# EVALUACIÓN DE PROBIOTICOS COMERCIALES PARA EL USO EN CULTIVO DE LANGOSTINOS PENEIDOS

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Trabajo realizado en Oceanic Institute, Waimanalo, Hawaii, USA, bajo la dirección del Dr. Shaun M. Moss y del Dr. Albert G. J. Tacon.

Y presentando como requisito parcial para la obtención del Título de Máster Universitario International en Acuicultura, otorgado por la Universidad de Las Palmas de Gran Canaria (ULPGC), el Instituto Canario de Ciencias Marinas (ICCM) y el Centro de Altos Estudios Agronomicos del Mediterráneo de Zaragoza (CIHEAM).

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EVALUATION OF COMMERCIAL PROBIOTICS FOR USE IN PENAEID SHRIMP CULTURE.

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The experiment was conducted at the Oceanic Institute, Waimanalo, Hawaii, USA, under the supervision of Dr. Shaun M. Moss and Dr. Albert G. J. Tacon.

I am submitting my work as partial requisite to have the Título de Máster Universitario International en Acuicultura, certified by the Universidad de Las Palmas de Gran Canaria (ULPGC), the Instituto Canario de Ciencias Marinas (ICCM) and the Centro de Altos Estudios Agronomicos del Mediterráneo de Zaragoza (CIHEAM).

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# List of Abbreviation

ABS - Absorbance;

$CO_2$ - Carbon dioxide;

DO - Dissolved oxygen;

FCR - Feed Conversion Ration;

$H^+$ - Hydrogen ion;

$H_2CO_3^-$ - Carbonic Acid;

$H_2O$ - Water;

$H_2S$ - Hydrogen sulfide;

$HCO_3^-$ - Bicarbonate;

n.d. - Not detectable levels

$NH_4^+$ - Oxidize ammonium;

Nitrate-N - Nitrate-Nitrogen;

Nitrite-N - Nitrite-Nitrogen;

$NO_2^-$ - Nitrite;

$NO_3^-$ - Nitrate;

$O_2$ - Molecular Oxygen;

OI - Oceanic Institute;

Proline Aqua. Bact. Conc. - Proline Aquaculture Bacteria Concentrate;

ratio C:N - ratio carbon:nitrogen;

SPSS - Statistical Package for Social Sciences;

TAN - Total Ammona Nitrogen;

UV - Ultra-violet rays.

# ABSTRACT

Shrimp aquaculture represents 5.8 % of the global aquaculture production by weight, and one of the most important aquaculture commodities by value due to it's high market value. However, during recent years the industry has been severely impacted by disease outbreaks and shrimp losses, including increased antibiotic usage and pathogen virulence. One possible solution to this crisis could be the possible use of microbiological techniques, and in particular the use of probiotics, to displace pathogenic bacteria by competitive processes.

This research was undertaken at the Oceanic Institute (Hawaii, USA) with the aim of identifying commercial probiotics which could increase shrimp growth, disease resistance against pathogens, and/or improve the culture environment.

To accomplish this research, four indoor laboratory experimental trials were conducted. One experiment tested the commercial probiotic "Engest" and three experiments screened and tested two other commercial potential nitrifying probiotic products, "Microtack 22 L" and "Proline Aquaculture Bacteria Concentrate".

The use of Engest, did not contribute to improved feed efficiency or shrimp growth. The nitrifying probiotic Microtack 22 L, commonly used to reduce nitrogen compounds in seawater, could not be administrated under emergency elevated ammonia concentrations in seawater. The "Proline Aquaculture Bacteria Concentrate", on the other hand showed satisfactory results with respect to the reduction of ammonia to nitrate in clean seawater and

presented no negative effects on shrimp growth, feed efficiency or health. Nevertheless, more research should be carried out with this product, because it has raised some questions regarding the nitrification of nitrite to nitrate under both clean seawater and green water shrimp raceway conditions.

The use of probiotics in shrimp culture can minimize water exchange and consequently reduce environmental impacts. However, considerably more investigations under controlled laboratory and farm conditions should be undertaken to identify other probiotics that work under different environments, and that also can be easily used and incorporated under large pond farming conditions and management.

# 1. INTRODUCTION

## 1.1 Aquaculture Overview

The world's human population is increasing every year, in 2004 it increased about 1.16 percent and it is estimated by the U.S Census Bureau (2005) that in 2025 there will be almost 8 billion people on earth and by the same year half of the world's seafood demand will be met by aquaculture (Moriarty, 1999). In 2002, FAO (2004) estimated that the world average consumption of seafood per capita per year was 16.2 kg, this value corresponds to 15.9 percent of the animal protein intake by the human population. Over the last 40 years, there has been an increase of 3.6 percent per annum in fish demand. Due to the high market demand, fishing pressure has increased during the last decade, and as a result the number of overexploited and depleted fishery stocks has been increased (FAO, 2004).

In general terms aquaculture is the controlled production, growth and commercialization of aquatic organisms (including both animals and plants) with commercial importance in fresh, brackish or marine waters. As a research activity, the aim of aquaculture is to study these aquatic organisms (their biology and their relationship with the environment) to have a better production performance. As an industry, aquaculture was required to increase and develop in the 90's, with little scientific knowledge, to accompany the fish demand and the fisheries' inability to supply the market. Aquaculture's contribution of seafood per capita has increased from 0.7 kg in 1970, 2.1 kg in 1998 and to 6.4 kg in 2002 (FAO, 2002; 2004).

Total aquaculture production by weight in 2002 was 29.9 percent of the total global fisheries landings, with the major aquaculture volume production being finfish, crustaceans and mollusks in freshwater environments (57.7 percent), followed by seaweeds, mollusks and finfish in marine environments (36.5 percent). However, due to the high market value, the production of aquatic organisms in brackish water (for example Penaied shrimp), makes them the major value product in the aquaculture market, constituting 5.8 percent of global production by weight and a 15.9 percent by value (FAO, 2004).

All over the world, aquaculture is starting to help the poor household by implanting rural aquaculture systems, since there are a large number of aquatic organisms that can be farmed, unlike the terrestrial farming where the production is based on a limited number of animal and plant species. The production of aquatic organisms is also having an important role in the development of new areas and countries. For example, in Asia, inland aquaculture is seen as an important source of food security, the omnivorous/herbivorous fish or filter-feeding fish being the most produced species. In Europe and predominantly in America, aquaculture is oriented towards the export market, with the production of high-value products, mainly carnivorous species. In spite of this, the development and growth of aquaculture in Africa, and in particular within sub-Saharan African countries, has been largely overlooked (FAO, 2004).

Although still facing some major difficulties, such as access to technology and financial resources, environmental impacts and diseases, aquaculture is seen as an important tool to increase global seafood production, provide fish to non-coastal communities, supplying high-value exports and seed stock for stock enhancement, as well as producing cultured bait for fisheries (FAO, 2004).

## 1.2 Shrimp Aquaculture History

Shrimp aquaculture is widespread throughout the tropical world, expanding during the 1980s, mostly from South America and Asia to North America in 1990s (Figure 1).

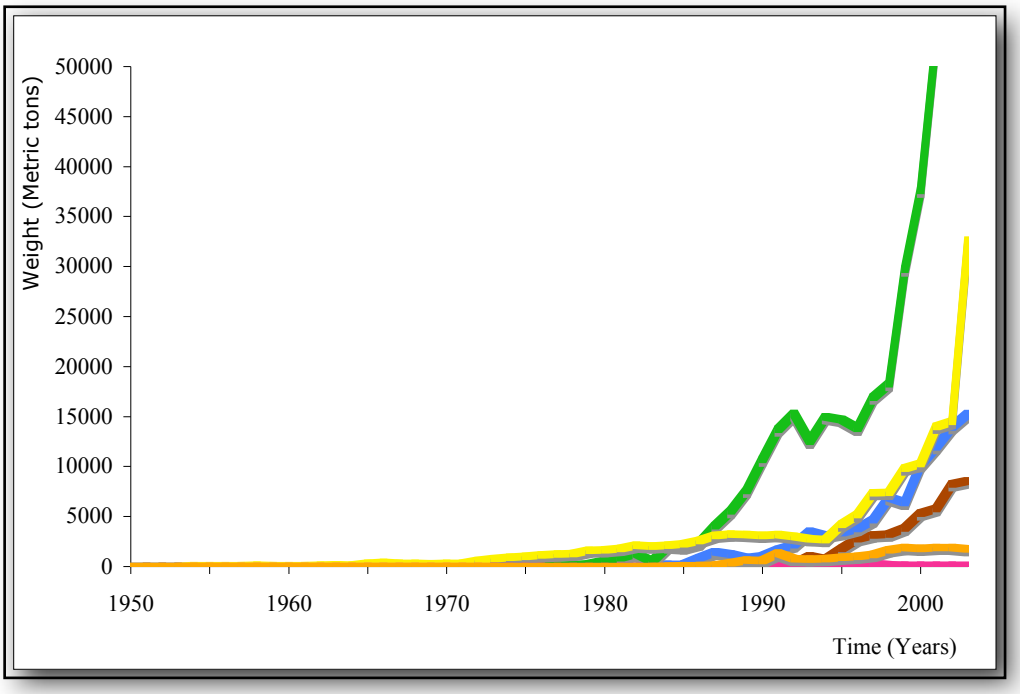


Figure 1. Global aquaculture shrimp production over the years by regions (*in* FAO 2005).

Legend: Africa; America, North; America, South; Asia; Oceania; Europe.

This expansion was based on the abundance of wild seed, static supplies of shrimp from capture fisheries and on the high-profits (Fast & Menasveta, 2000). From the 16.1 kg per capita seafood available for consumption in 1997, 4 kg was shellfish supply, including 2.2 kg of mollusks, 0.4 kg of cephalopods and 1.4 kg of crustaceans. The increase in crustacean per capita consumption from 0.4 kg in 1961 to the 1.4 kg in 1997 was largely due to the increased production of shrimps and prawns from aquaculture. In 2003, the total global aquaculture production of cultured shrimp was estimated to be 1 804 932 metric tons (Table I).

Table I. Global production of farmed shrimp in 2003 (*in* FAO 2005).

| Species | Metric tons |
|---|---|
| *Fenneropenaeus chinensis* | 195 385 |
| *Fenneropenaeus indicus* | 31 560 |
| *Fenneropenaeus japonicus* | 2 289 |
| *Litopenaeus merguiensis* | 79 338 |
| *Litopenaeus spp.* | 78 018 |
| *Litopenaeus stylirostris* | 2 289 |
| *Litopenaeus vannamei* | 723 858 |
| *Penaeus monodon* | 666 071 |
| Total | 1 804 932 |

The actual challenge of global and shrimp aquaculture is to reduce feed costs, improve feed conversion efficiency and minimize environmental impacts. Until recently, the intensive aquaculture systems relied on high rates of water exchange to flush out wastes and maintain water quality. To accomplish the challenge of minimizing the environmental impacts and the global problem in water demand, the water from aquaculture systems should be preserved and the wastes should be controlled. As an example, Asian farmers stock shrimp in smaller ponds and at higher intensive densities, when compared to Latin America farmers (Moss, 2002). These different methods of production allowed the Asian shrimp farmers to adopt biosecurity measures, at lower cost and with lower environmental impact. More recently, several North American scientists have been developing integrated aquaculture systems that employ zero water exchange and therefore less environmentally pollution and more biosecure in terms of disease occurrence. This system is based on four important interrelated elements that are being

optimized, including: genetics, engineering, feed and microbial ecology (Moss, 2002).

The biosecurity systems developed by the Asian farmers and by the North American scientists are especially important considering the devastating disease problems that have plagued the global shrimp farming industry, mostly due to bacteria, especially the luminous bacterium *Vibrio harveyi*, and viruses (Moriarty, 1999; Moss, 2002).

The use of antibiotics as prophylactics or in the treatment of diseases caused by pathogenic microorganism (such as bacteria, fungi and protozoa parasites) has lead to an increase of multiple resistance bacteria and more virulent pathogens (Moriarty, 1999), and also contributed to environmental imbalances (Kautsky *et al.*, 2000). Some authors have studied the resistance of bacteria to antibiotics. According to Moriarty (1999), after treating ponds with antibiotics, bacteria and algae die, however some days later a rapid increase of *Vibrio* sp. was observed. This is to be expected as marine vibrios have fast growth rates, and the treatment will decrease the number of competitors for nutrients by killing algae, thus increasing food resources. The bacteria surviving after the treatment are more resistant to antibiotics and also more pathogenic. Furthermore, the need to avoid the transfer of antibiotic resistance to human's pathogens led to a reduction in the use of antibiotics (Moriarty, 1999). As an example of bacterial resistance, Vaseeharan *et al.* (2004) studied antibiotic resistance of 40 isolates of *Listonella anguillarum*-like bacteria and 100 % were found to be resistant to ampicillin, 80 % to chlortetracycline and 60 % were sensitive to erythromycin.

The solution could lie in the field of microbial ecology, since the use of beneficial bacteria may provide broader-spectrum and greater non-specific disease protection by

displacing pathogenic bacteria using competitive processes and serological immunity enhancement, preventing also the animal from virus (Wyban *et al.*, 1992; Sung *et al.*, 1996). Adding selected bacterial species to large aquaculture ponds can change them and may be a better solution than administering antibiotics (Moriarty, 1999).

## 1.3. Probiotics

Parker (1974) was the first using the term probiotic as "organisms and substances which contribute to intestinal microbial balance". However Fuller (1987) restricted the term probiotic to gram-positive bacteria (*Lactobacillus*), because, at this time, the interest was particularly centered on terrestrial organisms. He thus defined probiotic as a "live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance". Conversely in aquaculture, the intensive interaction between the culture's environment and the host implies that a lot of probiotics are obtained from the culture's environment and not directly from feed (Verschuer *et al.*, 2000). Having this in consideration, Verschuer *et al*. (2000) proposed a new definition: "Probiotic is a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment". The health of the animals is thus improved by the complete removal or decrease in the population density of pathogens, and by improving water quality through the more rapid degradation of waste organic matter (Moriarty, 1999). Bacteria delivering essential nutrients to the host without being active in the host or without interacting with other bacteria, with the environment of the host or with the host itself are not included in the definition (Verschuer *et al.*, 2000).

Other authors Maeda *et al.* (1997), Moriarty (1998), Gatesoupe (1999) defined three categories of microbial preparations, including:

- Biocontrol: Method of treatment that uses antagonistic microorganisms to reduce or kill the pathogens established in the aquaculture environment;
- Probiotic: Only the strains of bacteria that are transient or resident in the gastrointestinal tract and act as antagonistic to pathogens;
- Bioremediation: This concept refers to the treatment of pollutants or waste by the use of microorganisms that break down the undesirable substances.

In this study, the term probiotic will be used as proposed by Verschuer *et al*. (2000).

### 1.3.1. How to develop a probiotic?

Probiotics are added to aquaculture production systems in order to modify or manipulate the microbial communities in the water and sediment, to reduce or eliminate selected pathogenic species of microorganisms, and generally, the ultimate goal of probiotics is to improve growth and survival of the target cultured species (Verschuer *et al*., 2000).

In the intensive aquaculture techniques, the preservation of a stable permanent microbial community should not be considered, because a tank is disinfected and cleaned before stocking and the cultures are discontinuous. Besides the sudden increases in nutrients due to exogenous feeding do not provide the right environment to establish those

communities (Verschuer et al., 2000). In the development of probiotics the deterministic factors (salinity, temperature, oxygen concentration, quantity and quality of the feed) and the stochastic factors (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) should be considered (Moriarty, 1999; Verschuer *et al*., 2000).

The development and research of safe probiotics is a long multi-step and multi-disciplinary process. It requires empirical and fundamental research, full-scale trials and an economic assessment of its use (Verschuer *et al.*, 2000). First it is necessary to acquire the strains of bacteria. This process could be done by isolation and purification of bacteria located in the pond and/or in the shrimp, or by searching in the market for probiotic companies that have products containing strains of bacteria, since few companies have a full trial experiment of their own products. The second step of the research is to conduct a small-scale test *in vivo* and *in vitro* as a screening of the different probiotics, and evaluate their efficacy. If the probiotics works, the next step is the pathogenicity test against the host. The absence of pathogenicity and infectivity is a requisite of probiotic safety. This step is very important, although the differentiation of a bacteria strain as probiotic, commensal, opportunistic or pathogenic is difficult because the association between bacteria and invertebrates remains largely unknown (Verschuer *et al.*, 2000). A bacterial single administration (acute) toxicity test and a repeated-administration (chronic) toxicity test, under normal or stress conditions of the host, will provide information on toxicity. The safety of a bacterial strain may be evaluated by considering questions such as whether invasion of the host by the bacteria leads to infection, whether infection results in severe outcome, and the effect of associating the bacteria to the host (Ishibashi & Yamazaki, 2001).

After a guarantee that the probiotic is safe to the organism, the subsequent experiment should be an *in vivo* pilot-scale test. This test should guarantee that the probiotic will work within commercial size ponds (Verschuer *et al.*, 2000). Once the probiotic is in the market as a certified and tested product, it is required that hatcheries and farmers maintain an appropriate quality control to avoid contamination by other bacteria. In addition, the existence of a large amount of bacteria can result in spontaneous or induced mutations as well as changes in the genetic stability of the safe bacteria. Consequently the probiotic's required activity can be reduced or, under extreme situations it can change into a pathogenic bacteria. Therefore the last step of the development of probiotics, should be to develop monitoring tools for routine use in farms (Verschuer *et al.*, 2000). Verschuer *et al*. (2000), Kowalchuk and Stephen (2001) believed that molecular tools might be the most appropriate tools to develop.

After the development of the probiotic, there are four categories to commercialize it, including:

- *Non-viable probiotics*: where the bacteria are dead;
- *Freeze-dried probiotics*: where the bacteria will die rapidly if not stored in a very cool location;
- *Fermentation products*: where the product of the probiotic is obtained through fermentation;
- *Live probiotic*: where the bacteria should have a guarantee of shelf life and a protocol for counting the organisms that it carries.

