner of the contour plots, representing animals with 0.32 g or more (Figs. 6.6 C, D). For other ISD, the important weight gain occurred in the last 10 days of each experiment, with *L. vannamei* postlarvae growing well in the three highest ISD treatments. *F. brasiliensis*, on the other hand, grew poorly at the higher ISD, but growth was more homogeneous throughout the ISD range. These weight gain patterns are better represented in the final individual average weight from both experiments shown in Figures 6.8 C and D. Postlarvae kept at the lowest ISD were 2 and 3 times larger than postlarvae from higher ISD treatments

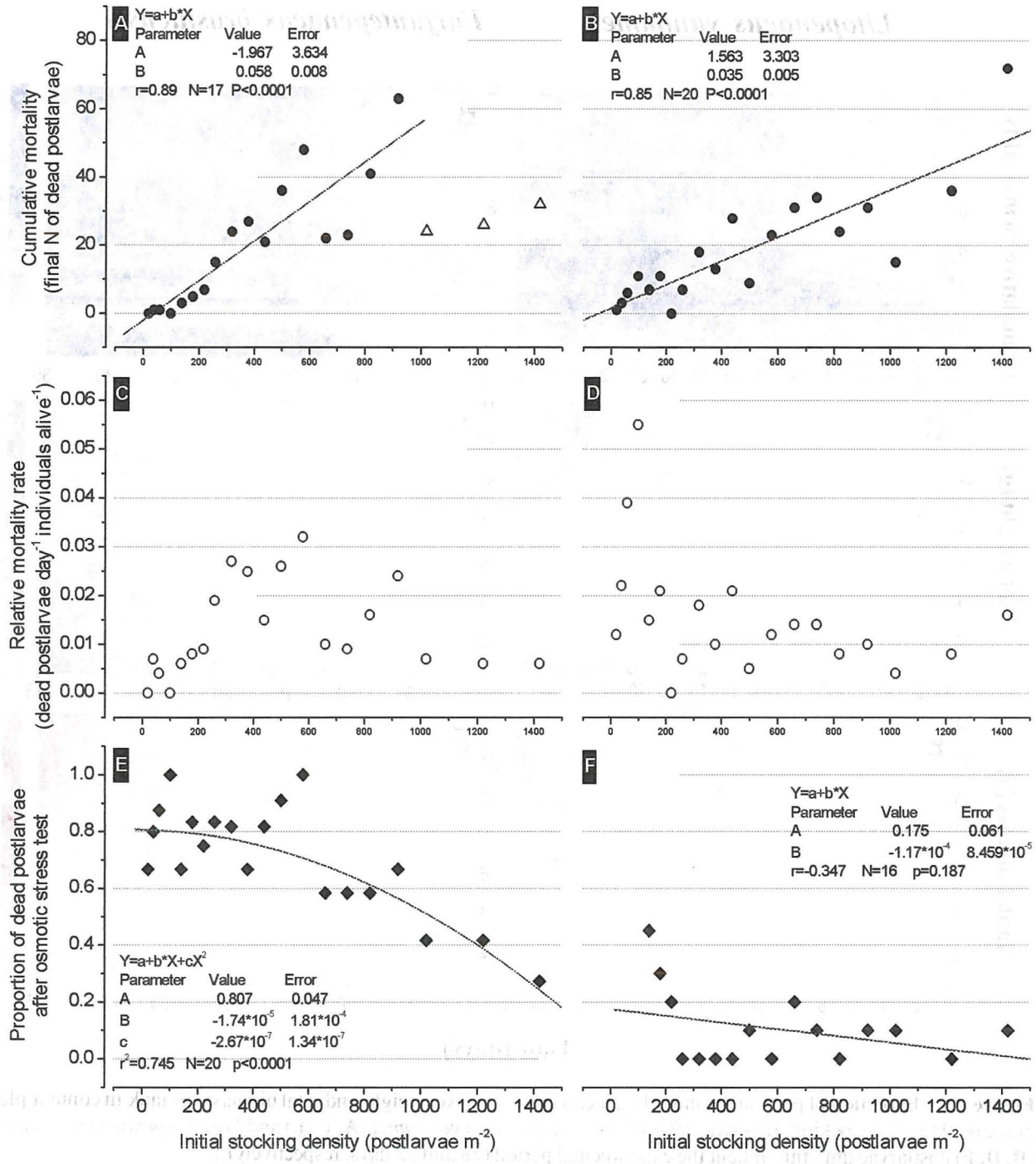


Figure 6.7 – Relation between initial stocking density and cumulative mortality, relative mortality rate and resistance to osmotic stress tests for *Litopenaeus vannamei* and *Farfantepenaeus brasiliensis* postlarvae. Different symbols for the last three points in *L. vannamei* Figure **A** represent unusual or unexpected observations – see text for discussion.

with few exceptions. For *L. vannamei*, if the unusual large weight gains of the three highest ISD are not included, final weight after 28 days shows once more an exponential decay in relation to ISD (Fig. 6.9 C), a pattern very similar to *F. brasiliensis* equivalent observations (Fig. 6.9 D), that strongly suggests that increasing ISD reduces shrimp postlarvae growth. However, despite a significant increase in variance, the three highest ISD *L. vannamei* tanks produced in average larger animals than almost all other tanks (Fig.

6.9 C). Potential explanations for these three unexpected observations are suggested and discussed separately in the discussion section.

Relative growth rate of postlarvae followed similar exponential decay patterns to those of final weight gain, indicating that animals grew proportionally slower as ISD increased (Fig. 6.9 E, F). However, for *Litopenaeus vannamei*, although this relationship was true for experimental tanks with ISD from 20 to 920 postlarvae m^{-2}, tanks with the three highest ISD presented relative growth rates as high as 0.16 mg day^{-1} individual weight $^{-1}$ (Fig. 6.9 E). Once more, potential explanations for these three unexpected observations are suggested and debated separately in the discussion section.

Relation between postlarvae size and available area

Assuming that the total length of a shrimp postlarvae is directly related to its ventral surface area projected on the bottom of a tank, an equivalent for each tank specific total "occupied" area was estimated adding the lengths of all postlarvae and dividing by the tank average bottom area at the beginning and end of the experiments. These results are plotted in Figure 6.10 A and B for *Litopenaeus vannamei* and

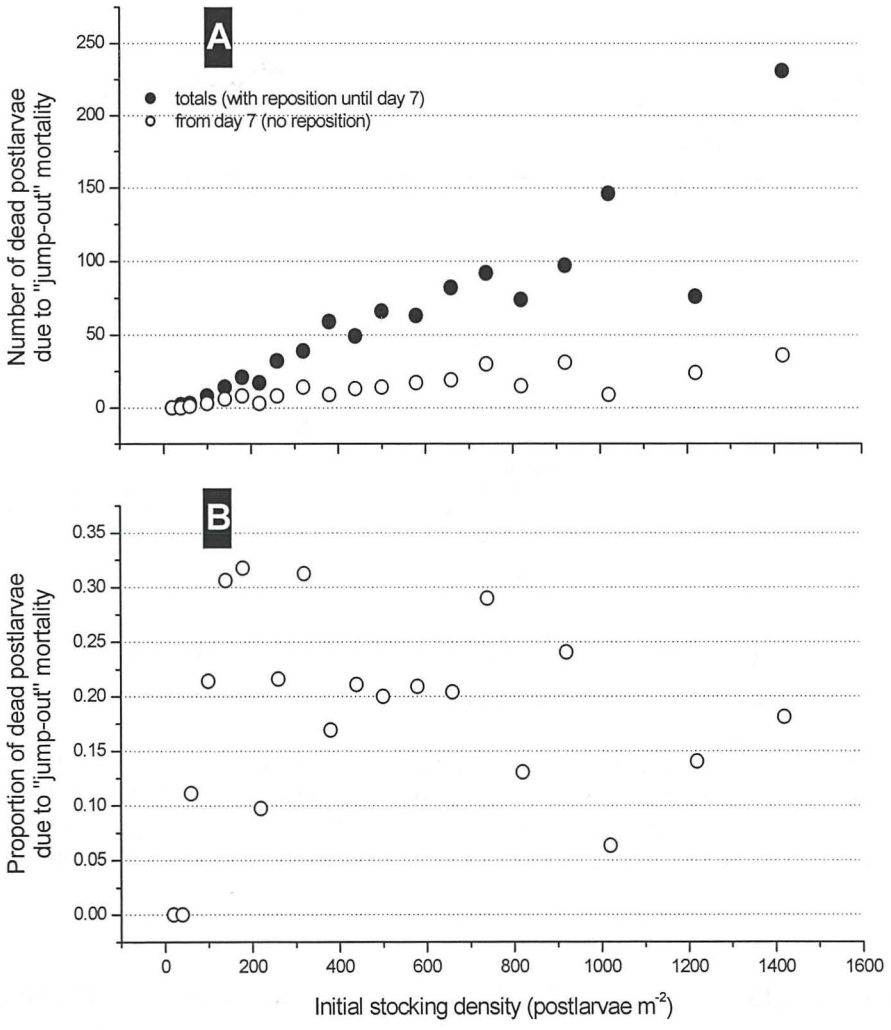


Figure 6.8 – *Farfantepenaeus brasiliensis* postlarvae "jump-out" mortality. **A** – Dead postlarvae over the entire experimental period (32 days) with reposition of dead animals in the first 7 days, and with no reposition during the last 26 days. **B** – Proportion of the initial number of postlarvae per tank that died due to "jump-out" mortality over the period of no-reposition.

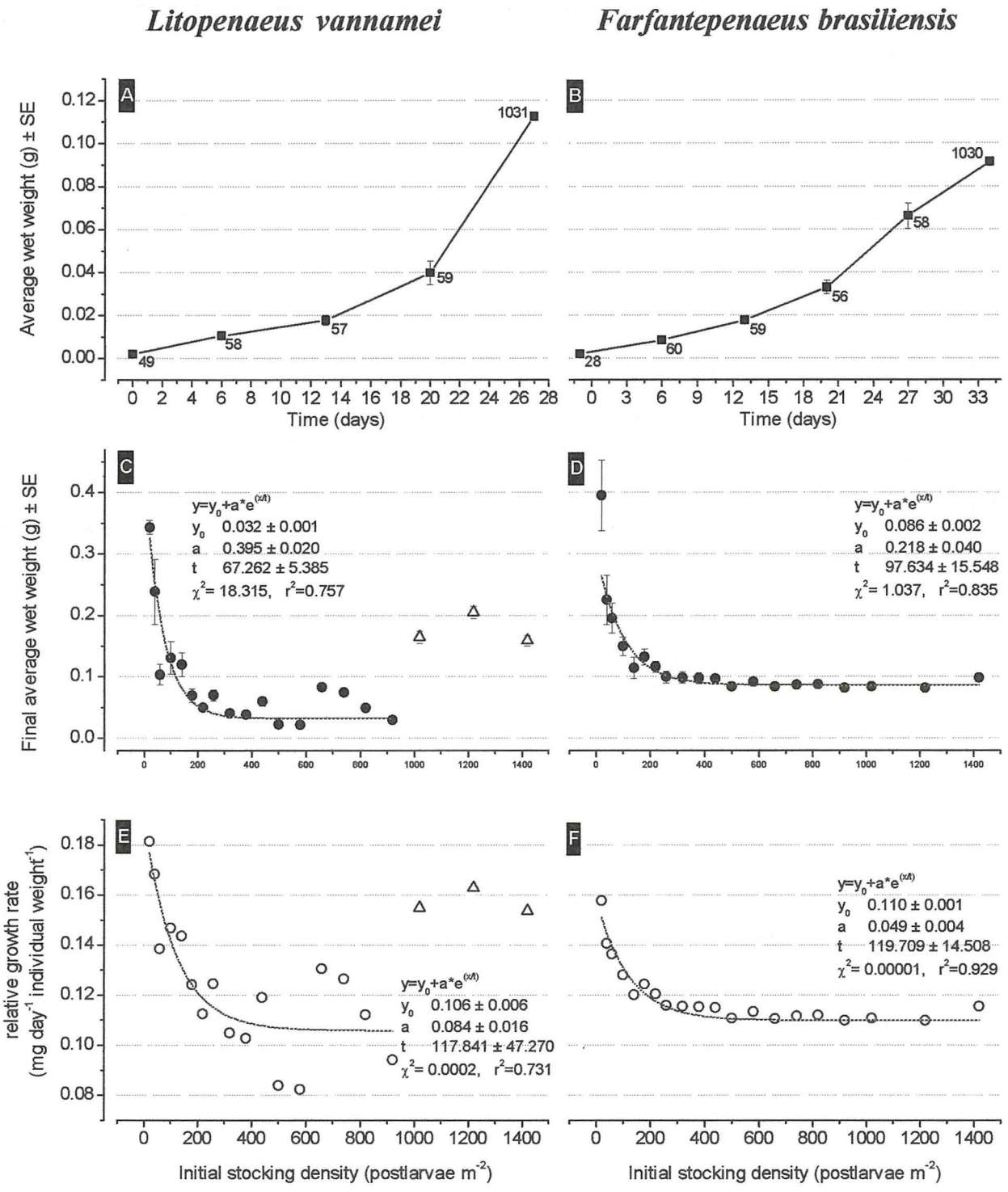


Figure 6.9 – (**A**, **B**) Increase in mean individual wet weight of postlarvae with time; data for all treatments was pooled. (numbers represent sampled animals); (**C**, **D**) final mean individual wet weight per tank; and (**E**, **F**) relative growth rate along the initial stocking density gradient for *Litopenaeus vannamei* and *Farfantepenaeus brasiliensis* postlarvae. Different symbols for the last three points in *L. vannamei* Figures **C** and **E** represent unusual or unexpected observations – see text for discussion.