### 1.3.2. When to start using probiotics?

Verschuere *et al.* (1997) monitored three identical culture series of *Artemia* juveniles. They reported that the development of microbial community in the first days of the experiment in the water of the three series was clearly distinct. Instead of allowing spontaneous primary colonization of the rearing water by bacteria accidentally present, Verschuer *et al*. (2000) proposed that the water could be preemptively colonized by the addition of probiotic bacteria, a microbial community consequently appearing, controlling the stochastic factors. A single addition of a probiotic culture may suffice to achieve colonization and persistence in the host and/or in its environment, providing that the probiotic culture is well adapted to the prevailing environmental conditions (deterministic factors). Otherwise if the host or its environment already carries a well-established and stable community, the probiotic will have to be supplied on a regular basis to achieve and maintain its artificial dominance (Verschuer *et al.*, 2000). This suggestion is based on the ecological processes of competitive exclusion, and enough is known to argue that it is possible to change species' composition by this principle (Smith & Davey, 1993).

In theory, bacteria can colonize the nauplii V stage of the shrimp, because it already undertakes anal drinking, even before opening is mouth. However more research and investigation is necessary on this area, to identify a probiotic that will allow the rapid growth and development of the shrimp, as well as facilitating the development and growth of different strains of bacteria, to avoid the excess of ammonia and nitrate in the water (Simões *et al.*, 2002).

It could also be interesting, in more advanced studies, to seek substances specifically digestible by candidate probiotics (prebiotic), which might not only antagonize pathogens but also stimulate the tissue defense of the host and proliferate the growth of the probiotic (Gatesoupe, 1999; Bomba *et al*., 2002).

### 1.3.3. How should a probiotic act?

The ways in which the bacteria can act in the host are described below, and include:

- Production of inhibitory compounds;
- Competition for chemicals or available energy;
- Competition for adhesion sites;
- Enhancement of the immune response;
- Improvement of environment quality;
- Interaction with phytoplankton;
- Direct food source and
- Improvement of additional digestive capabilities.

These various mechanisms of action should be tested to select the candidate probiotic, but rearing experiments remain necessary to prevent the emergence of resistant strains, a recognized risk of antibiotic treatments. However this possibility should not be underestimated, because it is particularly important to search for diversified antagonistic properties, which may lower the risk of multi-resistance (Gatesoupe, 1999).

Undoubtedly, only one strain of bacteria cannot act in all the ways described. It is also improbable that several bacteria will act in only one of the mechanisms. For example, a probiotic could act in the suppression of a pathogen and at the same time act as a supplement food resource, contribute to the digestion of food in the guts, or act as a hepatopancreas microbial, preventing the adhesion of pathogenic microorganisms (Verschuer *et al.*, 2000). Some authors agree that the efficacy of probiotics could be potentiated. This proposition is based on making one of the mechanisms more intensive or extending the range of mechanisms of the probiotic organism. According to Bomba *et* al. (2002) the efficacy of probiotics may be enhanced by the following methods:

- Selection of more efficient strains of microorganism;
- Gene manipulation;
- Combination of a number of strains of microorganism;
- Combination of synergistical bacteria and their compounds;
- Prebiotics.

Moreover, in order to improve the efficacy of probiotics in aquaculture, it is necessary to obtain further knowledge about their different mechanisms of action.

#### 1.3.3.1. *Production of inhibitory compounds*

The production of inhibitory compounds by microbial populations refers to the release of chemical substances that have a bactericidal or bacteriostatic effect on other microbial population (Verschuer *et al.*, 2000). These microbial populations are also called

antagonistic bacteria, and it seems to be common among marine bacteria. Most marine antagonistic strains are members of the *Pseudomonas-Alteromonas* and/or *Vibrio* groups (Lemos *et al.*, 1985; Nair *et al.*, 1985).

Bacteria can also be antagonistic to viruses (Kamei *et al*., 1987; Kamei *et al*., 1988; Direkbusarakom *et al.*, 1998) therefore they could be efficient for the biocontrol of viral diseases (Maeda *et al.*, 1997). The antagonism is mediated not only by antibiotics but also by other inhibitory compounds, either single or in combination, including: hydrogen peroxide (Ringø & Gatesoupe, 1998), siderophores (Gram & Melchiorsen, 1996), bacteriocins, lysozymes, proteases (Verschuer *et al.*, 2000) and the alteration of pH values by the prodution of organic acids (Sugita *et al.*, 1997).

These mechanisms are highly dependent on the experimental rearing conditions, which maybe different *in vitro* and *in vivo*. To date none of the published studies has shown unequivocally that the production of inhibitory compounds is the cause of the observed *in vivo* probiotic activity of the strains (Verschuer *et al.*, 2000). As a result, the expression of antagonism *in vitro* is not a sufficient criterion to select candidate probiotics (Riquelme *et al.*, 1997), nor is the absence of antagonism sufficient to rule the strain out (Rico-Mora *et al.*, 1998). So future research in this field is required.

#### 1.3.3.2 *Competition for chemicals or available energy*

Competition for chemicals or available energy can theoretically play an important role in the composition of the microbiota of the intestinal tract or of the environment of cultured aquatic species (Verschuer *et al.*, 2000). In practice it is difficult for microbial

ecologist to apply the principles of competition to natural situations. Some reports were made with bacteria competing for carbon and nitrogen, sulphur and iron.

It is known that heterotrophic bacteria can compete with each other to synthesize protein from organic carbon and ammonia compounds. However, bacteria are inefficient at composting organic materials that are either too carbonaceous or too nitrogenous (high protein vegetable meals). So it is crucial that organic carbon and ammonia be suitable for bacterial utilization and so the ratio C:N in the pond should be around 20:1. Generally, it is the carbon that acts as a limiting factor in this synthesis.

One of the most important chemical competitions is for iron, because most organisms require it. Iron availability in animal tissues may be a virulence factor for pathogens. Harmless bacteria that can produce siderophores could be used as probiotic to compete with pathogens whose pathogenicity is known to be due to siderophore production and competition for iron (Verschuer *et al.*, 2000). Siderophores are low molecular weight, ferric ion-specific chelating agents (Neilands, 1981), which can dissolve precipitated iron and make it available for microbial growth. The ecological significance of siderophores resides in their capacity to search an essential nutrient from the environment and deprive competitors of it (Verschuer *et al.*, 2000).

Similar to the detection of inhibitory compounds, the detection of siderophores *in vitro* production does not necessarily mean that they are produced in significant amounts *in vivo* in order to have a significant biological control effect. The evidence for the participation of competition for chemicals or available energy of free iron or siderophores in the mode of action of probiotics is still circumstantial (Verschuer *et al.*, 2000).

#### 1.3.3.3. *Competition for adhesion sites*

The competition for adhesion sites or colonization and the transient bacteria may also be efficient as a probiotic, since the bacterial adhesion to tissue surface is important during the initial stages of pathogenic infection (Krovacek *et al*., 1987). The inhibition effect of bacterial strains against other bacteria has been demonstrated in terrestrial animals. However, in aquatic species the research in competition for adhesion sites, as a way of probiotic action, is still not well known (Verschuer *et al.*, 2000).

The adhesion of bacteria to the host can be made by two mechanisms. It can be nonspecific, based on physicochemical factors, or it can be specific, involving adhesion molecule sites on the surface of adherent bacteria and receptor molecule sites on epithelial cells of the host (Salminen *et al*., 1996). The candidate to probiotic must be introduced at the accurate dose, continuously or semi-continuously, and for the best result it is necessary to evaluate the persistence of the probiotic in the gut of the shrimp or in the sediment (Gatesoupe, 1999; Simões *et al.*, 2002). Simões *et al*. (2002) observed that the shrimp releases the bacteria in every "ecdisis" and being the estimated time of 10-20 minutes, which is similar to the multiplying time of *Vibrio*, under optimal conditions. Therofore, almost all of the bacteria ingested by the shrimp will be released before they can multiply. Even when bacteria can multiply inside of the shrimp, they will be released if they are placed after the medium intestine. The bacteria will be enclosed in the peritrophic membrane and will be no longer in contact with the intestinal wall. For this reason, the putative probiotic must survive in the water column and/or in the sediment to allow the

colonization of the environment, changing the microbial ecology of it, and as already referred, changing the intestinal microbiota (Simões *et al.*, 2002).

Even with few published studies about the use and application of yeasts as probionts in aquaculture, it is known that they also have a great potential to adhere and colonize the guts of the shrimp (Gatesoupe, 1999; Burgents *et al.*, 2003).

1.3.3.4. *Enhancement of the immune response*

Immunostimulants are chemical compounds that activate the immune systems of animals and render them more resistant to infections by viruses, bacteria, fungi, and parasites (Raa, 1996). There are some reports that bacterial compounds can act as immunostimulants in shrimp, but only specific cell compounds or non-living cells were used (Verschuer *et al*., 2000).

Two types of immunostimulants have received the most attention in shrimp aquaculture, namely:

- Fragments of bacterial cell walls (lipopolysaccharide-LPS; Hansen & Olafsen, 1999; Tacon, 2000; Burgents *et al*., 2003);

- β-Glucans (from several fungal or algal species; Raa, 1996). Glucans are reported to enhance disease resistance by stimulating nonspecific components of the immune system or by improving processing and presentation of antigens during specific adaptive immune responses (Cuzon *et al*., 2000).

Shrimp possess a non-specific immune response, which means that immunostimulation may provide only short-term protection against specific pathogenic microorganisms (Anderson, 1992; Sung *et al.*, 1996). And if the immune system of the shrimp is always under prevention, the shrimp will be too stressed, and probably will be affected by other diseases.

1.3.3.5 *Improvement of environmental quality*

Most of the commercial probiotic such as: "Bacteria", "Bactipost", "Biostart", "BRF-1A", "BRF-13A", "PB-32", "PBL-44", "Liqualife", "Microbial and Enzymic Products", "Microtack 22 L", "PondPro-VC Probiotic", "Proline Aquaculture Bacteria Concentrate", improve the environment of the aquatic organism and consequently improve animal health. These probiotics generally contain nitrifying bacteria and/or *Bacillus* spp. (Gatesoupe, 1999) and de-nitrifying bacteria. The nitrifying bacteria are gram-negative bacteria, mostly rod-shaped and ranging 0.6 – 4 microns in length. As obligate chemolithotrophs, the nitrifying bacteria have restricted ecological niches and have not been detected in the gastrointestinal tract of animals (Gatesoupe, 1999). As a result it is believe that they do not compete for adhesion sites. These bacteria have their activity in the sediment (benthic nitrification) or in the water column (pelagic nitrification), and it is possible that they can compete for space with pathogenic bacteria in the aquatic environment. The most used bacteria in nitrition, the first step of nitrification (equation 1), are included in the genera *Nitrosospira*, *Nitrosomonas*, *Nitrosococcus*, *Nitrosolobus* and *Nitrosovibrio* (Suzuki *et al*., 1974). These bacteria possess the genes to encode two essential enzymes to oxidize ammonium ($NH_4^+$) to nitrite ($NO_2^-$) which are: ammonia monooxygenase and hydroxylamine oxidoreductase (Arp *et al.*, 2002). *Nictrobacter*,

*Nitrococcus*, *Nitrospira* and *Nitrospina* (Watson, 1971; Watson *et al*., 1986; Meincke *et al*., 1989) are the bacteria responsible for the oxidation of nitrite to nitrate ($NO_3^-$) (equation 2) .Only un-ionized ammonia and nitrite are toxic to the shrimp. At concentrations of 0,5 mg/l of un-ionized ammonia, shrimp start presenting lethal and sub-lethal effects and at 1,0 mg/l strong lethal effects are shown (personal communication C. Otoshi, 2005). Only the nitrate is not considered toxic unless it accumulates in high concentrations (Morrison *et al.* 2004). The reactions that occur with the nitrifying bacteria are shown in the equations below.

Ammonia oxidizer bacteria:

$$55\ NH_4^+ + 76\ O_2 + 109 HCO_3^- \rightarrow C_5H_7O_2N + 54 NO_2^- + 57 H_2O + 104 H_2CO_3 \quad (1)$$

Nitrite oxidizer bacteria:

$$400 NO_2^- + NH_4^+ + 4 H_2CO_3 + HCO_3^- + 195\ O_2 \rightarrow C_5H_7O_2N + 3 H_2O + 400\ NO_3^- \quad (2)$$

Since little energy is produced from these reactions, nitrifying bacteria have evolved to become extremely efficient at converting ammonia and nitrite. Most of the energy is used for fixing $CO_2$ via the Calvin cycle and little energy remains for growth and reproduction. As a consequence, they have a very slow reproductive rate. Under optimal conditions, *Nitrosomonas* spp., may double every 7 hours and *Nitrobacter* spp. every 13 hours (Watson, 1971; Bock *et al*., 1989), but it is more likely that ammonia-oxidizers will take 26 hours and nitrite-oxidizers bacteria will take 60 hours to double (Shilo & Rimon, 1982; Belser, 1984). Nitrifying bacteria reproduce by binary division and none of the Nitrobacteraceae is able to form spores, as they have a complex cytomembrane that is surrounded by a slime matrix. Unlike heterotrophic bacteria, nitrifying bacteria cannot

survive any drying process. In water, they can survive short periods of adverse conditions by using stored materials within the cell, and when these materials are depleted, bacteria die (Hagopian & Riley, 1998).

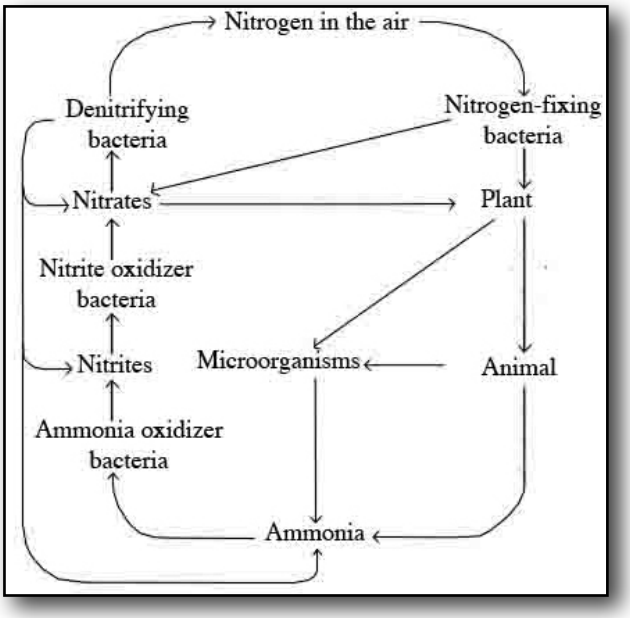


Figure 2. Nitrogen cycle, *in* http://library.thinkquest.org/C003763/images/planet/ncycle.gif .

The nitrification process is an important pathway of the nitrogen cycle (Figure 2), and influences the primary productivity of the aquatic system. This process modifies the form of nitrogen released during the decomposition of organic matter, it shunts nitrogen to denitrification pathways and it competes with heterotrophic bacteria for dissolved oxygen supplies (Henriksen & Kemp, 1988). Nitrifying bacteria are commonly used in biofilters, however studies showed that if they were added directly to the pond or tank the activation time of the filter would be reduced. Also these bacteria could be added to tanks or ponds when incidental increase of ammonia or nitrite levels is observed (Verschuer *et al.*, 2000).

*Bacillus* spp. are also reported as being able to improve water quality, even so the published evidence is poor (Lin, 1995; Rengpipat *et al.,* 1998). These gram-positive bacteria are generally more efficient in converting organic matter to $CO_2$ than gram-negative bacteria. The gram-negative bacteria are more efficient in converting organic carbon to bacterial biomass or slime (Stanier *et al*., 1963). Maintaining a high level of

*Bacillus* spp. in the aquatic system, it is possible to minimize the increase of dissolved and particulate organic carbon during the culture cycle, promoting more stable phytoplankton blooms trough the increased production of $CO_2$.

In ponds where some strains of *Bacillus* spp. were introduced, the survival of prawns was improved (Moriarty, 1998; Gatesoupe, 1999; Decamp *et al*., 2005). The conclusion was that the treatment had decreased the proportion of pathogenic luminous *Vibrio* spp. in the sediments, and to a lesser extent, in the water column. However, it is not known if only the improvement of the environmental quality was responsable for the survival of the prawn, or if there was any change in the intestinal microbiota, which helped as well in the survival of them.

The use of sulphur oxidizing and reducing bacteria in ponds can avoid the gill of shrimp clogging due to the precipitation of $H_2S$ compounds. These bacteria can convert sulphur and sulphur related compounds, in non-harmful compounds, so the shrimp can grow better and in good health conditions (Devaraja *et al.*, 2002).

1.3.3.6. *Direct food source and improvement of additional digestive capabilities*

The ingestion of bacteria can also serve as a direct food source or improve digestive capabilities, by releasing exogenous enzymes to the host, by supplying essential nutrients that are lacking in the host's diet (vitamins and amino acids; Moss *et al*., 2000) or even by preconditioning refractory materials into mire digestible forms (Moss *et al.*, 2000; Moss *et al.*, 2001).

## 1.4. Disease

"Disease is a condition of a living animal that impairs performance of a vital function and can be induced by environmental and nutrional factors or infection by pathogenic microorganisms" (Gallo, 1991).

Most shrimp diseases found in aquaculture are related with poor management in hatcheries and ponds, and very few are recorded as a direct consequence of protozoans (Bachère, 2003). For example, within hatcheries at the high larval densities, excess of food or poor food quality, are associated with larval mortalities by favoring contamination with either heterotrophic bacteria or with pathogenic bacterial strains like *Pseudomonas* (Colwell & Sparks, 1967; Brown, 1981), *Aeromonas* (Riquelme *et al.*, 1996) and *Vibrio*, (the latter being one of the most harmful pathogenic bacteria for mollusk and crustaceans larvae and juveniles). The presence of *Vibrio harveyi, Vibrio vulnificus* and *Vibrio splendidus* has been associated to larval mortalities in *Penaeus monodon* and *Litopenaeus vannamei* (Baticados *et al.*, 1990; Song & Lee, 1993; Karunasagar *et al.*, 1994; Robertson *et al.*, 1998). Moreover, in shrimp nursery or growout ponds, *Vibrio* spp. like *V. damsela* (Song *et al.*, 1993), *V. alginolyticus* , *V. parahaemolyticus* (Lightner*,* 1992), *V. penaeicida* (Costa *et al.*, 1998) are responsible for disease outbreaks.