Farfantepenaeus brasiliensis, respectively. Both species start with a very similar relation between available and occupied area that was linearly related to the increase of ISD due to homogeneous postlarvae length. At the end of the experimental periods results were influenced by both mortality and growth of the animals in each tank. *L. vannamei* postlarvae at ISD between 200 and 600 m^{-2} did not increase the relation

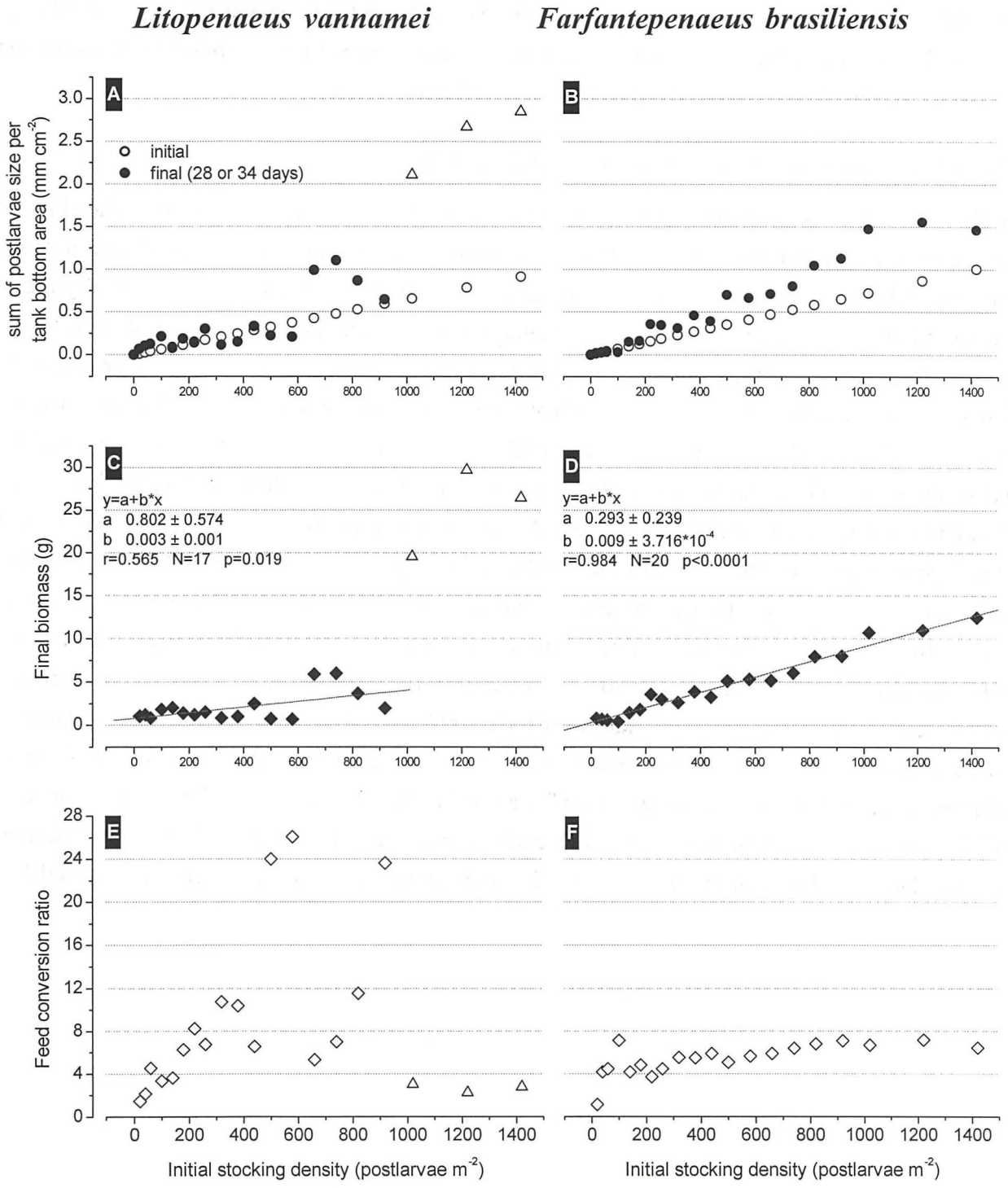


Figure 6.10 – (**A**, **B**) Relation between the sum of the length of all postlarvae and mean tank bottom area at the beginning and end of experiments; (**C**, **D**), final tank biomass; and (**E**, **F**) feed conversion ratio along the initial stocking density gradient for *Litopenaeus vannamei* and *Farfantepenaeus brasiliensis* postlarvae. Different symbols for the last three points in *L. vannamei* Figures **A**, **C** and **E**, represent unusual or unexpected observations – see text for discussion.

between occupied and available area due to high mortalities and reduced final lengths (Figs. 6.6 A and 6.9 C). The three highest ISD tanks showed an important increase in the occupied area by postlarvae due to the low mortalities and large final lengths (Figs. 6.6 A and 6.9 C). For *F. brasiliensis*, the large final weight of postlarvae from the lowest ISD tanks (< 200 postlarvae m^{-2}) was not enough to compensate the large

mortalities, and the occupied area remained practically the same as at the beginning of the experiment (Figs. 6.6 B and 6.9 D). Since from ISD larger than 200 postlarvae m^{-2}, growth and mortality were almost constant (Figs. 6.7 B, D and 6.9 D), the increase in occupied area was linear.

Final biomass and feed conversion ratio (FCR)

For both *Litopenaeus vannamei* and *Farfantepenaeus brasiliensis,* increase in tank population biomass through time can be followed along the ISD range in Figures 6.6 E and F. Despite the shorter period of growth (28 days), *L. vannamei* harvested a total of 110 g, whilst *F. brasiliensis* reached only 94 g after 34 days of culture. This important difference was mainly due to the large numbers and weight of the animals from the three highest ISD *L. vannamei* tanks. For *L. vannamei*, final biomass yield per tank was approximately constant in tanks with 20 to 600 animals m^{-2}, mainly due to the heavier animals from the lower ISD treatments (Fig. 6.10 C). Biomass in tanks with ISD above 600 animals m^{-2}, however, increased markedly as ISD increased and reached more than 20 g per tank. For *F. brasiliensis* the final biomass yield per tank showed a linear relation with ISD (Fig. 6.10 B). A similar relation can also be found for *L. vannamei* if the three highest ISD treatments are excluded from calculations (Fig. 6.10 C). If, however, these unusual observations are considered, then the overall pattern of increase can be described with a simple power function (Fig. 6.10 C, not draw). The FCR of both species increased in relation to ISD (Figs. 6.10 E, F). *L. vannamei* postlarvae reached a peak at 26.1 and showed more variation in the feed assimilation efficiency with three tanks above 20, clearly indicating overfeeding, probably due to incorrect survival assessment and consequent exaggerated weekly feed adjustment. *F. brasiliensis* postlarvae showed a more stable pattern of increase in the FCR with a tendency to stabilize around FCR 7. These results indicate poor assimilation efficiency at ISD above 40 postlarvae m^{-2}. Once again such conclusions were possible with the exclusion of the three highest ISD *L. vannamei* treatments. These three tanks showed FCRs between 2.25 and 3.

DISCUSSION

Initial stocking density

Several studies using postlarvae of different penaeid species show survival and growth data results from a broad range of initial stocking densities, up to 350 postlarvae m^{-2} (AQUACOP 1984; Sandifer *et al.* 1988; Hopkins *et al.* 1993; Williams *et al.* 1996; Palomino *et al.* 2001). Samocha *et al.* (1998a) report stocking densities of 700 *Litopenaeus setiferus* postlarvae m^{-2} (PL_6) with 78% survival and 0.071 g day^{-1} growth rate after 120 days. The range of initial stocking densities tested in the present study goes to extreme values of 1420 postlarvae m^{-2}. Best average weight gain per animal and growth rate results were achieved with densities below 200 postlarvae m^{-2} for both species (Figs. 6.6 C, D and 6.9 C, D, E, F) and equally for *Litopenaeus vannamei* survival (Figs. 6.6 A, 6.7 A, C). For *Farfantepenaeus brasiliensis*, these were the densities at which heavier mortalities occurred (Fig. 6.6 B, 6.9 D), although a random effect would have a bigger impact on the % mortality outcome due to the small number of animals at such lower densities (<200 postlarvae m^{-2}, equivalent to 28 experimental animals). Above 200 postlarvae m^{-2} there was no advantage in terms of growth for either species, although mortality was unexpectedly lower even at much higher densities. Such high numbers of surviving animals result in an interesting final biomass situation. If early postlarvae are readily available, the results presented here suggest that the higher the stocking density, the higher the biomass yield (Fig. 6.10 C, D), despite reduced feed conversion ratio, which above 200 postlarvae m^{-2} remains practically constant for both species (Figs. 6.8 I, J). Such results are in accordance with the biomass linear relation to stocking density reported by Sandifer *et al.* (1988), although they only report values up to 30 postlarvae m^{-2}. Unexpected low mortality, high final average weight and high relative growth rate of the three highest *L. vannamei* ISD tested should be interpreted with caution since they are outside the general trend seen in other tanks and *F. brasiliensis* results, and may represent occasional favourable conditions. Further experiments are needed to confirm the repeatability of these observations.

Toxicity of non-ionised ammonia

Present results are explicable by assuming that apart from bacteria in the biofilter, active nitrifying bacterial films also developed in tanks contributing to the nitrification process. Correlation between ISD and the consecutive alternation of rise, peak and fall of total ammonia, nitrite and nitrates concentration, confirms indirectly the presence of active nitrifying bacterial films specific to each tank (Figs. 6.4, 6.5). It is therefore hypothesised that the differences in the magnitude of concentration peaks and their occurrence in time varied among tanks due to the establishment of nitrifying bacterial communities at different stages of development or maturity. Assuming constant flow in and out of tanks, the presence and quantity of nitrogen compounds depends strongly on the quantity of food given (degradation by heterotrophic bacteria), number of animals present (excretion), and the maturity stage of the nitrifying bacterial community (extra-production and degradation), amongst other factors (Forster 1974; Mevel & Chamroux 1981; Huguenin & Colt 1992; Wheaton *et al.* 1994). The differential production and consumption of such compounds must be directly associated to ISD, and it is believed that variations in their availability conditioned the nitrifying bacterial flora in each tank.