Fungi represent potential pathogens for cultured crustaceans, particularly for stressed or immuno deficient individuals, as shown by the filamentous fungus *Lagedinium* sp. that affects the larval stages of shrimp and lobster (Crisp & Bland 1989) or fungi from the *Fusarium* genus.

In reality, difficulties in controlling diseases in marine aquaculture come partly from the poor management control and partly from the lack of knowledge on the diversity of pathogens and differences in susceptibility that affect the shrimp in each developmental stage (Bachère, 2003).

## 1.5 Objective

### 1.5.1 Aim of the present study

The present study is integrated in the North American shrimp farming system being developed at the Oceanic Institute (Waimanalo, Hawaii, USA), which is a super-intensive production of shrimp with the least environmental impact possible. The system based in the four interrelated elements is being developed. The engineering element is developing the water recirculating system and the filtration system that allow the system to reuse the water. The genetic department had conducted a selective shrimp breeding program over the past years, with species selection focusing on shrimps with faster growth and increased disease resistance. The studies conducted in the feed section have been showing reduced feed nutrient loss from the pellets and consequently reduced environmental waste.

The aim of this study is incorporated in the fourth element of this North American system. This system presents as optimal water for the production of shrimp an eutrophic water, full of microorganisms that arbitrarily colonized the water. However seasonal spikes of ammonia and nitrite concentrations in the raceway tanks have been observed, which are not desirable for the growth of the shrimp. The solution of partial water exchange of the

raceway tanks, which was being used at the Institute to reduce the ammonia and nitrite concentration, is not appropriate in an aquaculture farm, and the biofilters used in this system requires the use of a specialized technician and increased of costs.

With the aim of reducing the costs and improving the growth performance of the shrimp, the feed section and the microbial community segment is interested in exploring other nutritional supplements which will promote good shrimp performance under the stressful conditions of an intensive aquaculture system.

The objective of this experiment was to compare commercial probiotic tools that can help in the controlled colonization of the raceway water and help in the shrimp growth, in the improvement of environmental conditions and/or shrimp pathogen resistance.

Table VI - Mean, standard deviation and range for temperature, salinity, DO and pH for control and probiotic treatments. There were three replicates per treatment.

| Treament | Temperature (˚C) | | Salinity (ppt) | | DO (mg/l) | | pH | |
|---|---|---|---|---|---|---|---|---|
| | AM | PM | AM | PM | AM | PM | AM | PM |
| Control | 26.98 ± 0.01[a] | 28.36 ± 0.03[a] | 32.85 ± 0.01 | 32.83 ± 0.02[a] | 5.75 ± 0.06 | 5.55 ± 0.11 | 7.75 ± 0.01 | 7.75 ± 0.01 |
| | (26.98 - 26.99) | (28.32 - 28.38) | (32.84 - 32.86) | (32.82 - 32.85) | (5.70 - 5.81) | (5.43 - 5.64) | (7.75 - 7.76) | (7.74 - 7.76) |
| Engest | 26.77 ± 0.04[b] | 28.21 ± 0.08[b] | 32.87 ± 0.01 | 32.87 ± 0.02[b] | 5.66 ± 0.06 | 5.50 ± 0.09 | 7.75 ± 0.01 | 7.70 ± 0.01 |
| | (26.74 - 26.81) | (28.12 - 28.28) | (32.86 - 32.88) | 32.86 - 32.89) | (5.61 - 5.72) | (5.42 - 5.60) | (7.75 - 7.76) | (7.73 - 7.75) |

– Means in the same column with different letters are significant different ( $p < 0.05$)

**Table VII** - Mean and standard deviation for stocking weight, harvesting weight, weight gain, FCR and survival for control and probiotic treatments. There were three replicates per treatment.

| Treatment * | Stocking weight (g) | Harvesting weight (g) | Weigth gain (g) | FCR | Survival (%) |
|---|---|---|---|---|---|
| without probiotic | 1.72 ± 0.04 | 6.66 ± 0.64 | 4.94 ± 0.69 | 1.90 ± 0.44 | 90.00 ± 17.32 |
| with probiotic | 1.71 ± 0.04 | 6.97 ± 0.46 | 5.26 ± 0.50 | 1.78 ± 0.21 | 90.00 ± 10.00 |

* There was no significant difference between treatments ($p > 0.05$).

# 2 METHODOLOGY

## 2.1 Feed additive product experiment

### 2.1.1 Probiotic Specifications

The feed additive product (Engest product) was obtained from Microtack (Microtack Organic Aquaculture & Wastewater Treatment Supplies, Baxel Co., Bangkok, Thailand). This provider stats that Engest is a feed additive, to mix with the feed pellets, containing a highly concentrated source of enzymes designed to activate and nourish the beneficial digestive tract, which in turn improves digestion and assimilation of shrimp's feed (improving feed conversion ratio, average weight body gain and assist shrimp defense to withstand highly stressful conditions). As reported in the product guideline, it contains the following enzymes: protease, amylase, cellulase, lipase, pectinase, beta-glucanase, hemi-cellulase. In order to determine the efficacy of the commercial feed enzyme additive in the improvement of additional digestive capabilities, the experiment was conducted at the Oceanic Institute (OI), Waimanalo, Hawaii, USA (Figure 3) in an indoor laboratory,


Figure 3. View of Oceanic Institute, Waimanalo, Hawaii, USA.

where experiment conditions were controlled for a 40 day period. To accomplish these objectives, two treatments were evaluated in triplicate – one with the Engest and the control.

### 2.1.2 Experimental design

Six 45 l rectangular dark-blue plastic tanks (Figure 4) with a cover net were cleaned with chlorine and freshwater. Filled with seawater, and fitted with air pipes, air-stones and a drainage system. The tanks were filled with seawater at 32 ppt of salinity and the flow-through water system was set at ten tank water exchanges per day. The seawater was provided from the deep well placed at OI. The air-stones were placed on the bottom of the tank as well as the water pipe. The drain system was located in the surface, opposite to the water pipe and the air-stone (Figure 5). All the tanks were designed to have the same water circulation and water aeration pattern .

Figure 4. 45 l rectangular dark blue plastic tank, used in the experiment.

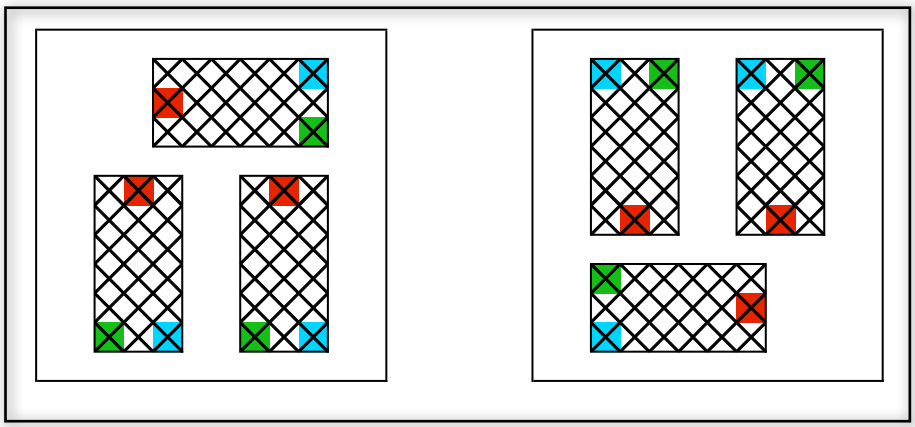

Figure 5. Schematic representation of the water and air flow system of the tanks.

Legend: Water in; Air flow; Water out.

The day after cleaning, the tanks were stocked with 1.71 ± 0.19g of *Litopenaeus vannamei* at a density of 10 shrimp per tank (equivalent to 380 g/$m^3$). The shrimp were individually weighed, with only those that weighed between 1.3 g and 2 g being chosen. The shrimp were obtained from a flow-through pond at the OI, where they had been fed on commercial shrimp pellet (Zeigler Brothers Inc. USA;Shrimp growth hyper-intensive 35% protein diet. 2.4 mm) and the shrimp were free of specific pathogens as listed by the U.S. Marine Shrimp Farming Program. The shrimps were moved from the pond to a 50 l tank, and after weighting were moved to the respective 45 l tank, with animals not being fed on the day of transfer.

At the start of the experiment the shrimp were fed twice daily (08:00/16:00 h), at a fixed rate of 8% of total biomass per day, with the treatment feed being the same Zeigler pellets. This amount of feed was adjusted daily in an attempt to keep a slight excess of feed on the tank bottom.

The Engest was added to the pellets before weighting the feed, following the product instructions and adjusted to 20 g of feed (Table II). During the feeding time, the water and airflow were stopped in all tanks for 45 minutes. This procedure was undertaken so that the product still remained attached to the pellets and was able to be ingested by the shrimps. The uneaten feed, faeces and exuvae's were removed by siphoning the tank bottom immediately prior to each morning feeding. The feed was collected and sieved through 1mm, 500µm and 300 µm sieve and placed on aluminum plates, which were then dried at 100 ˚C for 24 h. After this period, the pellets were weighed.

Table II. Provider's application rate of Engest Ultra for shrimp grow-out aquaculture and rate adjustment for experience application.

| | Amount of feed (g) | Volume of seawater to mix (ml) | Engest Ultra (mg) |
|---|---|---|---|
| Provider | 1000 | 50 | 5000 |
| Experience | 20 | 1 | 100 |

At the end of the experiment total shrimp body weight was recorded and statistical analysis undertaken, as describe below.

## 2.2 Nitrification probiotic screening experiment

### 2.2.1 Probiotic specification

The nitrifying probiotics were obtained from two companies. The product Microtack 22 L – Biological Aquaculture Nitrifier was obtained from Microtack (Microtack Organic Aquaculture & Wastewater Treatment Supplies, Baxel Co., Bangkok, Thailand) and the product Proline Aquaculture Bacteria Concentrate was obtained from Aquatic Eco-Systems (Aquatic Eco-systems, Inc. Apopka, Florida USA). Both probiotic products were claiming to have a culture of *Nitrobacter* spp. and *Nitrossomonas* spp. and help in the nitrification/de-nitrification process in the water. However, the Proline product reportedly had a shelf life of three months and the provider said that it was a live bacteria product. The Microtack 22 L product reportedly had a shelf life of two years and contained a fermentation product of *Nitrobacter* spp. and *Nitrosomonas* spp., as well as some waste digestive enzymes and specially selected strains of enzymes producing bacteria.

Both probiotic products were stored and applied following the respective manufacturers's recommendations. The Microtack 22 L product was stored in a room under 38 ˚C, in a dark closet and the Proline product was stored in the refrigerator between 0 – 4 ˚C. The Microtack provider recommended to mix 1 l of product with 50 l of pond water, and then to spray the diluted product to 10000 $m^3$ pond, with the provider advising to use between 5-50 times more under emergency situations. By contrast the North American provider recommended to use one cup of product per 150 gallons of pond water under emergency situtations. Both products were calculated to be used in 1 l beakers (Table III).

Table III. Provider's application rate of Microtack 22 L and Proline Aquaculture Bacteria Concentrate for shrimp grow-out aquaculture under emergency ammonia situations (rate adjustment by experience).

| | Water volume | Mix seawater | Probiotic Volume |
|---|---|---|---|
| Microtack 22 L | 10 000 000 l | 50 l | 1 l |
| Experience | 1 l | 5 µl | 0.1 µl |
| Proline Aqua. Bact. Conc. | 2 400 gallon<br>9 085 l | | 1 gallon<br>3.8 l |
| Experience | 1 l | | 0.417 µl |

### 2.2.2 Experimental design

The experiment was conducted for ten days, within an indoor laboratory located at the OI. Twenty-five plastic beakers of 1 l were filled with water at 16 ppt salinity and fitted with air stones, pipelines and aluminum covers. To reduce the water to this salinity, 500 ml of fresh water and 500 ml of salt water were used; the seawater was previously warmed up

to 30 ˚C, filtered (5mm, 1 mm and sand filters) and then sterilized by passing over ultra-violet rays (UV). The air stones were adjusted within each beaker so that the air flow did not have a shaking effect on the water surface. The beakers were labeled from 0 to 24, as well with the un-ionized ammonia concentration and with the name of the product added (Table IV).

Table IV. Beakers label information of nitrification probiotic screening experiment.

| Beaker number | Treatment | Un-ionized Ammonia Concentration (mg/l) | Probiotic |
|---|---|---|---|
| 0 (Blank) | 0 | 0 | 0 |
| 1, 2, 3 | 1 * | 0.5 | 0 |
| 4, 5, 6 | 2 * | 1.0 | 0 |
| 7, 8, 9 | 3 | 0.5 | Microtack 22 L |
| 10, 11, 12 | 4 | 1.0 | Microtack 22 L |
| 13, 14, 15 | 5 | 0.5 | Proline Aqua. Bact. conc. |
| 16, 17, 18 | 6 | 1.0 | Proline Aqua. Bact. conc. |
| 19, 20, 21 | 7 * | 0 | Microtack 22 L |
| 22, 23, 24 | 8 * | 0 | Proline Aqua. Bact. conc. |

* Control treatments

### 2.2.3 Treatments

Un-ionized ammonia standards were established at 1 mg/l and 0.5 mg/l, since these values correspond to the lethal and sub-lethal levels for shrimp. To prepare the treatments at the desired concentration of un-ionized ammonia, the salinity, pH and temperature were measured previously in a pre-mix water beaker and the ammonia concentration required was calculated using an Excel spreadsheet that computes the concentration of un-ionized

ammonia in seawater, as a function of total ammonia, pH, temperature and salinity (Hansson, 1973; Whitfield, 1974; Emerson *et al*., 1975; Khoo *et al*., 1977; Millero, 1986). The master ammonia solution was prepared at 5 g/l of ammonium chloride in a 0.5 l beaker, and Milli Q water was used. From this beaker 6.5 ml were measured to fill the treatments at 1 mg/l of un-ionized ammonia and 3.25 ml to the treatments at 0.5 mg/l of it. To avoid phosphate being the limitary factor it was added 4 mg/l of sodium phosphate to all treatments.

For this experiment five of the nine treatments were control (Table IV). All the treatments were made in triplicates, with the exception of the blank treatment.

### 2.2.4 Bacteria counting

For the bacteria counting analysis, water samples from each beaker were collected on day 0 and day 8, to sterile 15 ml Falcon tubes and immediately fixed with formaldehyde to a final concentration of 2-4 %, during at least one hour. An appropriate amount of sample was subsequently filtered onto 0.2 μm black polycarbonate membrane and washed with sterile water. The samples that were not filtered immediately were stored in the refrigerator until filtering and counting.

One of the triplicate samples was used to prepare a slide with the 4,6-diamindino-2-phenylindole Vecta-shield method (DAPI Vecta-shield method, Porter & Feig 1980), since this is a method that accurately enumerates whole water microbial samples. Once the slide was ready, it was moved between places in a dark box and stored in the refrigerator when

necessary, as this method does not allow for the slide to stay under light or in warm temperature for a long time.

Bacteria were counted using an Olympus Epifluorescent Microscope (Olympus BX 51, with WU Filter) and total bacterial counts were computed as the average of twenty different random fields, ensuring that the entire slide was covered.

## 2.3 Nitrite oxidizer experiment

This experiment was conducted for the reason that major problems were being observed with high water nitrite concentrations than with high ammonia concentrations in the raceways at OI (Figure 6). With the aim of observing the nitrification process starting from the nitrite pathway, it was determined to use the product with the best performance from the nitrification probiotic screening experiment.

Figure 6. View of the shrimp raceway tanks at OI.

### 2.3.1 Experimental design

The laboratory conditions were similar to the nitrification probiotic screening experiment. The experiment was conducted for eleven days in an indoor laboratory located at OI, using 1 l plastic beakers fitted with air stones, pipelines and aluminum covers, as described previously. Six beakers were filled with clean filtered seawater at a salinity of 18 ppt and the remainder filled with raceway water with a salinity of 18 ppt. The raceway water was eutrophic green water full of microorganisms and was exhibiting a nitrite concentration of 15 mg/l. The filtered clean seawater was previously warmed up to 30 ˚C, filtered (5 mm, 1 mm and sand filters) and then sterilized by UV irradiation. The air stones were adjusted within each beaker so that the air flow did not have a shaking effect on the water surface. The beakers were labeled from 1 to 18, as well as nitrite concentration used and if it was clean filtered seawater or raceway water (Table V).

Table V. Beakers label information of nitrite oxidizers experiment

| Beaker number | Treatment | Nitrite concentration (mg/l) | Proline Aqua. Bact. conc. | Water source |
|---|---|---|---|---|
| 1, 2, 3 | 1 * | 15 | 0 | Clear water at 18 ppt |
| 4, 5, 6 | 2 | 15 | 1x | Clear water at 18 ppt |
| 7, 8, 9 | 3 * | 15 | 0 | Raceway water at 18 ppt |
| 10, 11, 12 | 4 | 15 | 1x | Raceway water at 18 ppt |
| 13, 14, 15 | 5 | 15 | 10x | Raceway water at 18 ppt |
| 16, 17, 18 | 6 | 15 | 100x | Raceway water at 18 ppt |

* Control treatments

### 2.3.2 Treatments

This experiment was subdivided into two similar experiments. One experiment was made with the raceway water where the nitrite-N was set at 15 mg/l and the second was with filtered clean seawater and at the same nitrite-N concentration. The experiments were settled with six treatments in triplicate and with two treatments (beakers 1 – 3 and 7 – 9) as control treatments (Table V).

The raceway water treatments were established with the control (without probiotic), with one time probiotic concentration suggested by the provider, with ten times and with one hundred times. These concentrations were proposed due to the results obtained in a pre-experiment (data not shown).

To prepare the clean seawater treatments at the desired concentration, a nitrite solution was prepared at the concentration of 800 mg/l in a 0.5 l beaker using Milli Q water. 18.75 ml of this solution were added to the clean seawater beakers. To avoid phosphate being the limiting factor, 4 mg/l of sodium phosphate were added to all clean seawater beakers.