The un-ionised form of ammonia (NH_3-N) is considered to be toxic to most aquatic life (Armstrong *et al.* 1978; Chin & Chen 1987) and its accumulation together with nitrite in culture systems with unconditioned biological filters, or little water exchange, causes mortality and affects growth of cultured animals (Colt & Armstrong 1979; Spotte & Adams 1983). Hence, this discussion concentrates on the analysis of these two toxic chemicals. Water from the reservoir flowing through the tanks was not enough to dilute or maintain non-ionised ammonia and nitrite concentrations as low as in the control, and consequently concentrations rose linearly with ISD (Figs. 6.4 and 6.5 A, C). In addition, the water from previous trials used to maintain the continuity of the bacterial flora in the recirculation system seems to have contributed to the initial higher-than-normal concentrations of total ammonia (2 – 4 mg L^{-1}, Fig. 6.4 B, 6.5 B). The pH is the determining factor in the equilibrium between the un-ionised and the ionised ammonia forms (Whitfield 1974; Johansson & Wedborg 1980), and is thus closely related with the levels of ammonia toxicity. Therefore, the initial high pH values in the *Litopenaeus vannamei* experiment (8.2 until day 5, Fig. 6.2 G) explain the early rise and peak of non-ionised ammonia at day 5, seven days before the maximum concentrations of total ammonia were reached (Fig. 6.4, A, B). The same occurred in the *Farfantepenaeus brasiliensis* experiment, where a rise of pH on day 5 (Fig. 6.2, H) produced a peak of non-ionised ammonia fraction at the higher ISD, although it was of considerably less amplitude (Fig. 6.5 A). Palomino et al (2001), working with *Litopenaeus setiferus*, observed that the un-ionised ammonia fraction and nitrite concentrations increased with time and stocking density, attaining the highest values in shrimp stocked at 350 postlarvae m^{-2} and 0% water exchange (0.23 and 6.7 mg L^{-1}, respectively) and the lowest at 50 shrimp m^{-2} and 18% water exchange (0.007 and 0.7 mg L^{-1}, respectively). Chen and Tu (1991), working with *Penaeus monodon* large postlarvae (1.43 ± 0.03 cm), determined that a sub-lethal non-ionised ammonia fraction concentration as low as 70 µg L^{-1} reduced growth (weight gain) by 50% in *P. monodon* postlarvae over an 8-week experimental period. Lin *et al.* (1993), similarly, found that the susceptibility of *Marsupenaeus japonicus* to non-ionised ammonia increased during and just after the moult in larvae, post-larvae and juveniles. In the present study concentration of non-ionised ammonia in the majority of tanks was above such values throughout the experimental periods with postlarvae of all tanks experiencing non-ionised ammonia fraction concentrations 100 times higher during the first days, with some tanks attaining even higher concentrations (Fig. 6.4 and 6.5 A). The observed non-ionised ammonia maximum values (1.12 and 0.73 mg L^{-1} for *L. vannamei* and *F. brasiliensis*, respectively) were within previously reported values of LC_{50} (concentration at which 50% of the population dies within a specific period of time; Fig. 6.11A). However, percentage mortality of *L. vannamei* postlarvae subjected to non-ionised ammonia concentrations of 0.95 mg L^{-1} or above was always below 10% at such peak concentrations time, demonstrating the apparent innocuous effect of these high concentrations on survival. For *F. brasiliensis*, on the other hand, percentage mortality at the time of non-ionised ammonia peak concentrations (0.50 mg L-1 and above) was between 20 and 40% (Figs. 6.5 A and 6.6 B). It was expected that such high values would have had a strong effect on survival and growth of *L. vannamei* and *F. brasiliensis* postlarvae. For *F. brasiliensis* postlarvae, maximum non-ionised ammonia fraction was, indeed, inversely related to final wet weight and relative growth rate (Table 6.2). However, for *L. vannamei*, despite higher concentrations, the maximum un-ionised ammonia fraction concentration was not linearly related to any zootechnical variable (Table 6.2). Toxicity studies that use LC_{50} tend to overestimate concentration effects because acclimation periods are not used. Sprague (1969) argues that the short-term LC_{50} values (especially 24-h LC_{50}) can be very misleading, and recommends that toxicity should be described in terms of incipient LC_{50} or lethal threshold concentrations (levels of toxicant which are lethal for 50% of the individuals

exposed for periods that are sufficiently long for the acute lethal action to cease – the level beyond which 50% of the population cannot live for indefinite time). In the present study, shrimps were exposed to highest observed non-ionised ammonia concentrations only for short periods, and after gradual acclimation. The postlarvae physiological stress resistance, shown in Figure 6.7 E, F, increases significantly in animals that were exposed to higher concentrations of both non-ionised and total ammonia, suggesting that these animals were more capable of dealing with an osmotic stress situation. Moreover, the resistance of postlarvae to ammonia and nitrite toxicity also increases as they grow, as a result of the development of the osmoregulatory system (Dr. Rosas pers com.). LC_{50} values in Figure 6.11 only show results of animals exposed for 72 – 96 h. However, all shrimps in the present study were exposed for approximately 13 days to concentrations of non-ionised ammonia equivalent or higher than the lowest value of LC_{50} reported, yet the highest observed mortality was below 50%. These results constitute further evidence of Sprague's argument, and demonstrate that early postlarvae can withstand and acclimate to relatively high concentrations of non-ionised ammonia for long periods of time without major loss in survival and growth. Hence, intensification of postlarva stocking density to increase biomass production is possible under recirculation conditions, even if the animals are exposed to relatively high concentrations of non-ionised ammonia fraction.

Toxicity of nitrite

Nitrite is known to convert normal haemoglobin of fish to methaemoglobin, which is incapable of binding with oxygen causing hypoxia and cyanosis (Lewis & Morris 1986). Crustaceans, however, contain haemocyanin instead of haemoglobin that can be transformed into methaemoglobin (Russo 1985). The active binding place of haemocyanin contains two copper ions that change from the un-oxidised (deoxihaemocyanin) to the oxidised form (oxihaemocyanin) in the presence of oxygen (Chen & Cheng 1996). Tahon *et al.* (1988) have demonstrated that the reaction of nitrite with deoxihaemocyanin is 15 times faster than with oxihaemocyanin in the *Astacus leptodactilus* crayfish haemolymph, suggesting that the oxygen transport capacity of the haemocyanin is greatly reduced in the presence of nitrite.

Nitrite can accumulate to millimolar concentrations in crustacean haemolymph against a considerable concentration gradient, react with haemocyanin forming methaemocyanin, and have other physiological effects, such as impairment of gill chloride ion uptake and haemolymph pH balance depending on environmental pH, available oxygen and chloride and potassium ions concentration in the haemolimph and in the water (Harris & Coley 1991; Jensen 1995; Chen & Cheng 1995; Chen & Lee 1997). Some authors, however, observed that haemocyanin still had its oxygen binding properties in the presence of nitrite (Needham 1961; Wickins 1976). Jensen (1990) submitted the crayfish *Astacus astacus* to different nitrite concentrations and observed that the molecule was rapidly accumulated in the extracelular spaces inducing a haemolymph pH increment (alkalosis) due to the increase in CO_2 which changes to HCO_3. However, Chen and Cheng (1996) observed that the *Marsupenaeus japonicus* juvenile haemolymph pH decreases with an increase of the nitrite water concentration, which, according to the authors, is related to the formation of HNO_2. Armstrong *et al.* (1976), suggested that the action of nitrite on oxygen transport may be more critical during ecdysis when oxygen consumption increases (Passano 1960). Mawatari and Hirayama (1975) measured the tolerance of *Neptunus trituberculatus* crab larvae during the moulting period to ammonia, nitrite and nitrate and found a remarkable decrease in resistance to ammonia and nitrite during this period. Concentrations above

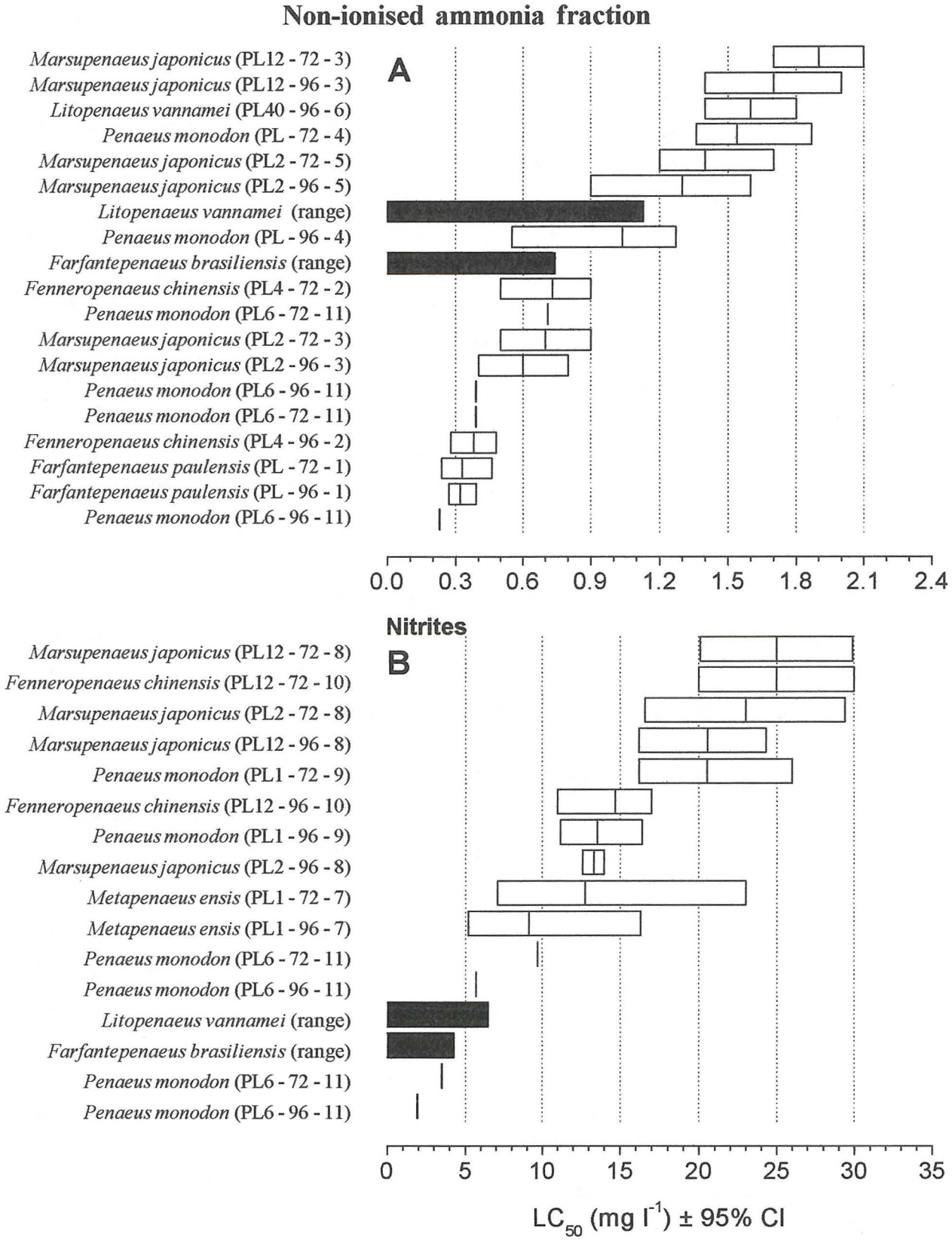


Figure 6.11– Values of lethal concentration of non-ionised ammonia fraction (**A**) and nitrite (**B**) that kills 50% of populations of early postlarvae of several penaeid species within a specific time period (LC_{50} ± 95% CI). Black bars represent the non-ionised ammonia fraction and nitrite range of concentrations observed in the present study expressed in the same units. Since some values were geometrically inferred from graphs with logarithmic scales, some range limits are not symmetrical. "PL" stands for postlarvae; following numbers are 1) the development stage, 2) the time period used for that specific LC_{50} trial, and 3) the number corresponding to the reference: **1** (Ostrensky & Wasielesky 1995); **2** (Chen & Lin 1991); **3** (Chen *et al.* 1989); **4** (Chin & Chen 1987); **5** (Lin *et al.* 1993); **6** (Lin & Chen 2001); **7** (Chen & Nan 1991); **8** (Chen & Tu 1990); **9** (Chen & Chin 1988a); **10** (Cheng & Chen 1994); **11** (Chen & Chin 1988b).

1.8 mg L^{-1} of nitrite seem to adversely affect the rate of metamorphosis to postlarvae of *Fenneropenaeus indicus* (Jayasankar & Muthu 1983), suggesting that the resistance to this toxicant seems to decrease during moult periods. This is particularly relevant for postlarval stages, since they have a higher moulting frequency during early development. Nitrite concentration in the present *Litopenaeus vannamei* experiment peaked at days 16 - 18 in tanks with the lowest ISD (Fig. 6.4 C), whilst, at the same time, mortality further increased in the same tanks (Fig. 6.6 A). Furthermore, the significant negative correlation between the maximum attained nitrite concentration and the number of dead postlarvae at the end of the experimental period ($r = -0.538$, $p = 0.012$) suggests that the nitrite maximum concentration was responsible for the death of *L. vannamei* postlarvae from tanks with approximately 150 to 650 postlarvae m^{-2}. Final postlarval weight was also reduced around these ISD (Fig. 6.9 C). In the *Farfantepenaeus brasiliensis* experiment, not only were nitrite concentrations considerably lower throughout, but it accumulated towards the end of the experimental period and in the higher ISD tanks (Fig. 6.5 C). Final wet weight and relative growth rate were significantly inversely related to the maximum nitrite concentrations reached in the tanks (Table 6.2). Such observations can, perhaps, be explained by a reduced moult rate induced by sub-lethal concentrations of nitrite.

Water quality and initial stocking density

Mortality of *Litopenaeus setiferus* postlarvae reported by Palomino *et al.* (2001) could not be associated with oscillations in water quality variables dependent on initial stocking density. Similarly, Hopkins *et al.* (1993) found no relation between mortality and water quality for *L. setiferus* cultured under different stocking densities. Thus, despite the fact that initial stocking density affects water quality, it may have little effect upon mortality unless extreme concentrations of toxicants are reached, such as the ones reported in the present study for nitrite in the *Litopenaeus vannamei* trial. On the other hand, initial stocking density seems to have an indirect sub-lethal effect on shrimp postlarvae and reflects mainly on growth rates. This effect has been attributed to the synergistic relation between environmental factors (dissolved oxygen, ammonia, nitrite, temperature and pH) and crowding, with the consequent competition for food and space (Williams *et al.* 1996). Sub-lethal concentrations of ammonia and nitrite, together with dissolved oxygen, temperature and salinity, have shown to reduce oxygen consumption, ammonia excretion and consequently the growth rate of several penaeid postlarvae species (Rosas *et al.* 1997; Martinez *et al.* 1998; Rosas *et al.* 1999; Alcaraz *et al.* 1999a; Alcaraz *et al.* 1999b). In the present study, water quality variables generally correlated well with ISD and helped explain the observed patterns in both species' zoo-technical performance variables with the exception of the three highest *L. vannamei* ISD tanks. To explain the unpredicted high survival and growth rates at these tanks it is proposed that the gradual acclimation and momentary exposure to non-ionised ammonia and nitrite toxic concentrations increased tolerance to toxic situations. In addition extra nutrition was available from the natural bacterial coating of debris and less cannibalism occurred during moulting due to burrowing and hiding amongst accumulated bottom debris.