Due to previous results with the clean seawater treatments (see results section), these beakers were maintained for the necessary time so that the nitrite concentration decreased. Inorganic carbon (30 mg/l of sodium bicarbonate) was added to the beakers number 2, 3, 5 and 6 and organic carbon (20 mg/l of sucrose) to the beakers 1 and 4. They were maintained with this carbon source for 11 more days. Since no modifications on the nitrite concentration were observed (see results section) ammonia was added to all the

beakers. The experiment was conducted for another 5 days, when the probiotic was added again, at the providers concentration, to the treatment with the product. During this period the nutrient analysis continued with the same schedule as before.

## 2.4 Effects of commercial probiotic on shrimp

### 2.4.1 Experimental design

For this experiment the same 45 l rectangular dark-blue plastic tanks with a net cover from the feed additive experiment were used. Eight tanks were cleaned with chlorine and the air-stones on the bottom of the tanks were also settled. All the tanks were designed to be identical. As this experiment was conducted in a batch system, there was no water recirculation system in the tanks and so a full water exchange was made every ten days. The tanks were always filled with 40 l water at 18 ppt salinity. The trial was conducted for thirty-one days, in an indoor laboratory located at OI.

On the day that the tanks were cleaned, a flow-through system was set to prevent chlorine from remaining in the tanks. The following day, the *Litopenaeus vannamei* juveniles originating from a flow-through system, with a salinity of 32-34 ppt and a specific pathogens free status was stocked at a similar density in all eight tanks. Due to the shrimp were coming from a different salinity, a three day acclimation period was used. After this period the shrimp were then weighed and stocked at a density of 5 shrimp per tank (equivalent at 153.75 g/$m^3$; shrimp weighting 1.23 ± 0.23 g, and with only those weighting between 0.80 g and 1.70 g being selected).

At the beginning of the experiment, the treatment feed was the same Zeigler pellets as used in the previous experiment and the shrimps were fed twice daily (08:00/16:00) at 8% of total biomass per day. This amount of feed given was adjusted daily in an attempt to keep a slight excess of feed on the tank bottom. The uneaten feed, faeces and exuviae's were removed by siphoning the tank bottom immediately prior to each morning feeding. The siphoning was made into a bucket using a filter on it (300 μm), and so it was possible to remove all solids from the water and return the water back to the experimetnal tanks. Buckets, filters and siphoning hoses were cleaned using fresh water.

### 2.4.2 Treatments

Using the product with the best performance of the nitrifies probiotic screening experiment, two treatments were settled, in quadruplicate, including a control and the treatment with the probiotic. The tanks were settled in two rows, side by side. The probiotic was added to the tanks every seven days with a full water exchange undertaken in all tanks after ten days. For this, a holding tank was used, a similar 45 l rectangular tank with clean water at 18 ppt, settled with air stones. This tank was used to keep the shrimp as long as the tank of the experiment was being cleaned. Only when the first tank was cleaned and settled again with the shrimps and with the same conditions as in the beginning of the experiment, the shrimps of the second tank were moved to the holding tank, and the second tank was cleaned. The control tanks were the first to be cleaned up and then the water of the holding tank was changed before starting cleaning the tanks that had the probiotic, to prevent any contamination. The tanks were washed and cleaned with Alcanox. After the

tanks were cleaned, the feed was added to all of them including the probiotic to the tanks with the treatment.

## 2.5 Analytical analysis

### 2.5.1 Environmental analysis

#### 2.5.1.1 *Water quality*

Water quality analysis was similar for all experiments. However due to the experimental design employed or due to technical problems, there were some differences between the analyses undertaken.

*Feed additive product experiment* - The water quality analyses were made twice daily, and were registered during the water and airflow stop period. The temperature, the salinity, the dissolved oxygen (DO) concentration and the pH, were recorded using the probe "YSI 650 MDS" (Yellow Springs Instrument Company, Yellow Springs, Ohio, USA). The DO reading was also registered with the probe "YSI 550 DO" (Yellow Springs Instrument Company, Yellow Springs, Ohio, USA), because of technical problems in the "YSI 650 MDS" probe.

*Nitrification probiotic screening experiment* – Water temperature, salinity, DO concentration and pH were measured daily for each beaker using a "YSI 556 MPS" probe (Yellow Springs Instrument Company, Yellow Springs, Ohio, USA). Between beakers the probe was washed with freshwater to avoid cross contamination.

The alkalinity of the water was measured the day before adding the ammonium chloride solution to the beakers (this value was settled to all the beakers, as the initial alkalinity value), and at the end of the experiment. It was also measured when the pH values started to drop. The alkalinity was measured by mixing 25 ml of a premix water with phenolphthalein indicator powder and bromcresol green-methyl red indicator powder and titrated with 0.0016 ± 0.0008 N HCl to permanent change of color, using the digital titrator (model 16900) of Hach (Hach Company, Loveland, Colorado, USA,). Sodium bicarbonate was added to increase the alkalinity to the initial values in the beakers that showed a drop of the pH.

*Nitrite oxidizer experiment* – Water temperature, salinity, DO concentration and pH were measure daily for each beaker. At the beginning the probe "YSI 556 MPS" (Yellow Springs Instrument Company, Yellow Springs, Ohio, USA) was used. However due to technical problems with the probe, it was changed to "YSI 550 DO" probe (Yellow Springs Instrument Company, Yellow Springs, Ohio, USA) to measure the temperature and the DO concentration. The pH was measured using the probe "Accumet AP 61 pH meter" (Fischer Scientific, Pittsburgh, Pennsylvania, USA) and the salinity was measured using a temperature compensator refractometer (Aquatic Eco-systems, Inc. Apopka, Florida USA). Between beakers the probes were washed with freshwater to avoid cross contamination.

The alkalinity of the clean seawater was measured at the beginning of the experiment from premix water, setting the value to all treatments. At the end of the experiment and when the pH dropped, the alkalinity was individually measured. The alkalinity of the clean seawater was measured by the same method used in the probiotic

screening experiment. The alkalinity of the raceway water was measured from the raceway tank, being the value settled for all the beakers. To measure the alkalinity of the raceway water, the method was the same as used in the "Nitrification probiotic screening experiment", but 100 ml of this water was mixed with phenolphthalein indicator powder and bromcresol green-methyl red indicator powder and titrated with 0.016 ± 0.008 N HCl to permanent change of color, using the same digital titrator.

*Effects of commercial probiotic on shrimp* - Water temperature, salinity, DO and pH were measured daily for each tank. The temperature and the DO were measured using a "YSI 550 DO" probe (Yellow Springs Instrument Company, Yellow Springs, Ohio, USA), the pH was measured using the probe "Accumet AP 61 pH meter" (Fischer Scientific, Pittsburgh, Pennsylvania, USA) and the salinity was measured using a temperature compensated refractometer (Aquatic Eco-systems, Inc. Apopka, Florida USA). Between tanks the probes were washed with freshwater to avoid cross contamination.

2.5.1.2. *Nutrient analysis*

*Total Ammonia Nitrogen analyses*

The Total Ammonia Nitrogen (TAN) analysis was made following the Salicylate Method (method 10031) of Hach. This method, adapted by Hach, is a variation of the Phenate Method, but with the advantage of being free from mercury salts and phenol. In this method, ammonia compounds in the water are initially combined with hypochlorite to form monochloramine, which then reacts with salicylate to form 5-aminosalicylate. The oxidation of 5-aminosalicylate is carried out in the presence of nitroferricyanide (yellow-

colored compound), resulting in the formation of indosalicylate, a blue-colored compound. A green-colored solution results from this reaction, and the intensity of the color is directly proportional to the ammonia concentration in the water (Hach Water Analysis Handbook. Hach Company).

*Method description* — This analysis was made by filling one test tube with 5 ml of Milli Q water (blank) and one test tube per sample with 5 ml of sampling water, previously filtered. Once the test tubes were filled up, the Colorimeter DR/890 (Hach Company, Loveland, Colorado, USA) was turned on and the program 67 for TAN analysis was set. The absorbance (ABS) mode button was choose, placing the absorbance automatically to 655 nm and the timer was set to twenty minutes. For each test tube, a pack of ammonia salicylate reagent for 5 ml sample and one pack of ammonia cyanurate reagent for 5 ml sample were opened. Quickly and carefully all the packs of ammonia salicylate were introduced in the test tubes. When the first pack of ammonia cyanurate was added, the enter button of the colorimeter was pressed to start the countdown. When all the tubes had both reagents, they were capped and turned upside down for one minute. Following this minute and making sure that all the reagents were dissolved, the tubes sat undisturbed for the rest of the time. Once the time finished, the tubes were red. The blank tube was used to zero the colorimeter and in order all the test tubes were red, and the ABS values registered. The test tube was cleaned before being inserted in the colorimeter. If the ABS value was higher then 600 nm, the samples were diluted. Once the value was between 0 – 600 nm, the concentration of TAN was calculated, by inserting this value into the formula obtained from the calibration standard curve.

*Nitrite-Nitrogen analyses*

The nitrite-nitrogen (nitrite-N) analyses were made following a modification of the classical brown ring test for nitrate using ferrous sulfate adapted by Hach. This method (method 8153) consists in the formation of an acidic medium ferrous sulfate that reduces nitrogen to nitrite. The ferrous ions from the solution combine with the nitrous oxide to form a brown-colored complex ion. The color intensity is in direct proportion to the nitrite present in the water.

*Method description* — This analysis requires one test tube with 5 ml of Milli Q water (blank tube) and one test tube per sample with 5 ml of water. On a separate test tube rack the samples were duplicate. After the test tubes were filled with the water, the packs with the nitrite reagent powder - NitriVer 2 for 5 ml samples, were opened. The colorimeter was settled to program 59, and the ABS mode was chosen. The timer was set for ten minutes by pressing the timer button. The powder was added to the no-duplicate test tubes, as well as in the TAN analysis, the timer started the countdown when the first tube was filled with the Nitriver 2. Once all the tubes had the powder reagent in, they were capped and the tubes were inverted six times. The test tubes were settled undisturbed for the remaining time, after ensuring that there was no powder on the walls. Once the time finished, the test tube to be inserted in the colorimeter was cleaned. The blank tube of the duplicates was inserted in the colorimeter and the zero button was pressed. The blank tube with the powder was red, and the ABS value was the blank value. For each sample, the duplicate test tube was used to zero the colorimeter. After all the tests tube were read, the ABS values were inserted in the standard formula obtained from the standard curve calibration. The nitrite

concentration was obtained, but before, the concentration of the blank value was subtracted from each concentration sample value to correct the error made by the interference that powder makes to the water.

*Nitrate-Nitrogen analyses*

The nitrate-nitrogen (nitrate-N) analyses were made using NitraVer 5 high range method (method 8039), which is a modification of the Cadmium reduction method made by Hach. This reaction uses cadium metal to reduce nitrate to nitrite ions. In the acidic medium with sulfanilic, the nitrite ions react and form an intermediate diazonium salt which in the presence of gentisic acid, forms an amber-colored compound. The color intensity is directly proportional to the nitrate concentration.

*Method description* — This analysis is similar to the nitrite-N analysis, duplicate test tubes filled with 5 ml of Milli Q water and waters samples were also used. The nitrate reagent powder - NitraVer 5 for 5 ml samples, were opened and the colorimeter was settled to program 51. After selecting the ABS mode, the timer button was pressed to set one minute time. The powder was added to the no-duplicate tests tube and they were capped and only after this the timer was started. During this minute the tests tube were continuously shaken. After standing for five minutes, the test tubes were then read, as in nitrite-N analysis. The duplicate blank test tube was used to zero the colorimeter and the ABS value (blank value) obtained from the blank test tube with the powder. The duplicate samples were used to zero the colorimeter and the respective sample was red. The concentration of nitrate was obtained by inserting the ABS values of each sample into the standard formula, after subtracting the concentration of the blank value.

*Experiments*

To proceed with nitrogen analysis in the different experiments all samples must be filtered before being analyzed. For each treatment, one syringe was used to collect the water and for each beaker one glass fiber GF/F 0.7 μm filter was used. This nitrogen analyses allows 8 samples to be tested at the same time. For each nitrogen analysis test the standard calibration curve was made and the formula of the trendline was determined.

*Nitrification probiotic screening experiment* - The nitrification performance was analyzed with the Hach test kit (Hach company, Loveland, Colorado, USA) for TAN, nitrite-N and nitrate-N. On the day the ammonium chloride solution was added to the beakers (day 0) and in the last day (day 8) the TAN, nitrite-N and nitrate-N were tested. In the remaining days just TAN and nitrite-N were tested. To do the TAN, nitrite-N and nitrate-N analysis, 30 ml of water from each beaker was collected and filtered to a falcon test tube, and to do only the TAN and nitrite-N analysis only 20 ml of water were collected.

*Nitrite oxidizer experiment* - The nitrification performance was analyzed with the Hach test kit for TAN, nitrite-N and nitrate-N. In the day sodium nitrite was added to the beakers (day 0) and in the last day (day 11) the TAN, nitrite-N and nitrate-N analysis were tested. In the other days just TAN and nitrite-N were tested. The TAN analysis was not carrie out when after three consecutive analysis, the concentration of TAN was observed to be low. The nitrate analysis was conducted in the clean seawater treatments prior to the addition of a new treatment to the beakers.

*Effects of commercial probiotic on shrimp* - The analysis of TAN and nitrite-N was made twice a week for four weeks. It was assumed that every time the water was exchanged the TAN and nitrite-N concentration was the same as in the beginning of the experiment. The nitrate-N on the water tanks was only measured when an increase of the nitrite-N concentration was observed.

### 2.5.2 Statistical analysis

At the end of the "Feed additive product experiment" and "Effects of commercial probiotic on shrimp experiment", the total shrimp body weight was recorded and growth rate was calculated, as well as shrimp survival. The feed conversion ratio (FCR) was also calculated in the "Feed additive product experiment".

Using the Statistical Package for Social Sciences for Macintosh OS X (SPSS version 11.0.4), an Anova statistical analysis (Sokal & Rohlf, 1981) was made to evaluate water parameters, FCR, survival, shrimp growth and bacteria counting for the respective experiments. The significant level was established to be 0.05. The required data was transformed to make their analysis possible and the experiments that needed a Tukey analysis were made to identify homogenous treatments.

# 3. RESULTS

## 3.1. Feed additive product experiment

*Water quality analysis*

The water quality parameters had no significant difference between treatments, with the exception of the morning and afternoon temperature and the afternoon salinity, ($p < 0.05$). The morning temperature of the control varied from 26.98 to 26.99 ˚C and the treatment with Engest went from 26.74 to 26.81 ˚C. The afternoon temperature varied from 28.32 to 28.38 ˚C, in the control and from 28.12 to 28.38 ˚C in the treatment with Engest. The control showed a salinity range of 32.82 – 32.86 ppt and for the treatment with Engest was 32.86 – 32.89 ppt (Table VI).

*Statistical analysis*

There was no significant difference in the initial and final weight of the shrimp between treatments, neither was any difference for the FCR value. The survival was 90% for both treatments (Table VII).

## 3.2. Nitrification Probiotic Screening experiment

*Water quality analysis*

The water quality parameters showed a significant difference between treatments, this being established in subset homogenous groups, indicated by letters in Table VIII. The variation of highest and lowest temperature treatments was 0.29 ˚C. The treatments showed a difference between the highest and the lowest salinity of 0.69 ppt and the DO difference was of 0.49 mg/l. The treatments that are responsible for the pH difference between all the treatments are the treatments with Proline Aquaculture Bacteria Concentrate and un-ionized ammonia at 0.5 and 1.0 mg/l (treatments 5,6). These treatments presented a pH average of 7.96 and 7.82, respectively and the rest of the treatments presented an overall pH average of 8.07 (Table VIII).

Table VIII. Mean, standard deviation and range for temperature, salinity, DO concentration and pH for controls and treatments with probiotics. There were three replicates per treatment,

| Treatment | Temperature (˚C) | Salinity (ppt) | DO (mg/l) | pH |
|---|---|---|---|---|
| 0 | 28.97 | 16.00 | 5.30 | 8.08 |
| 1 | 28.94 ± 0.02 $^{a,b}$ | 16.01 ± 0.24 $^{b}$ | 5.63 ± 0.07 $^{c}$ | 8.08 ± 0.03 $^{c}$ |
| | (28.92 - 28.96) | (15.87 - 16.29) | (5.55 - 5.70) | (8.05 - 8.12) |
| 2 | 28.87 ± 0.03 $^{a}$ | 15.73 ± 0.04 $^{a}$ | 5.65 ± 0.05 $^{c}$ | 8.09 ± 0.01 $^{c}$ |
| | (28.83 - 28.89) | (15.69 - 15.76) | (5.59 - 5.70) | (8.08 - 8.10) |
| 3 | 29.03 ± 0.06 $^{b,c}$ | 15.86 ± 0.05 $^{a,b}$ | 5.54 ± 0.08 $^{b,c}$ | 8.05 ± 0.02 $^{c}$ |
| | (28.99 - 29.10) | (15.82 - 15.91) | (5.45 - 5.60) | (8.04 - 8.08) |
| 4 | 29.04 ± 0.05 $^{b,c}$ | 15.61 ± 0.01 $^{a}$ | 5.41 ± 0.10 $^{a,b}$ | 8.05 ± 0.00 $^{c}$ |
| | (29.01 - 29.10) | (15.59 - 15.62) | (5.30 - 5.51) | (8.05 - 8.06) |
| 5 | 29.10 ± 0.02 $^{c}$ | 15.84 ± 0.06 $^{a,b}$ | 5.43 ± 0.07 $^{a,b,c}$ | 7.96 ± 0.01 $^{b}$ |
| | (29.08 - 29.12) | (15.77 - 15.87) | (5.36 - 5.48) | (7.94 - 7.97) |
| 6 | 29.06 ± 0.04 $^{c}$ | 15.66 ± 0.01 $^{a}$ | 5.23 ± 0.03 $^{a}$ | 7.82 ± 0.02 $^{a}$ |
| | (29.02 - 29.10) | (15.65 - 15.67) | (5.21 - 5.26) | (7.79 - 7.83) |
| 7 | 29.03 ± 0.03 $^{b,c}$ | 16.04 ± 0.06 $^{b}$ | 5.44 ± 0.08 $^{a,b,c}$ | 8.06 ± 0.01 $^{c}$ |
| | (28.99 - 29.05) | 15.97 - 16.09) | (5.38 - 5.53) | (8.05 - 8.07) |
| 8 | 29.02 ± 0.04 $^{b,c}$ | 16.04 ± 0.04 $^{b}$ | 5.50 ± 0.10 $^{b,c}$ | 8.08 ± 0.04 $^{c}$ |
| | (28.97 - 29.06) | (16.00 - 16.08) | (5.41 - 5.61) | (8.05 - 8.12) |

- Means in the same column with different letters are significant different ( $p < 0.05$)

*Nutrient analysis*

In what concerning to the concentration of TAN, nitrite-N and nitrate-N the control treatments showed no variation (Figure 7, 8) and the nitrate-N concentration was low or under detectable levels (Table IX).