Competition for food

Reduced space probably augments competition for food as the probability of losing a captured pellet when encountering another animal should increase with ISD. However, presumable ingestion of abundant debris may compensate. The decline in dissolved oxygen (despite increased aeration) observed towards the end of the experiments (Fig. 6.2 C, D) and at the higher ISD treatments (Fig. 6.3 C, D) was most certainly due to higher oxygen consumption from larger shrimp postlarvae, but could also be related to intense microbial activity, resulting from the degradation of increased suspended solids, especially in tanks with higher ISD. Such decline in oxygen concentration was proportional to the increment in ISD, and correlated strongly to the large quantities of suspended solids (Fig. 6.3 I, J). Therefore, as ISD increased, animals had a greater chance to re-ingest debris coated with microrganisms, which may provide extra nutrients apart from the ones in the diet. This clearly increased the assimilation efficiency as reflected in the low FCR of the three highest *L. vannamei* ISD tanks (Fig. 6.10 E). These extra nutrients may have given an advantage to the animals in the tanks at the three highest ISD enabling them to cope with the elevated concentrations of non-ionised ammonia, total ammonia and nitrite they experienced in comparison with other tanks.

Competition for space

High concentrations of ammonia and nitrite were not solely responsible for the observed mortality values. The "jump-out" mortality previously described was clearly a consequence of density-induced stress due to reduced space demonstrated in Figures 6.10 A and B, rather than poor water quality. This reaction was more intense for *Farfantepenaeus brasiliensis* but diminished after a week, indicating some acclimation to overcrowding conditions. This reaction was also a consequence of the small area of the tanks used. In tanks with larger areas, the probability of a postlarvae sticking on a tank wall after a tail-flip escape response decreases considerably. Furthermore, as organic mats developed at the bottom of the tanks with higher ISD, many animals were observed digging and hiding among the debris. When mats were disrupted and re-suspended, larger particles settled and accumulated in small "hills" where postlarvae buried themselves, especially in tanks with 660 and over postlarvae m^{-2}. Such accumulation was particularly large in the three tanks with highest ISD. This behaviour may provide an advantage for recently moulted animals avoiding cannibalism.

Temperature, salinity and dissolved oxygen

Average temperature was higher in both tanks with low and high ISD (Fig. 6.3 A, B). Because high temperatures accelerate postlarvae metabolism and affect final weight gain, this temperature pattern increases the difficulty in interpreting changes in postlarvae growth in relation to ISD. However, changes in postlarval growth rate in relation to ISD were more pronounced than changes in temperature (Fig. 6.9 C, D), and despite a moderate correlation to final weight, tank average temperature was not correlated to relative growth rate. These results suggest that other factors played more important roles in determining the final weight gain pattern than did the average water temperature. Variations in salinity were within the accepTable limits for penaeid postlarvae (Parado-Estepa 1998). Average oxygen concentrations never dropped below 5 mg O_2 L^{-1} and were maintained well within normal physiological conditions (Dr. C. Rosas, pers. Comm.).

CONCLUSIONS

Despite the pre-conditioning of water, peaks of non-ionised and total ammonia, nitrite, nitrates and phosphates were observed, following common nitrifying bacterial community dynamics and indicating that the system organic load exceeded the biofilter's capacity and that continuous flow through tanks should have been even greater. The temporal high concentrations of such chemicals are of concern, but can be mitigated by the observed gradual acclimation, an increase in tolerance as animals grow and relatively short exposure times when the biofilter is functioning optimally and a nitrifying bacterial community is allowed to establish in the culture tanks. Mortality increased with ISD, but the relative mortality rate seemed to be independent from ISD. Both growth and relative growth rate decrease exponentially with ISD. The yield of biomass increased with ISD, but conversion ratio augmented with ISD. Despite the reduced available area and the expected problems related to competition for food and space, the unusual observations of reasonable weight gain and relatively low mortalities at the highest tested densities (1100 postlarvae m^{-2} and above) for *Litopenaeus vannamei* can be explained by gradual acclimation and momentary exposure to non-ionised ammonia and nitrite toxic concentrations, the extra nutritional value from re-ingestion of debris coated with bacteria and less cannibalism during moulting due to burrowing and concealment amongst accumulated bottom debris. Data presented here can be used for the mathematical modelling of the carrying capacity of shrimp postlarvae nursery production systems.

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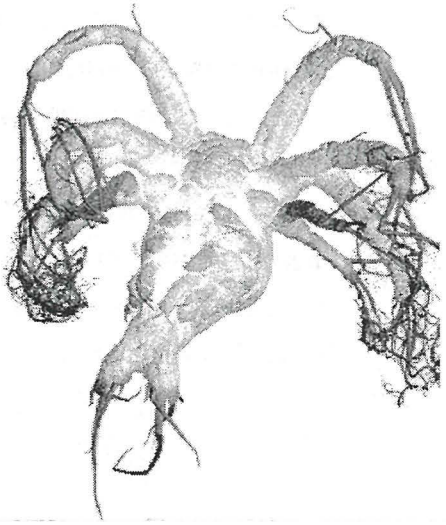
CHAPTER 7
General Discussion



Enhanced survival and growth of penaeid shrimp larvae on microparticulate diets when these larvae are reared in microbially matured seawater is validated. The beneficial effect of a single dose of live microalgae for larval stages feeding upon artificial diets was equally validated. Results indicate that the presence of live microalgal cells improves survival, development and growth as opposed to the addition of either water where the microalgal cells were filtered out, or water with just microalgal culture enrichment nutrients. The larval response to the addition of microalgae as an initiation feed when microdiets (MD) are to be used changes with the microflora composition of the water. Beneficial or detrimental effects can be observed as early as the first two larval development stages, indicating the critical importance of microalgae during the "first-feeding" larval stages. Induction of microbial composition changes in culture water through different mechanical, chemical and biological water treatments produced significant impacts on the larval capacity to survive and moult into the next larval stages as early as during first two larval stages. These results suggest that microalgae can be used as an effective method to manage microbial populations in culture water and that the microbial control strategy used with success for fish larvae (Vadstein *et al.* 1993;Skjermo *et al.* 1997), can be applied to crustacean larvae using microbial matured seawater.