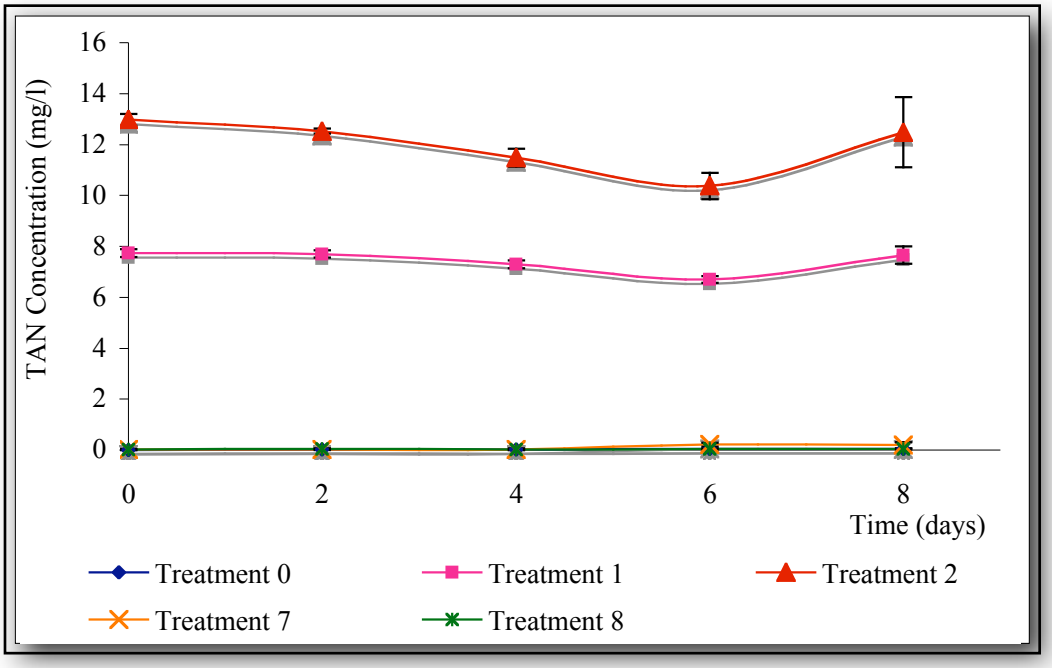


Figure 7. TAN concentration evolution over time of control treatments. There were three replicates for all treatments, except for treatment 0 that had one replicate. Error bars represent standard deviation around mean of the treatments.

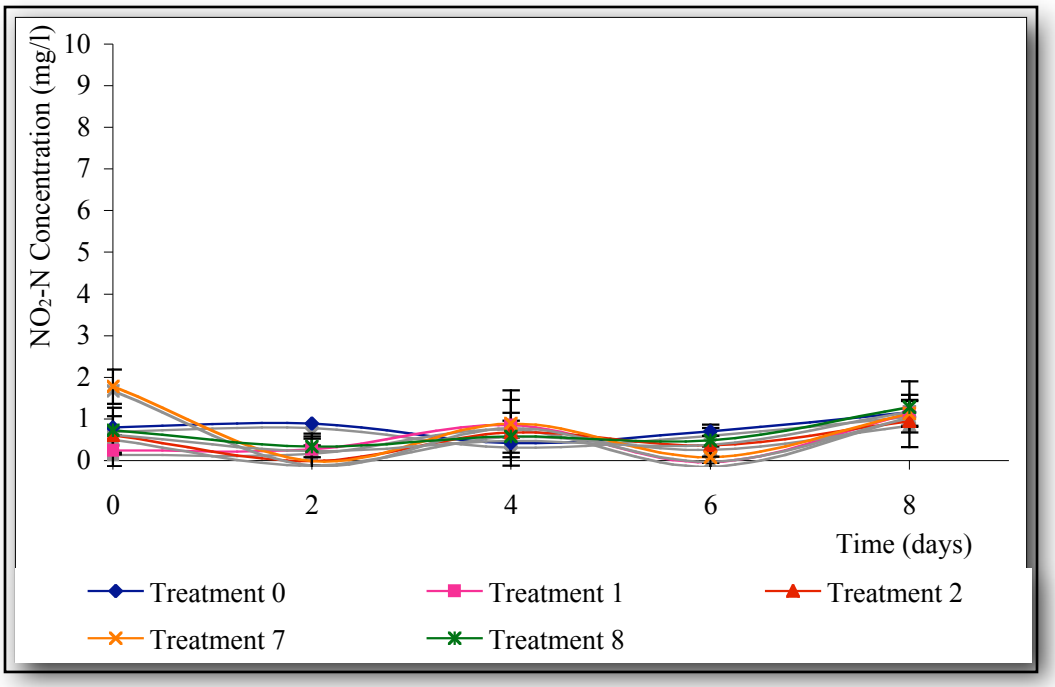


Figure 8. Nitrite-nitrogen concentration evolution over time of control treatments. There were three replicates for all treatments, except for treatment 0 that had one replicate. Error bars represent standard deviation around mean of the treatments.

Table IX. Nitrate-N concentration (mg/l) in day 0 and in day 8 of all the treatments. There were three replicates for all treatments, except for treatment 0 that had one replicate.

| Treatment | Day 0 | Day 8 |
|---|---|---|
| 0 | n.d. | n.d. |
| 1 | n.d. | n.d. |
| 2 | n.d. | n.d. |
| 3 | 0.002 | n.d. |
| 4 | 0.318 | n.d. |
| 5 | n.d. | 3.298 |
| 6 | n.d. | 2.031 |
| 7 | n.d. | n.d. |
| 8 | n.d. | n.d. |

n.d. - Not detectable levels

The treatments with Microtack 22 L showed a minor variation in both un-ionized ammonia concentrations, starting with 7.152 and 11.742 mg/l and finishing the experiment with 6.644 mg/l and 11.540 mg/l of TAN (Figure 9). The nitrite-N concentration had also a slight variation, the 0.5 and the 1.0 mg/l of un-ionized ammonia treatments started with 0.454 and 0.761 mg/l and finished with 0.792 and 0.638 mg/l, respectively (Figure 10). The nitrate-N concentration was under detectable levels (Table IX).

The treatments with Proline Aquaculture Bacteria Concentrate and un-ionized ammonia at 0.5 and 1.0 mg/l presented a variation in all nitrogen analysis. Both treatments showed a decrease in the concentration of TAN at the fourth day, from 6.458 mg/l to 4.883 mg/l in the treatment with 0.5 mg/l of un-ionized ammonia and 12.245 mg/l to 9.027 mg/l in the treatment with 1.0 mg/l of un-ionized ammonia. In the last day the concentration of TAN was low in both treatments (Figure 9). An increase of nitrite-N concentration from 0.946 mg/l to 2.913 mg/l and 1.284 mg/l to 2.513 mg/l, respectively was observed. The nitrite-N in the treatment with 0.5 mg/l of un-ionized ammonia had a maximum of 4.634 mg/l at the sixth day, showing a decrease in the last day. However, in the treatment with 1.0 mg/l of un-ionized ammonia the nitrite concentration did not decreased (Figure 10). The nitrate-N passed from no detectable levels to detectable levels in the last day in both treatments, being 3.298 ± 0.227 mg/l in the treatment with 0.5 mg/l of un-ionized ammonia and 2.031 ± 1.318 mg/l in the other treatment.

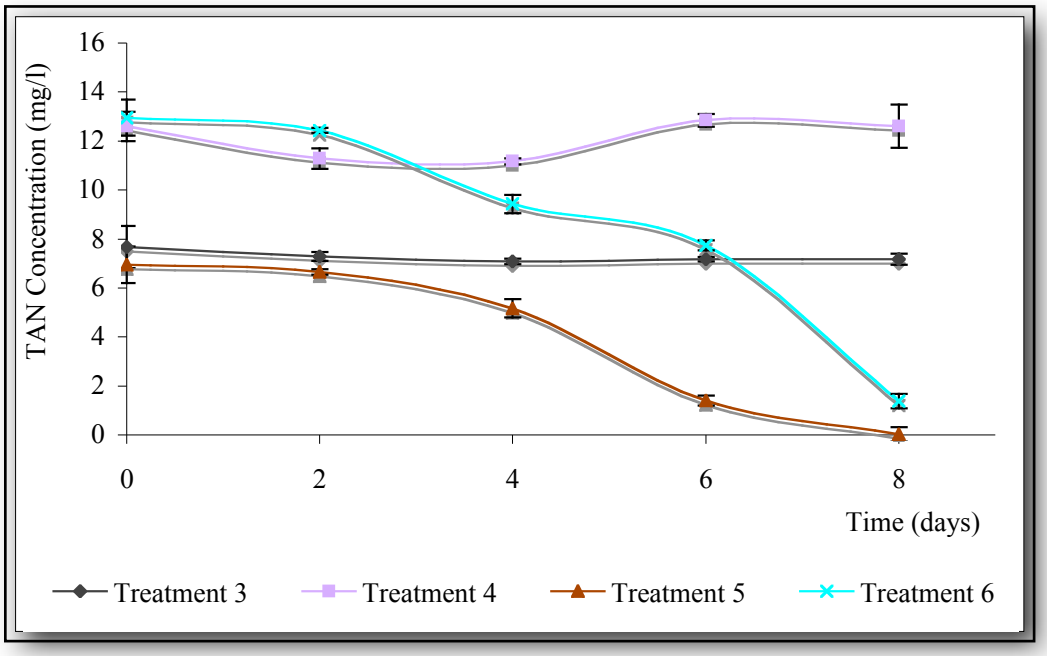


Figure 9. TAN concentration evolution over time of control treatments. There were three replicates for all treatments. Error bars represent standard deviation around mean of the treatments.

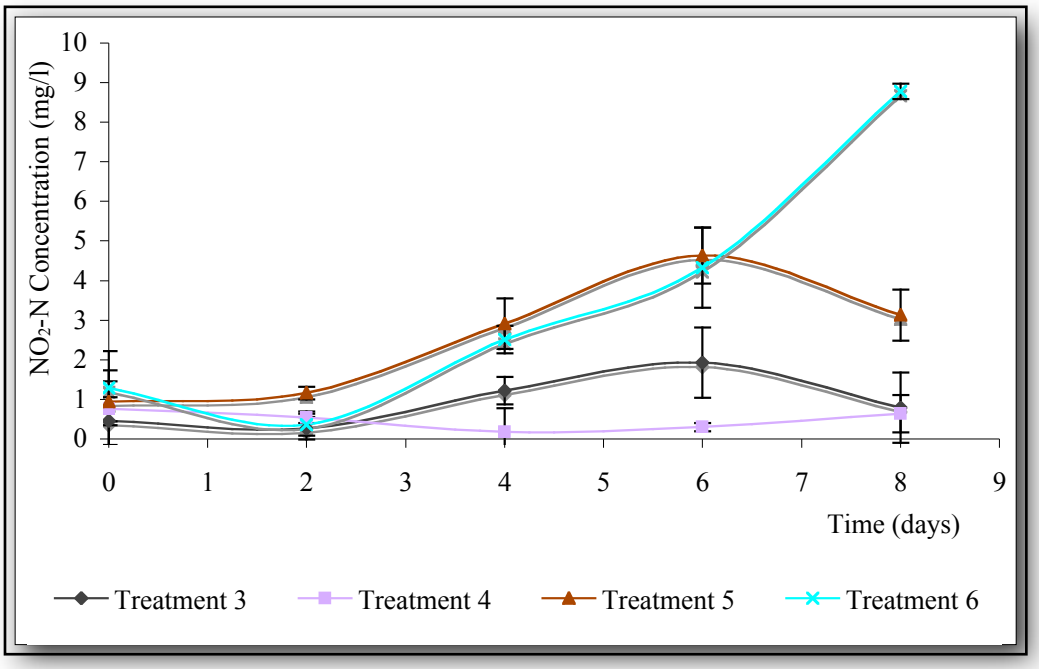


Figure 10. Nitrite-nitrogen concentration evolution over time of control treatments. There were three replicates for all treatments. Error bars represent standard deviation around mean of the treatments.

*Bacteria counting*

Bacteria counting it is verify that the treatments with probiotics had higher concentration of bacteria. However at the end of the experiments, all the treatments presented a reduction in bacteria concentration, with the exception of treatments 5 and 6 where it was not posible to count the bacteria on the water samples (Figure 11).

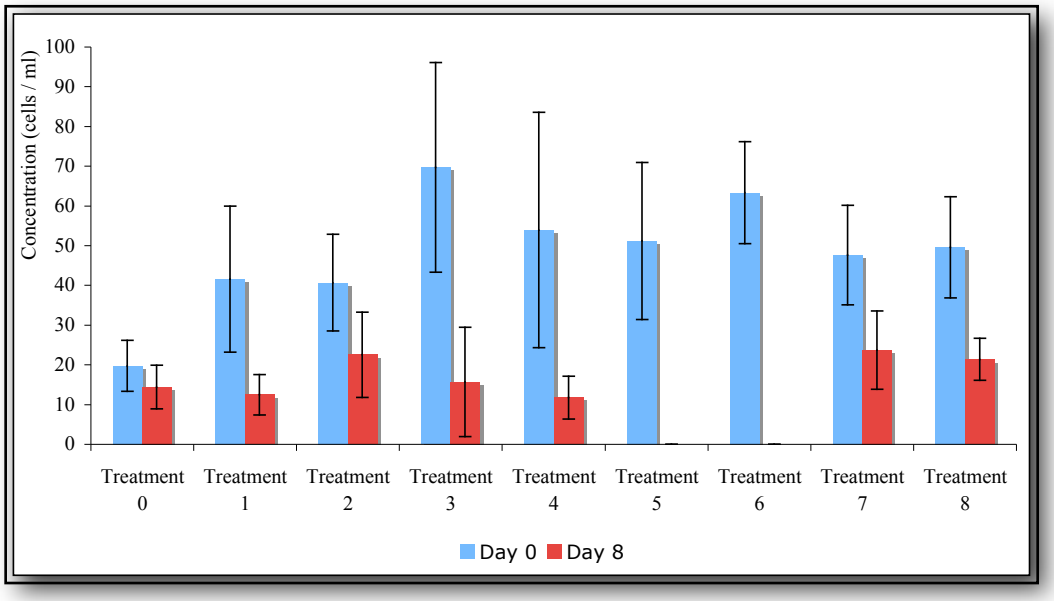


Figure 11. Means of bacteria cells per milliliter in the different treatments, in day 0 and in day 8. Error bars represent standard deviation around mean of the treatments.

## 3.3. Nitrite oxidizer experiment

*Water quality analysis*

The results of the nitrite oxidizer experiment showed no significant difference between the clean seawater treatments in all the water quality parameters (Table X). There was also no

significant difference in the water quality parameters in the clean seawater treatments when the carbon source, ammonia and the probiotic were added (Table XI). However, it was in the raceway water treatments that a significant difference between the water quality parameters were found (Table X), with the exception of pH parameter, which had no significant differences between all treatments.

Table X. Mean, standard deviation and range for temperature, salinity, DO and pH for control and probiotic treatments. There were three replicates per treatment, except for the clean seawater

| Treatment | Temperature (˚C) | Salinity (ppt) | DO (mg/l) | pH |
|---|---|---|---|---|
| 0 | 28.97 | 16.00 | 5.30 | 8.08 |
| 1 | 27.82 ± 0.02 $^{a}$ | 16.13 ± 0.11 $^{a}$ | 6.62 ± 0.03 $^{b}$ | 8.25 ± 0.03 $^{a}$ |
| | (27.80 - 27.84) | (16.00 - 16.19) | (6.60 - 6.65) | (8.22 - 8.27) |
| 2 | 27.85 ± 0.03 $^{a,b}$ | 16.03 ± 0.05 $^{a}$ | 6.58 ± 0.06 $^{a,b}$ | 8.25 ± 0.02 $^{a}$ |
| | (27.83 - 27.88) | (15.97 - 16.07) | (6.54 - 6.65) | (8.23 - 8.27) |
| 3 | 27.96 ± 0.02 $^{b,c}$ | 16.11 ± 0.04 $^{a}$ | 6.39 ± 0.09 $^{a}$ | 8.22 ± 0.06 $^{a}$ |
| | (27.94 - 27.99) | (16.08 - 16.16) | (6.29 - 6.45) | (8.15 - 8.27) |
| 4 | 27.89 ± 0.09 $^{a,b}$ | 16.16 ± 0.01 $^{a}$ | 6.54 ± 0.04 $^{a,b}$ | 8.31 ± 0.02 $^{a}$ |
| | (27.82 - 27.99) | (16.16 - 16.18) | (6.50 - 6.57) | (8.29 - 8.33) |
| 5 | 28.06 ± 0.03 $^{c}$ | 16.36 ± 0.50 $^{a}$ | 6.38 ± 0.06 $^{a}$ | 8.23 ± 0.04 $^{a}$ |
| | (27.03 - 28.09) | (16.06 - 16.93) | (6.31 - 6.42) | (8.19 - 8.28) |
| 6 | 27.89 ± 0.04 $^{a,b}$ | 17.00 ± 0.01 $^{b}$ | 6.43 ± 0.15 $^{a,b}$ | 8.27 ± 0.05 $^{a}$ |
| | (27.85 - 27.92) | (17.00 - 17.01) | (6.28 - 6.57) | (8.21 - 8.32) |

– Means in the same column with different letters are significant different ( $p < 0.05$).

Table XI. Mean and standard deviation for temperature, salinity, DO and pH for control and probiotic treatment, after adding carbon (organic or inorganic), ammonia chloride and Proline Aquaculture Bacteria Concentrate. There were three replicates per treatment.

| | Treatment | Temperature (˚C) | Salinity (ppt) | DO (mg/l) | pH |
|---|---|---|---|---|---|
| Added carbon | 1 | 27.09 ± 0.06 | 16.00 ± 0.00 | 6.85 ± 0.05 | 8.39 ± 0.12 |
| | 2 | 27.17 ± 0.06 | 16.00 ± 0.00 | 6.82 ± 0.03 | 8.44 ± 0.05 |
| Added ammonia chloride | 1 | 26.63 ± 0.06 | 16.00 ± 0.00 | 7.07 ± 0.04 | 8.24 ± 0.10 |
| | 2 | 26.55 ± 0.15 | 16.00 ± 0.00 | 7.06 ± 0.05 | 8.27 ± 0.06 |
| Added Proline | 1 | 26.68 ± 0.05 | 16.00 ± 0.00 | 7.16 ± 0.02 | 8.20 ± 0.07 |
| | 2 | 26.55 ± 0.06 | 16.00 ± 0.00 | 7.19 ± 0.02 | 8.21 ± 0.07 |

*Nutrient analysis*

The raceway water showed no presence of TAN in the beginning of the experiment, although the treatment with one hundred times of Proline Aquaculture Bacteria Concentrate had TAN concentration present on the second day (4.753 ± 2.369 mg/l), which decreased immediately to low levels by the fourth day (Figure 12).

The nitrite-N, in all raceway water treatments started to decrease on the eighth day, showing the biggest difference between treatments on the last day. The probiotic at one hundred times showed the highest increase of nitrite-N (25.168 ± 3.790 mg/l) and was also the treatment with the lowest nitrite-N concentration (2.605 ± 1.218 mg/l) on the last day. The lowest variation of nitrite-N (5.287 mg/l) was observed in the treatment with only raceway water (Figure 13). The nitrate-N concentration detected in the raceway water treatments was proportional to the concentration of probiotic.

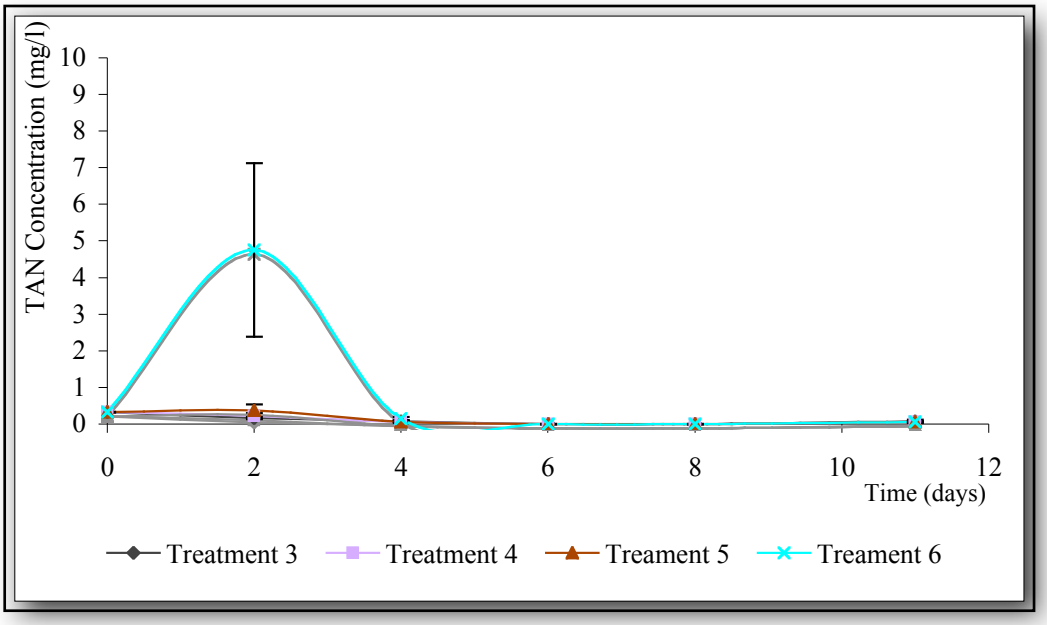


Figure 12. TAN concentration evolution over time of control and probiotic treatment at 15 mg/l of nitrite-N in raceway water. There were three replicates for all treatments. Error bars represent standard deviation around mean of the treatments.

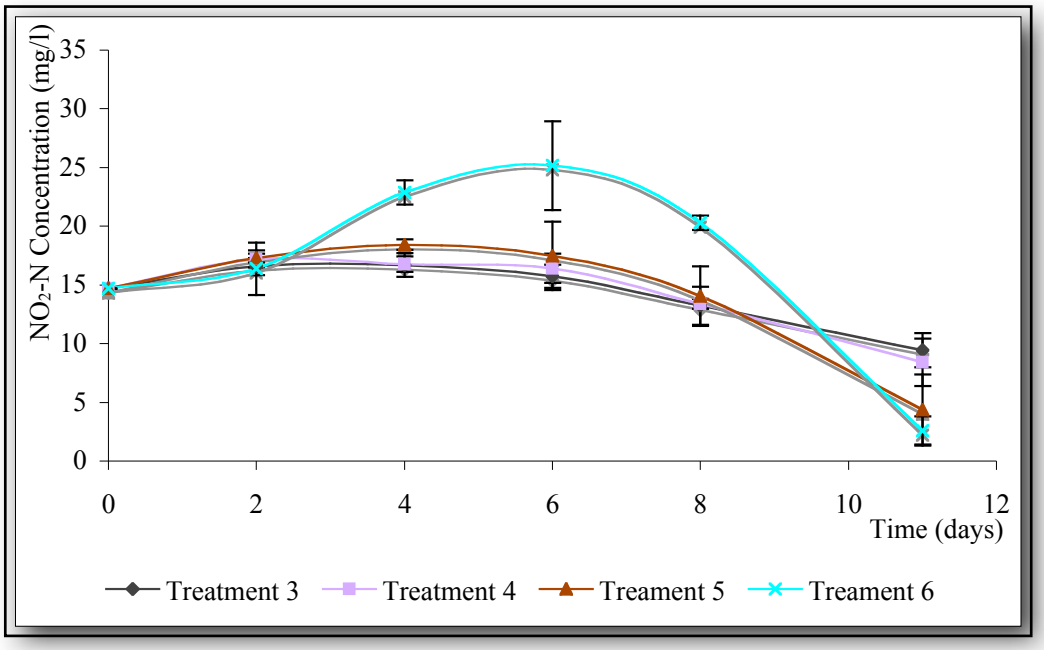


Figure 13. Nitrite-N concentration evolution over timer of control and probiotic treatment at 15 mg/l of nitrite-N in raceway water. There were three replicates for all treatments. Error bars represent standard deviation around mean of the treatments.

The control had present the lowest concentration of nitrate-N (5.893 ± 1.211 mg/l) and the treatment with probiotic at one hundred times had the highest concentration of nitrate (14.132 ± 3.142 mg/l). The treatment with probiotic at providers concentration had presented the nitrate-N at 9.138 ± 2.059 mg/l and the treatment with the probiotic concentration at ten times was 13.244 ± 3.466 mg/l (Table XII).

Table XII. Nitrate-nitrogen concentration (mg/l) and standard deviation in treatments with raceway water and different concentrations of Proline Aqua. Bact. Conc.

| | Day 0 | Day 11 |
|---|---|---|
| Treatment 3 | n.d | 5.893 ± 1.211 |
| Treatment 4 | n.d | 9.138 ± 2.059 |
| Treatment 5 | n.d | 13.244 ± 3.466 |
| Treatment 6 | n.d | 14.132 ± 3.142 |

n.d - Not detectable levels

The clean seawater treatments showed no TAN concentration until the day that ammonia chloride was added (day 24), in the control and in treatment with probiotic (Figure 14). After the ammonia chloride was added to both treatments, the probiotic treatments showed a decrease in TAN concentration and the control treatment was stabilized. In the probiotic treatment, it was detected that the beaker with organic carbon had 0.649 mg/l of TAN two days after adding the probiotic, while the beakers with inorganic carbon still had 3.975 ± 0.240 mg/l of TAN, and the control had 6.649 ± 0.495 mg/l of TAN concentration.

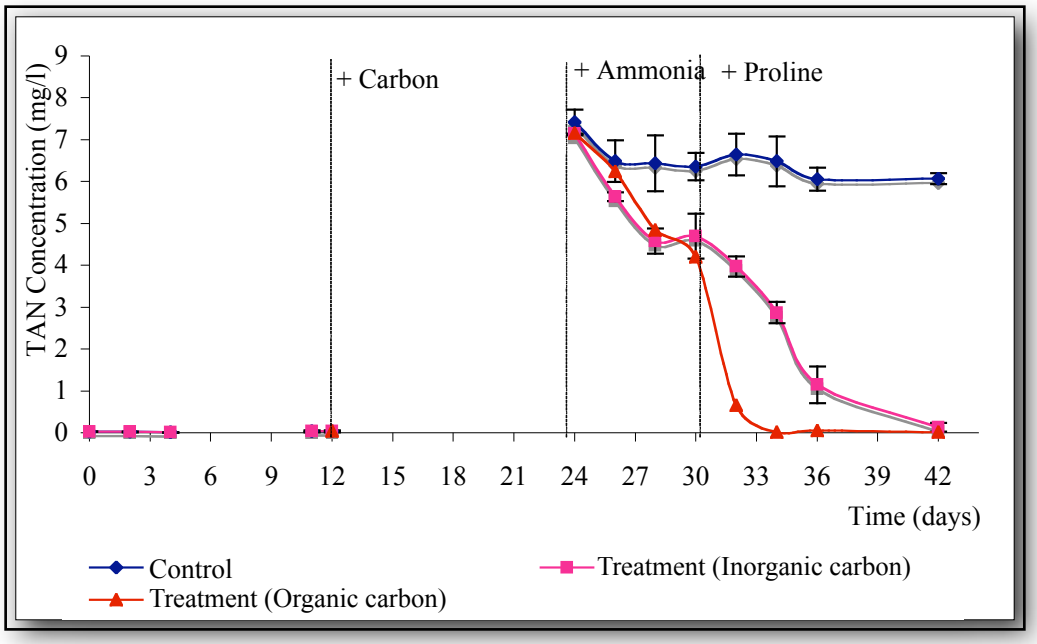


Figure 14. TAN concentration evolution over timer of control and probiotic treatment at 15 mg/l of nitrite and clean seawater. Three replicates for control treatment; Two replicate for treatment with inorganic carbon and one replicate for treatment with organic carbon. Error bars represent standard deviation around mean of the treatments.

The nitrite-N concentration was stable until the ammonia chloride was added, decreasing thereafter. When the probiotic was added, differences in nitrite-N concentration between the beakers with inorganic carbon and organic carbon of the treatment with probiotic were observed. The beakers with inorganic carbon presented a slow decrease over the time, from 14.763 ± 0.456 mg/l in the day the probiotic was added to 5.910 ± 2.152 mg/l in the last day. On the contrary, the beaker with organic carbon presented a slight increase of nitrite-N (17.931 mg/l) and in the last day a fast decrease of nitrite-N to 3.558 mg/l was observed (Figure 15). The control treatment presented no variation in the nitrite-N concentration, even after the carbon source and the ammonia were added (Figure 15).

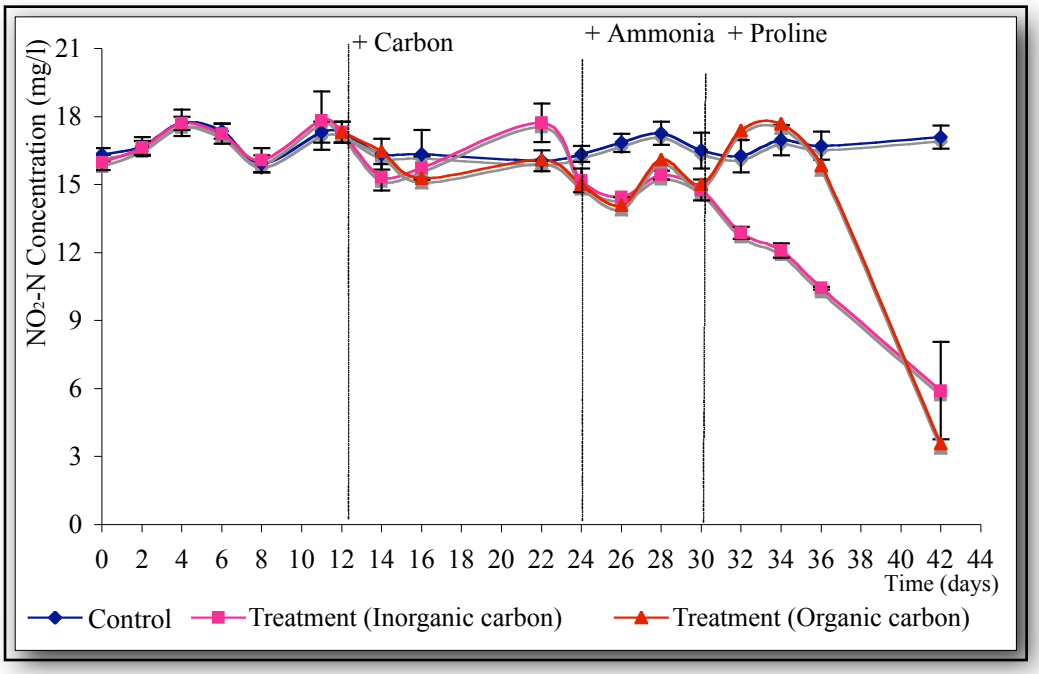


Figure 15. Nitrite-N concentration evolution over timer of the control and probiotic treatment at 15 mg/l of nitrite and clean seawater. Three replicates for the control and probiotic treatment; After the addition of carbon source, there were two replicates for the treatment with inorganic carbon and one replicate for the treatment with organic carbon,. Error bars represent standard deviation around the means of the treatments.

The nitrate-N concentration in the control treatment was below detectable levels in all the analysis (Table XIII). In the probiotic treatment, the nitrate-N was only detectable on the last day, being 11.045 mg/l and 9.305 ± 1.694 mg/l in the beaker with organic carbon and in the beakers with inorganic carbon, respectively (Table XIII).

Table XIII. Nitrate-nitrogen concentration mean (mg/l) and standard deviation of the clean seawater treatments at day 0, the day before adding the carbon source (day 11), in the day when ammonium chloride was added (day 24), the day that Proline Aqua. Bact. Conc.(day 30) and in the last day. There were three replicates for control and for treatment 2 (inorganic carbon) until day 12. From day 12 to day 42 there were two replicates for treatment 2-inorganic carbon and one replicate for treatment 2-organic carbon.

| | Day 0 | | Day 11 | Day 24 | Day 30 | Day 42 | |
|---|---|---|---|---|---|---|---|
| Treatment 1 | 1.406 | ± 0.147 | n.d. | n.d. | n.d | n.d. | n.d. |
| Treatment 2 (Inorganic Carbon) | 1.406 | ± 0.147 | n.d. | n.d. | n.d | 9.305 | ± 1.694 |
| Treatment 2 (Organic Carbon) | - | - | n.d. | n.d. | n.d | 11.045 | |

## 3.4. Effects of commercial probiotic on shrimp

*Water quality analysis*

There was no significant difference in temperature, salinity, DO and pH between treatments (Table XIV).

Table XIV. Mean and standard deviation for temperature, salinity, DO and pH for control and probiotic treatment. There were three replicates per treatment.

| Treatment * | Temperature (˚C) | Salinity (ppt) | DO (mg/l) | pH |
|---|---|---|---|---|
| Control | 26.22 ± 0.09 | 17.61 ± 0.19 | 6.51 ± 0.20 | 7.80 ± 0.01 |
| With probiotic | 26.20 ± 0.08 | 17.65 ± 0.03 | 6.70 ± 0.09 | 7.84 ± 0.03 |

* No significant difference between treatments ($p > 0.05$)

*Nutrient analysis*

The variation of TAN (Figure 16) and nitrite-N (Figure 17) concentration observed was similar in the tanks with probiotic and in the control tanks. The highest TAN concentration was reached in day 19, and it was 6.500 ± 1.902 mg/l for the treatment with probiotic and 5.570 ± 0.903 mg/l for the control. The lowest TAN concentrations matched with the days of water exchange (Figure 16). The nitrite-N concentration had low variation during the period of the experiment (Figure 17). Only in the last day of experiment was observed a divergence in TAN and nitrite-N concentration between treatments.

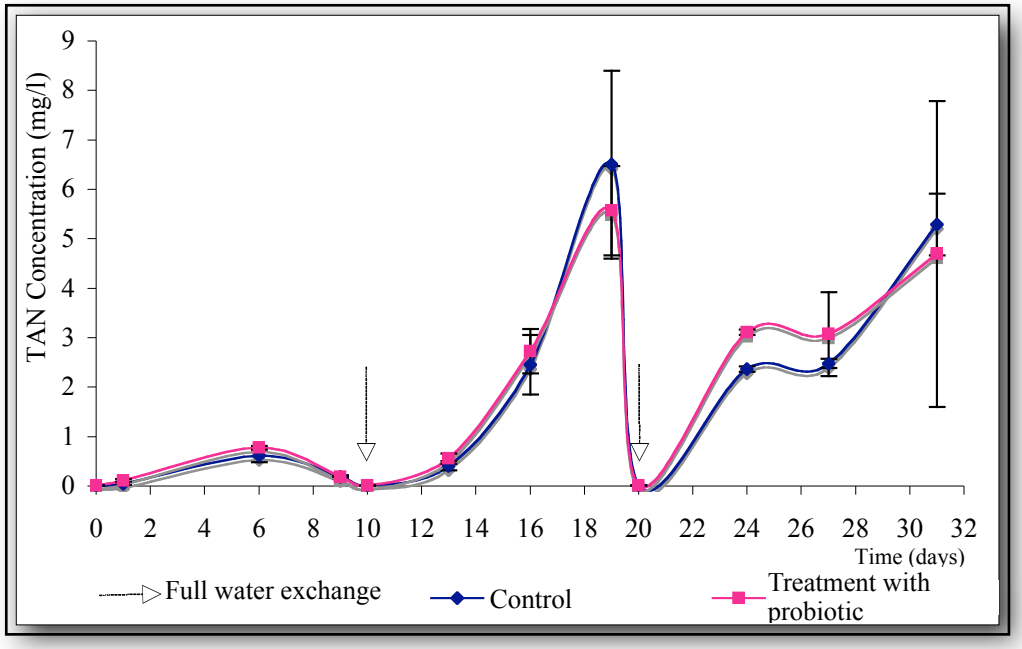


Figure 16. TAN concentration evolution over timer in control and probiotic treatments. There were four replicates per treatments. Error bars represent standard deviation around mean of the treatments.