Studies using powerful modern molecular biology techniques are needed to resolve the actual changes in the microflora and microbial composition when microalgae are added as live feed, either as a complement to MD or alone. For practical purposes, experiments should replicate normal hatchery feeding procedures. Variation due to different microalga species should also be assessed. Changes in nutrient availability during the differing growth phases in microalgae culture will assist in an understanding of the microbial population dynamics associated with the microalgae cultures. The addition of probiotic bacterial strains is of great interest and has great application potential, although little is known at present concerning the variations in the culture water microbial ecology that their use implies. Further research should focus on the microflora and microbial composition modifications when probiotic strians are added to culture water. As there is no "ideal" probiotic strain (Olafsen 2001), such studies should encompass more than one strain simultaneously, at a range of physio-chemical conditions similar to those found under commercial culture situations, and initially, without larvae.

Present studies have demonstrated the ingestion of bacteria present in the water column both alone or in conjunction with microalgal cells by first-feeding shrimp larval stages and provide insight on the fate of ingested bacteria as potential colonizers of the larval gut environment. Results from histological sections, scanning electron microscopy and *in vivo*, *in situ* real time observations of live fluorescent stained bacteria, suggest bacterial colonisation may start as early as $Nauplius_5$, since at this stage an anal pore is present and antiperistalsis movements occur which are capable of bringing bacteria present in the water column into the digestive tract. This hypothesis needs confirmation with observations using different bacterial strains and larval stages. The staining technique used in the present study, although innovative, still allowed stain to leach from bacterial cells, colouring larval tissues making consequent observation resolution difficult. New, more specific, staining techniques such as the incorporation of fluorescent protein genes in the bacterial strains of interest through plasmids or molecular genetics engineering need be tested. However, the use of simple fluorescent stains with different emission wave lengths still has great potential, especially when more than one bacterial strain is to be observed. Present study observations should be repeated with different larval stages,

using combinations of pathogenic, probiotic and innocuous bacterial strains, in order to describe and understand the different mechanisms in larval gut colonisation. In order to demonstrate bacterial attachment to the columnar epithelial cells of the larval gut lining, other techniques are needed, such as transmission electron microscopy observations. There is also the need to use high speed cinematography observations to redescribe the feeding mechanisms of shrimp larvae in fine detail, in order to understand their "options" in terms of food item selection, specially of small particles such as bacteria. These observations would improve understanding of crustacean larval feeding ecology and hence assist in the development of better larviculture procedures.

Present studies validate the hypothesis that a continuous closed recirculation system is a biologically viable alternative to standard batch exchange procedures in the commercial production of decapod crustacean postlarvae. It also provides valuable data than can be used in mathematical predictive models of recirculation systems. Although not yet completely cost-effective, the implementation of tight environmental laws in addition to recent major engineering and recirculation technology advances, and the need to increase crustacean hatchery biosecurity levels, will certainly support the use of such systems. Present studies also show that increased biomass yield can be obtained by increasing the initial stocking density, although survival, growth and feed conversion ratios are reduced in proportion to the stocking density used. Information here presented can be used for economics cost-benefit analysis of intensive recirculation systems.

Water quality variable fluctuations measured in the present recirculation system studies suggests that crustacean postlarvae are capable of acclimating to a gradual increase in potentially toxic substances such as ammonia and nitrite, as observed values were higher than reported "safe" concentrations. "Safe" levels are normally calculated by applying a 10% value of the LC_{50} (the concentration of a toxicant that kills 50% of the population over a determined period of time), although there is no valid biological reason behind that "10%" choice, as far as is known. Care is needed when analysing published results as 24 h LC_{50} are generally much higher than 96 h LC_{50}, and does not take into account the potential acclimation that the shrimp may have to a gradual increase in ammonia concentration. Perhaps the problem of sub-lethal nutrient concentrations is better addressed by using the Incipient LC_{50} or "lethal threshold concentration" (the level of toxicant which is lethal for 50% of the individuals exposed for periods sufficiently long for the acute lethal action to cease OR that level beyond which 50% of the population cannot live for an indefinite time), as suggested by Wickins (1976). There are, however, very few published results using such a concept, as there are few long term experimental studies which address the "sub lethal" effect of ammonia and nitrite toxic situations. The published "safe" levels for many species and animal sizes overlap tremendously and extend over a very wide range, making it difficult to determine a general "safe" level. Furthermore, most of the published "safe" levels are obtained under laboratory conditions which seldom replicate commercial tank operations. Attention should also be paid to the un-ionised ammonia fraction which has been reported to be the main "toxicant factor" as this depends greatly on the culture water pH values, although temperature and salinity are also important. There are several ammonia dissociation models published that take such variables in account, although most are for fresh water. Future studies should assess the sub-lethal effects of ammonia and nitrite concentrations.

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APPENDIX 1

In order to calculate the rates required to achieve 30, 60 and 90% water volume exchange of each tank per day, an exponential decay equation was used:

$$V_t = V_0 \times e^{rt} \tag{1}$$

Where V_0 is the total available water volume (L) in each tank, V_t is the remaining volume of water (L) that has not yet been replaced, r is the decay-rate constant and t is time. This means that in the period of time t, the remaining water volume that has not yet been replaced (V_t), depends on the initial total available volume in each tank (V_0) and on the value of a certain decay-rate constant, r. Solving for r we get

$$r = \frac{Ln(V_t/V_0)}{t} \tag{2}$$

The quantity V_t/V_0 is the proportion of water that has already been changed. If the proportion of water replacement is represented by x, then

$$V_t/V_0 = 1 - x$$

Substituting in equation (2) gives

$$r = \frac{Ln(1-x)}{t} \tag{3}$$

In a continuous recirculation system with an overflow, the flow f(ml min^{-1}) that enables x volume replacement is represented by

$$f = V_0 - V_t = V_0 - V_0 \times e^{rt}$$

This, for a unit time, can be rearranged as

$$f = V_0 \times \left(1 - e^{r}\right) \tag{4}$$

Substituting r from equation (3) into equation (4), the formula for calculating the flow rate given the percentage of water replacement, expressed as a proportion, can be written as

$$f = V_0 \times \left(1 - e^{Ln\left(\frac{1-x}{t}\right)}\right) \tag{5}$$