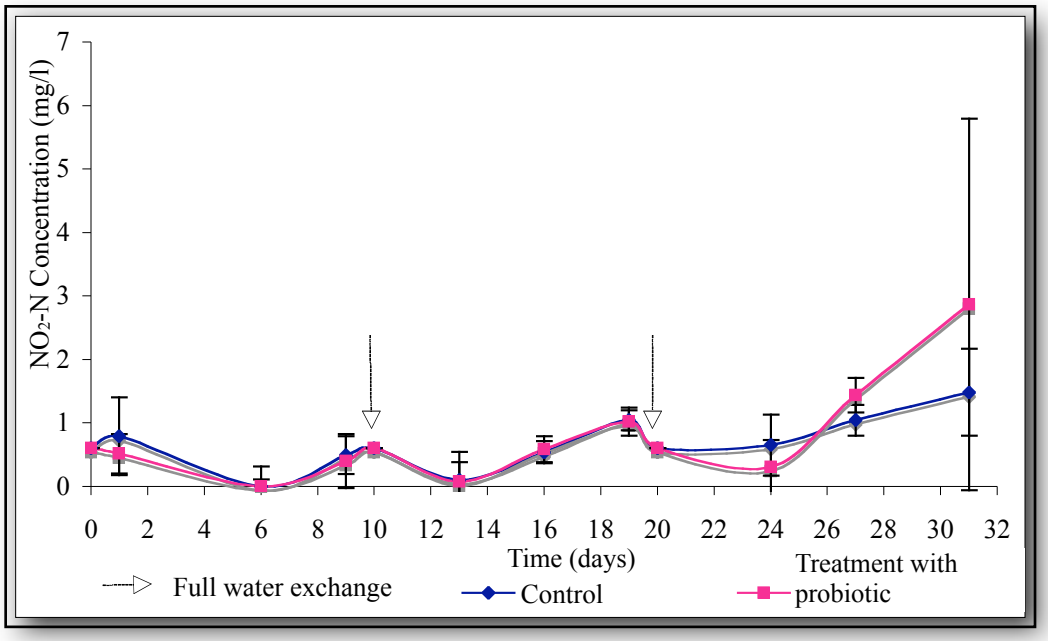


Figure 17. Nitrite-N concentration evolution over timer in control and probiotic treatment in the tanks. There were four replicates per treatments. Error bars represent standard deviation around mean of the treatments.

*Statistical analysis*

The stocking and harvesting weight presented no significant difference between treatments, with the growth of the control being 4.07 ± 0.55 g and that of the probiotic being of 3.86 ± 0.72 g (Table XV).

Table XV. Mean and standard deviation for stocking weight, harvesting weight, weight gain and survival for control and probiotic treatments. There were four replicates per treatment.

| Treatment * | Stocking weight (g) | Harvesting weight (g) | Weigth gain (g) | Survival (%) |
|---|---|---|---|---|
| control | 1.27 ± 0.05 | 5.34 ± 0.59 | 4.07 ± 0.55 | 100.00 ± 0.00 |
| with probiotic | 1.20 ± 0.09 | 5.06 ± 0.73 | 3.86 ± 0.72 | 100.00 ± 0.00 |

* No significant difference between treatments ($p > 0.05$)

# 4. DISCUSSION

## 4.1. Feed additive product experiment

The aim of this experiment was to evaluate the influence of the Engest in the North American aquaculture system, since according to the provider this product increases the digestion and assimilation of the shrimp's feed. The experimental design was made considering that the product had all the enzymatic supplements that the provider reported in the guideline, and for this reason there was no enzyme analysis.

To make sure that the results obtained after the use of probiotics or enzymes products are due only to their action, some conditions had to be implemented. This experiment was conducted in an indoor laboratory, using a flow-through system and small tanks, allowing a water quality control and similar conditions between tanks, and so avoiding the growth of microorganisms in the tank, which could change the results (Verschuer *et al*. 2000). Other studies (McIntosh *et al.* 2000; Sung *et al.* 2003; Vaseeharan *et al.* 2004; Decamp *et al.* 2005; Macey & Coyne 2005) carrie out on farms, did not have water control due to the fact that the ponds were locate outdoors. Their results were positive, suggesting that the probiotics had an effect on the shrimps. In these cases, we do not know if this was a false positive result because their conditions were not the right ones. They did not knew the water's microorganism community and there are no studies conducted under laboratory conditions showing a similar effect with the same probiotic.

The results obtained contradicted the statement of the product provider. According to the FCR values attained there was no significant statistical difference between treatments (Table VII), although the FCR average of the treatment with probiotic is 0.12 lower than in the control. Similarly, although the overall growth was higher in shrimp fed with the probiotic, there was no significant statistical difference between treatments.

The reason for this experiment be conducted with juvenile shrimp for one third of the regular time needed on a shrimp growth farm, was because according to some authors, like Verschuer *et al*. (2000) and Simões *et al*. (2002), the smaller the host the less established community will be there and the easier it will be for the probiotic and derived compounds to establish in the host. Besides the fact that it is during the first month of growth that the shrimp growths faster (personal communication C. Otoshi, 2005), supposedly, it should be during this time that the differences in growth are more evident. Since no differences were observed between treatments, there was no reason to submit the shrimps for gut enzymes/bacteria analysis and identify possible differences between treatments (Moss *et al*. 2001).

Another possible explanation why no significant statistical differences were observed between treatments, apart from the Engest action, could have been due to the instructions methods of the provider. There was no warranty that the shrimp had ingested the product due to the small quantity included within the feed and the leaching that was observed. When dealing with large aquaculture farm operations, there should be more easier and effective methods to use these sort of products. One possibility is to incorporate the product directly within the feed during feed manufacture; thus ensuring that when the product comes into contact with the water, the

enzymes are released slowly and by so doing increasing the probability of the shrimp to ingest the product and minimizing the leaching. A different possibility could be the insertion of a bacteria community in the pellet, instead of enzymes (Decamp *et al.* 2005). These bacteria would colonize the walls of the tanks or the water column, producing the enzymes in considerable quantities. The shrimp could then filter it or even ingest some of these bacteria, and so allowing the production of enzymes within the shrimp's digestive tract.

The significant statistical difference found in the value of the temperature and the salinity (Table VI) does not influence the FCR or the shrimp's growth. Between the treatment and the control the variation of temperature was of 0.25 ˚C in the morning and 0.26 ˚C in the afternoon. In what concerns to the afternoon salinity the difference was 0.07 ppt. These changes and differences are not thought to be enough to influence the shrimp's biology. Similarly the shrimp mortality observed was not believed to have been due to the product tested, but rather due to external conditions.

## 4.2. Nitrification probiotic screening experiment

This experiment was carrie out to screen commercial probiotics that were claiming to reduce nitrogen compounds and help the North American system to have a better shrimp production performance and at the same time to be more environmental friendly. This first experiment was conducted in clean seawater to observe if the commercial probiotics, by themselves, were working and which one had the best performance, following the steps presented by Verschuer *et al.* (2000).

The results showed that the Microtack 22 L probiotic did not decrease the TAN concentration in the treatments (Treatments 3, 4 - Figure 9), perhaps due to the fact that the volume of product specified by the provider to use under emergency situations was not the appropriate dosage level. The fact that the nitrifiers' bacteria are aerobic and chemoautotrophic, means that they have the ability to use inorganic substrates (nitrogenous compounds) as a source of energy and the final acceptor usually is the oxygen. Therefore the fermentation product of these bacteria, if it exits, cannot be used as a compound to oxidize ammonia to nitrate. Moreover no references were found in the bibliography regarding the use or production of a fermentation product by the nitrifiers' bacteria. It is known that some heterotrophic organisms are able to reduce the nitrogenous compounds in the absence of oxygen (Brune *et al.* 2004; Gutierrez-Wing *et al.* 2004; Morrison *et al.* 2004; Samocha *et al.* 2004), but a larger variety of end products are usually formed. Maybe, this probiotic was made with some other bacteria in higher amounts than the nitrifiers' bacteria, thus contributing to the apparent failure of the probiotic.

In conclusion the Microtack 22 L cannot be used under ammonia emergency situations since it does not present satisfactory results in a short period of time. To this product works, as the provider says, the dosage levels that should be added to the tanks should be restated or the technique by which it is made should be revised.

In the two treatments with Proline Aquaculture Bacteria Concentrate and un-ionized ammonia (Treatments 5,6), the TAN concentration reduced in less than a week (Figure 9), which is a reasonable result. Of particular note was the increase and decrease of nitrite (Figure 10) and,

as the last compound of the nitrite-oxidizer bacteria cycle, the increase of nitrate (Table IX).

The process of nitrification (Equation 1, 2) consumes a substantial amount of alkalinity, in the form of bicarbonate, affecting both hardness and pH stability. The equation 3 shows the alkalinity buffer system of the seawater.

$$H_2O + CO_2 \Leftrightarrow H_2CO_3 \Leftrightarrow HCO_3 + H^+ \Leftrightarrow CO_3 + 2H^+ \quad (3)$$

With the consumption of bicarbonate by the ammonia-oxidizer bacteria and by the nitrite-oxidizer bacteria, the buffer system was forced to release hydrogen ions due to the transformation of carbonic acid in bicarbonate, making the pH fall. The addition of sodium bicarbonate to the water aimed at compensating the bicarbonate consumed by the nitrifying bacteria, allowing the buffer system shift to left, increasing the pH value. In conclusion, as a direct effect of the probiotic, the statistical differences obtained in the pH values in those two treatments (Table VIII) were different from the other treatments. An effort was made to maintain all the treatments with the same pH, by adding sodium bicarbonate.

As in the previous experiment, it is assumed that the variation observed in the temperature (0.12 ˚C), salinity (0.5 ppt) or DO (0.49 mg/l; Table VIII) did not contribute to the differences obtained in the nutrient results between treatments and control (Figures 6 – 9).

The Proline Aquaculture Bacteria Concentrate seems to work as the provider states, however, it looks like this product has an initial period of four days before the bacteria starts working, which could be the normal adaptation time (Shilo & Rimon, 1982; Belser, 1984).

Nevertheless, more experiments should be carrie out to verify if this period is obligatory or if it is an adaptation period of the bacteria to the medium. Because under emergency situations the probiotic should act almost immediately after added to the water, otherwise it could be disastrous to the farm and result in a large loss of money, due to the high price of the shrimp in the market (FAO, 2004). A possible way to solve this problem could be the use of a bacteria solution previously prepared in a tank with high ammonia concentration, where the bacteria are not under the restricted conditions of energy source and temperature, as they are in the container.

The results of the bacteria analysis, confirm the results observed in the nutrient analysis. In all the treatments the concentration of bacteria per milliliter decreased (Figure 11), with the exception of the treatments 5, 6. It was not possible to proceed to the count of bacteria in these treatments because they formed layers on the walls of the beaker and the water sample was not homogeneous. To proceed to the count these treatments it was necessary to pass the sample by ultrasonic sound, and this was not possible to do. However a high bacteria concentration was observed on slides.

## 4.3. Nitrite oxidizers experiment

The major problem of the North American system is to reduce the nitrogenous compounds of the medium to less toxic forms, and in general the compound that causes the most problems is the nitrite. For this reason, the second experiment was conducted with the Proline Aquaculture Bacteria Concentrate, since it was the product with the best performance (previous experiment).

So two experiments were undertaken, one with clean seawater where similar results to the last experiment (Figure 11 and Table IX) were expected and another experiment with raceway water to see if the probiotic worked directly in this eutrophic water.

The results obtained during the firsts eleven days (Figure 14) in the clean seawater treatments, did not show the same performance of the probiotic as before. On the other hand, the results of the raceway water treatments, in the same period, showed a decrease of the nitrite-N concentration (Figure 13). Yet, this decrease in concentration occurred when the water started to be more translucent and layers of dead cells started to appear on the bottom of the beakers (data not shown). The nitrite-N concentration declined in the raceway water control, which means that this water has nitrite oxidizer bacteria, but somehow they were being inhibited by the phytoplankton existent in the water. It is unlikely that protozoas were killing the nitrite oxidizers' bacteria, since a proportional decrease of the nitrite in the raceway water treatments was observed, and if hypothetically they were being killed the nitrite would not decrease in the control raceway water.

The results are indeed a preoccupation on what this probiotic is concerned and in future investigations possible relations between the ammonia oxidizer bacteria and the nitrite oxidizer bacteria should be taken in consideration, as well as the interaction between the nitrifiers' bacteria and the phytoplankton.

Since a nitrite-N concentration decrease was observed in the raceway water experiment it was decided to finish this experiment (Figure 13). In the clean seawater experiment no decrease

in nitrite-N concentration was observed, so it was maintained and it was decided to make some trials on it (Figures 13,14), to help in the development of future experiments.

Inorganic carbon was added to two control beakers and two treatment beakers, because this was the first difference being identified between this experiment and the probiotic screening experiment. Moreover organic carbon was added to the others beakers, since a decrease in nitrite-N concentration occurred in the raceway water experiment when the phytoplankton died. It was thought that the organic carbon released by the phytoplankton was used as primary carbon energy source. Because there were no positive results in ten days (Figure 15), this hypothesis was rejected.

During the second trial (between day 24 and day 30) ammonia was added to all the beakers, to verify the existence of any association between the nitrite oxidizers bacteria and the ammonia oxidizers bacteria (Figure 14). The decrease of ammonia and the slight decrease of nitrite-N concentration in the treatment with probiotic and also with organic or with inorganic carbon (Figure 15), had allowed to reject the hypothesis pondered that the ammonia and nitrite oxidizer bacteria were dead. However, the results were not satisfactory.

In the last trial, from day 30 to day 42, the probiotic was added again in the provider's concentration to the treatment. With this addition, the slow nitrification process that was being observed increased (Figures 13, 14). It looks likes the ammonia oxidizer bacteria had a faster adaptation in the beaker with organic carbon, but the nitrite oxidizer bacteria were not able to follow this rhythm, and there was higher accumulation of nitrite in this beaker (Treatment 2 -

organic carbon; Figures 13,14). In the beakers with inorganic carbon, the ammonia oxidizer bacteria were not able to adapt rapidly (Treatment 2 - inorganic carbon; Figure 14) and the nitrite oxidizer bacteria were able to decrease the nitrite-N concentration (Treatment 2 - inorganic carbon; Figure 15), at the same progression that the ammonia oxidizer bacteria were oxidizing ammonia to nitrite.

These results show that it is not probable that an inhibitory proceeding of the nitration had happened in the initial week (Anthonisen *et al*., 1976; Charley *et al*., 1980; Audic *et al*., 1984; Both *et al*., 1992; Komaros *et al*., 1996; Cohen *et al*., 2005). It is more likely that an association existed between the nitrite oxidizers bacteria and the ammonia oxidizers bacteria. Additional experiments should be conducted to ascertain if it was obligatory for these bacteria to work together or if some chemical factor or nutrient that is released by the ammonia oxidizers bacteria is essential for the normal work of the nitrite oxidizers bacteria.

## 4.4. Effects of commercial probiotic on shrimp

The purpose of this experiment was to verify if the bacteria itself was toxic to the shrimp. So the water was maintained with the same probiotic and with no differences in the ammonia concentration between treatments, as often as possible. Yet the time the ammonia concentration would take to increase the levels that allow the probiotic to start working and to cause differences in ammonia concentration between treatments was not known.

A full exchange of the water containing the probiotic was made, to avoid the indirect

effect of water quality on the shrimp, as shown in the previous results. As it is known, if the shrimp has a better water quality, it will grow better.

Since this was a batch system, it was believed that to have less ammonia concentration in the water and to make it possible to maintain the water for a longer period of time the number of shrimps per tank had to be reduced. Realizing that with five shrimps per tank, if one died, that would represent a high mortality in the treatment. Because of that one more tank was added to each treatment to compensate the low number of shrimps.

The results of the previous experiment with the Proline Aquaculture Bacteria Concentrate seemed to indicate that the bacteria would start working between the tenth and the eleventh day. On the first week we realized that a ten day period with two additions of probiotic would be the maximum allowed by the system before the ammonia and nitrite concentrations started to differ (Figure 16, 17). The experiment finished after thirty-one days, and the last water exchange should have been on the thirtieth day, but it was delayed one day, as well as the nutrient analysis. Because of this, we were able to confirm the hypothesis of how long it would take for the nitrogen compounds concentration to start differing (Figure 16, 17).

After successive administrations of the probiotic at emergency concentrations (Ishibashi & Yamazaki, 2001), no differences were observed between treatments, nor negative effects of the bacteria on the shrimp. By the fact that these bacteria were not founded inside of organism (Gatesoupe, 1999) and due to results obtained, it seems to indicate that there is a very low possibility of toxicity of the probiotic to the shrimp.

# 5. CONCLUSION

All the products, enzymes and bacteria (probiotics), used in this study, arose some questions or did not work as the provider claimed.

Products like Engest that are used as an additive in the feed and have the function of promoting the growth or feed conversion ratio, should be conceived as a product of easy management in a large aquaculture facility and at the same time ensure that no leaching of the product will occur and the shrimp will ingest it. Another advantage would be the insertion of living bacteria (probiotic) in this kind of product, promoting the production the enzymes, the environment colonization and the competition for space between these bacteria and other microorganisms' pathogens.

In the nitrification experiments only the Proline Aquaculture Bacteria Concentrate probiotic presented results that, with future research, can help in the nitrification process in the North American system's water. This probiotic showed to work under our conditions, but more research should be carried out in the microbiology field. Because little is known about the nitrite-oxidizer bacteria, and it was in the nitration process where the water quality problem appeared, studies to identify the association between the ammonia-oxidize bacteria and nitrite-oxidize bacteria should be undertaken, as well as between the nitrifiers' bacteria and the phytoplankton.

Summing up, a lot of the studies made on the different probiotic mechanisms are not made in a multistep research. The research of the probiotic effect is done directly or in farm

conditions or under laboratory conditions, making it difficult to say if the product really works by itself and under different farm conditions. These experiments can be carried out simultaneously, allowing to know how the product works, if it works by itself and if it can be used under different farm conditions.

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# 7. APPENDIX

## 7.1 Appendix A - Feed additive product experiment

Table 1. Levene's Test for equality of variances of water quality, initial and final weight, weight gain, FCR and shrimp survival. FCR and survival values were transformed.

| | F | Sig. |
|---|---|---|
| Initial weight | 0.13 | 0.73 |
| Final weight | 0.49 | 0.52 |
| Weigth gain | 0.38 | 0.57 |
| Temperature AM | 3.52 | 0.13 |
| Temperature PM | 3.39 | 0.14 |
| Salinity AM | 0.00 | 1.00 |
| Salinity PM | 0.00 | 1.00 |
| DO AM | 0.01 | 0.93 |
| DO PM | 0.18 | 0.70 |
| pH AM | 0.00 | 1.00 |
| pH PM | 0.31 | 0.61 |
| FCR (Log10/Sqrt) | 1.80 | 0.25 |
| Survival Arcsin | 0.83 | 0.41 |

Equal variances assumed

Table 2. Independent t-test analysis for of water quality, initial and final weight, weight gain, FCR and shrimp survival. FCR and survival values were transformed.

| | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|
| | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | 95% Confidence Interval of the Difference | |
| | | | | | | Lower | Upper |
| Initial weight | 0.19 | 4.00 | 0.86 | 0.01 | 0.04 | -0.09 | 0.11 |
| Final weight | -0.68 | 4.00 | 0.53 | -0.31 | 0.46 | -1.58 | 0.96 |
| Weigth gain | -0.65 | 4.00 | 0.55 | -0.32 | 0.49 | -1.68 | 1.04 |
| Temperature AM | 10.22 | 4.00 | 0.00 | 0.21 | 0.02 | 0.15 | 0.27 |
| Temperature PM | 2.78 | 4.00 | 0.05 | 0.14 | 0.05 | 0.00 | 0.29 |
| Salinity AM | -2.45 | 4.00 | 0.07 | -0.02 | 0.01 | -0.04 | 0.00 |
| Salinity PM | -2.83 | 4.00 | 0.05 | -0.04 | 0.01 | -0.08 | 0.00 |
| DO AM | 1.92 | 4.00 | 0.13 | 0.09 | 0.05 | -0.04 | 0.21 |
| DO PM | 0.57 | 4.00 | 0.60 | 0.05 | 0.08 | -0.18 | 0.27 |
| pH AM | 0.00 | 4.00 | 1.00 | 0.00 | 0.00 | -0.01 | 0.01 |
| pH PM | 1.51 | 4.00 | 0.21 | 0.01 | 0.01 | -0.01 | 0.04 |
| FCR (Log10/Sqrt) | 0.39 | 4.00 | 0.72 | 0.04 | 0.11 | -0.25 | 0.33 |
| Survival Arcsin | 0.31 | 4.00 | 0.78 | 0.10 | 0.33 | -0.81 | 1.01 |

## 7.2 Appendix B. Nitrification Probiotic Screening Experiment

Table 2 - 10. Analysis of TAN, ammonia, un-ionized ammonia-N, nitrite and nitrate concentration (mg/l) mean values and standard deviation.

Table 2. Treatment 0

| Date | Day | TAN (mg/l) | Ammonia-N (mg/l) | Unionized Ammonia-N (mg/l) | Nitrite-N (mg/l) | Nitrate-N (mg/l) |
|---|---|---|---|---|---|---|
| 9/30/05 | 0 | 0.007 | 0.006 | 0.001 | 0.792 | n.d. |
| 10/2/05 | 2 | 0.014 | 0.013 | 0.001 | 0.884 | |
| 10/4/05 | 4 | 0.022 | 0.021 | 0.002 | 0.423 | |
| 10/6/05 | 6 | 0.034 | 0.031 | 0.003 | 0.700 | |
| 10/8/05 | 8 | 0.051 | 0.047 | 0.004 | 1.161 | n.d. |

Table 3. Treatment 1

| Date | Day | TAN (mg/l) | Std. deviation | Ammonia-N (mg/l) | Unionized Ammonia-N (mg/l) | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) | Std. deviation |
|---|---|---|---|---|---|---|---|---|---|
| 9/30/05 | 0 | 7.732 | 0.156 | 7.156 | 0.577 | 0.239 | 0.369 | n.d. | |
| 10/2/05 | 2 | 7.701 | 0.150 | 7.125 | 0.577 | 0.269 | 0.384 | | |
| 10/4/05 | 4 | 7.301 | 0.150 | 6.808 | 0.493 | 0.853 | 0.296 | | |
| 10/6/05 | 6 | 6.703 | 0.136 | 6.162 | 0.541 | -0.038 | 0.369 | | |
| 10/8/05 | 8 | 7.654 | 0.335 | 7.026 | 0.629 | 1.130 | 0.296 | n.d. | |

Table 4. Treatment 2

| Date | Day | TAN (mg/l) | Std. deviation | Ammonia-N (mg/l) | Unionized Ammonia-N (mg/l) | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) | Std. deviation |
|---|---|---|---|---|---|---|---|---|---|
| 9/30/05 | 0 | 12.974 | 0.236 | 12.028 | 0.946 | 0.607 | 0.461 | n.d. | n.d. |
| 10/2/05 | 2 | 12.526 | 0.098 | 11.639 | 0.887 | -0.007 | 0.540 | | |
| 10/4/05 | 4 | 11.488 | 0.357 | 10.635 | 0.853 | 0.669 | 0.784 | | |
| 10/6/05 | 6 | 10.372 | 0.510 | 9.512 | 0.860 | 0.361 | 0.416 | | |
| 10/8/05 | 8 | 12.484 | 1.379 | 11.487 | 0.997 | 0.946 | 0.628 | n.d. | n.d. |

Table 5. Treatment 3

| Date | Day | TAN (mg/l) | Std. deviation | Ammonia-N (mg/l) | Unionized Ammonia-N (mg/l) | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) | Std. deviation |
|---|---|---|---|---|---|---|---|---|---|
| 9/30/05 | 0 | 7.666 | 0.857 | 7.152 | 0.514 | 0.454 | 0.600 | 0.002 | 1.391 |
| 10/2/05 | 2 | 7.285 | 0.184 | 6.750 | 0.534 | 0.269 | 0.282 | | |
| 10/4/05 | 4 | 7.080 | 0.106 | 6.609 | 0.471 | 1.222 | 0.349 | | |
| 10/6/05 | 6 | 7.175 | 0.095 | 6.579 | 0.595 | 1.929 | 0.886 | | |
| 10/8/05 | 8 | 7.175 | 0.223 | 6.644 | 0.531 | 0.792 | 0.889 | n.d. | |

Table 6. Treatment 4

| Date | Day | TAN (mg/l) | Std. deviation | Ammonia-N (mg/l) | Unionized Ammonia-N (mg/l) | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) | Std. deviation |
|---|---|---|---|---|---|---|---|---|---|
| 9/30/05 | 0 | 12.597 | 0.595 | 11.742 | 0.855 | 0.761 | 0.969 | 0.318 | 0.969 |
| 10/2/05 | 2 | 11.284 | 0.423 | 10.437 | 0.847 | 0.546 | 0.141 | | |
| 10/4/05 | 4 | 11.174 | 0.125 | 10.418 | 0.755 | 0.177 | 0.600 | | |
| 10/6/05 | 6 | 12.849 | 0.256 | 11.834 | 1.015 | 0.300 | 0.106 | | |
| 10/8/05 | 8 | 12.602 | 0.885 | 11.540 | 1.061 | 0.638 | 0.473 | n.d. | |

Table 7. Treatment 5

| Date | Day | TAN (mg/l) | Std. deviation | Ammonia-N (mg/l) | Unionized Ammonia-N (mg/l) | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) | Std. deviation |
|---|---|---|---|---|---|---|---|---|---|
| 9/30/05 | 0 | 6.949 | 0.289 | 6.458 | 0.491 | 0.946 | 0.508 | n.d. | |
| 10/2/05 | 2 | 6.648 | 0.268 | 6.200 | 0.448 | 1.161 | 0.160 | | |
| 10/4/05 | 4 | 5.162 | 0.295 | 4.883 | 0.279 | 2.913 | 0.639 | | |
| 10/6/05 | 6 | 1.401 | 0.403 | 1.338 | 0.064 | 4.634 | 0.704 | | |
| 10/8/05 | 8 | 0.017 | 0.004 | 0.017 | 0.000 | 3.128 | 0.648 | 3.298 | 0.227 |

Table 8. Treatment 6

| Date | Day | TAN (mg/l) | Std. deviation | Ammonia-N (mg/l) | Unionized Ammonia-N (mg/l) | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) | Std. deviation |
|---|---|---|---|---|---|---|---|---|---|
| 9/30/05 | 0 | 12.956 | 0.740 | 12.245 | 0.711 | 1.284 | 0.933 | n.d. | |
| 10/2/05 | 2 | 12.431 | 0.109 | 11.724 | 0.708 | 0.361 | 0.282 | | |
| 10/4/05 | 4 | 9.429 | 0.374 | 9.027 | 0.402 | 2.513 | 0.349 | | |
| 10/6/05 | 6 | 7.741 | 0.196 | 7.503 | 0.238 | 4.327 | 1.012 | | |
| 10/8/05 | 8 | 1.381 | 0.289 | 1.381 | 0.000 | 8.784 | 0.192 | 2.031 | 1.318 |

Table 9. Treatment 7

| Date | Day | TAN (mg/l) | Std. deviation | Ammonia-N (mg/l) | Unionized Ammonia-N (mg/l) | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) | Std. deviation |
|---|---|---|---|---|---|---|---|---|---|
| 9/30/05 | 0 | 0.013 | 0.004 | 0.012 | 0.001 | 1.776 | 0.416 | n.d. | |
| 10/2/05 | 2 | 0.029 | 0.028 | 0.027 | 0.002 | -0.007 | 0.373 | | |
| 10/4/05 | 4 | 0.029 | 0.036 | 0.027 | 0.002 | 0.884 | 0.799 | | |
| 10/6/05 | 6 | 0.210 | 0.083 | 0.195 | 0.015 | 0.085 | 0.508 | | |
| 10/8/05 | 8 | 0.194 | 0.122 | 0.179 | 0.015 | 1.130 | 0.324 | n.d. | |

Table 10. Treatment 8

| Date | Day | TAN (mg/l) | Std. deviation | Ammonia-N (mg/l) | Unionized Ammonia-N (mg/l) | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) | Std. deviation |
|---|---|---|---|---|---|---|---|---|---|
| 9/30/05 | 0 | 0.021 | 0.020 | 0.020 | 0.001 | 0.730 | 0.540 | n.d. | |
| 10/2/05 | 2 | 0.039 | 0.002 | 0.035 | 0.003 | 0.331 | 0.244 | | |
| 10/4/05 | 4 | 0.023 | 0.016 | 0.022 | 0.002 | 0.577 | 0.384 | | |
| 10/6/05 | 6 | 0.050 | 0.024 | 0.047 | 0.004 | 0.484 | 0.384 | | |
| 10/8/05 | 8 | 0.042 | 0.020 | 0.039 | 0.004 | 1.284 | 0.614 | n.d. | |

## 7.3 Appendix C. Nitrite oxidizer experiment

Table 12-15. Analysis of TAN, nitrite-N and nitrate-N concentration (mg/l) mean values and standard deviation of the raceway water treatments with 15 mg/l of nitrite-N.

Table 12. Treatment 3

| Date | Day | TAN (mg/l) | Std. deviation | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) | Std. deviation |
|---|---|---|---|---|---|---|---|
| 10/17/05 | 0 | 0.330 | 0.000 | 14.717 | 0.000 | n.d. | |
| 10/19/05 | 2 | 0.171 | 0.032 | 16.561 | 0.720 | | |
| 10/21/05 | 4 | 0.074 | 0.008 | 16.684 | 1.012 | | |
| 10/23/05 | 6 | 0.000 | 0.000 | 15.731 | 0.998 | | |
| 10/25/05 | 8 | 0.000 | 0.000 | 13.241 | 1.621 | | |
| 10/28/05 | 11 | 0.081 | 0.036 | 9.430 | 1.465 | 5.893 | 1.211 |

Table 13. Treatment 4

| Date | Day | TAN (mg/l) | Std. deviation | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) | Std. deviation |
|---|---|---|---|---|---|---|---|
| 10/17/05 | 0 | 0.330 | 0.000 | 14.717 | 0.000 | n.d. | |
| 10/19/05 | 2 | 0.218 | 0.080 | 17.207 | 0.720 | | |
| 10/21/05 | 4 | 0.065 | 0.004 | 16.776 | 0.648 | | |
| 10/23/05 | 6 | 0.000 | 0.000 | 16.408 | 1.252 | | |
| 10/25/05 | 8 | 0.000 | 0.000 | 13.395 | 0.426 | | |
| 10/28/05 | 11 | 0.067 | 0.001 | 8.415 | 2.004 | 9.138 | 2.059 |

Table 14. Treatment 5

| Date | Day | TAN (mg/l) | Std. deviation | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) | Std. deviation |
|---|---|---|---|---|---|---|---|
| 10/17/05 | 0 | 0.330 | 0.000 | 14.717 | 0.000 | n.d. | |
| 10/19/05 | 2 | 0.367 | 0.173 | 17.299 | 0.369 | | |
| 10/21/05 | 4 | 0.067 | 0.031 | 18.436 | 0.436 | | |
| 10/23/05 | 6 | 0.000 | 0.000 | 17.483 | 2.906 | | |
| 10/25/05 | 8 | 0.000 | 0.000 | 14.041 | 2.543 | | |
| 10/28/05 | 11 | 0.057 | 0.020 | 4.358 | 3.031 | 13.244 | 3.466 |

Table 15. Treatment 6

| Date | Day | TAN (mg/l) | Std. deviation | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) | Std. deviation |
|---|---|---|---|---|---|---|---|
| 10/17/05 | 0 | 0.330 | 0.000 | 14.717 | 0.000 | n.d. | |
| 10/19/05 | 2 | 4.753 | 2.369 | 16.377 | 2.236 | | |
| 10/21/05 | 4 | 0.145 | 0.048 | 22.863 | 1.037 | | |
| 10/23/05 | 6 | 0.000 | 0.000 | 25.168 | 3.790 | | |
| 10/25/05 | 8 | 0.000 | 0.000 | 20.312 | 0.614 | | |
| 10/28/05 | 11 | 0.054 | 0.009 | 2.605 | 1.218 | 14.132 | 3.142 |

## 7.3 Appendix C. Nitrite oxidizer experiment

Table 12-15. Analysis of TAN, nitrite-N and nitrate-N concentration (mg/l) mean values and standard deviation of the raceway water treatments with 15 mg/l of nitrite-N.

Table 12. Treatment 3

| Date | Day | TAN (mg/l) | Std. deviation | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) | Std. deviation |
|---|---|---|---|---|---|---|---|
| 10/17/05 | 0 | 0.330 | 0.000 | 14.717 | 0.000 | n.d. | |
| 10/19/05 | 2 | 0.171 | 0.032 | 16.561 | 0.720 | | |
| 10/21/05 | 4 | 0.074 | 0.008 | 16.684 | 1.012 | | |
| 10/23/05 | 6 | 0.000 | 0.000 | 15.731 | 0.998 | | |
| 10/25/05 | 8 | 0.000 | 0.000 | 13.241 | 1.621 | | |
| 10/28/05 | 11 | 0.081 | 0.036 | 9.430 | 1.465 | 5.893 | 1.211 |

Table 13. Treatment 4

| Date | Day | TAN (mg/l) | Std. deviation | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) | Std. deviation |
|---|---|---|---|---|---|---|---|
| 10/17/05 | 0 | 0.330 | 0.000 | 14.717 | 0.000 | n.d. | |
| 10/19/05 | 2 | 0.218 | 0.080 | 17.207 | 0.720 | | |
| 10/21/05 | 4 | 0.065 | 0.004 | 16.776 | 0.648 | | |
| 10/23/05 | 6 | 0.000 | 0.000 | 16.408 | 1.252 | | |
| 10/25/05 | 8 | 0.000 | 0.000 | 13.395 | 0.426 | | |
| 10/28/05 | 11 | 0.067 | 0.001 | 8.415 | 2.004 | 9.138 | 2.059 |

Table 14. Treatment 5

| Date | Day | TAN (mg/l) | Std. deviation | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) | Std. deviation |
|---|---|---|---|---|---|---|---|
| 10/17/05 | 0 | 0.330 | 0.000 | 14.717 | 0.000 | n.d. | |
| 10/19/05 | 2 | 0.367 | 0.173 | 17.299 | 0.369 | | |
| 10/21/05 | 4 | 0.067 | 0.031 | 18.436 | 0.436 | | |
| 10/23/05 | 6 | 0.000 | 0.000 | 17.483 | 2.906 | | |
| 10/25/05 | 8 | 0.000 | 0.000 | 14.041 | 2.543 | | |
| 10/28/05 | 11 | 0.057 | 0.020 | 4.358 | 3.031 | 13.244 | 3.466 |

Table 15. Treatment 6

| Date | Day | TAN (mg/l) | Std. deviation | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) | Std. deviation |
|---|---|---|---|---|---|---|---|
| 10/17/05 | 0 | 0.330 | 0.000 | 14.717 | 0.000 | n.d. | |
| 10/19/05 | 2 | 4.753 | 2.369 | 16.377 | 2.236 | | |
| 10/21/05 | 4 | 0.145 | 0.048 | 22.863 | 1.037 | | |
| 10/23/05 | 6 | 0.000 | 0.000 | 25.168 | 3.790 | | |
| 10/25/05 | 8 | 0.000 | 0.000 | 20.312 | 0.614 | | |
| 10/28/05 | 11 | 0.054 | 0.009 | 2.605 | 1.218 | 14.132 | 3.142 |

Table 16 - 18. Analysis of TAN, nitrite-N and nitrate-N concentration (mg/l) mean values and standard deviation of control and treatment 2 with inorganic carbon or organic carbon.

Table 16. Control

| Date | Day | TAN (mg/l) | Std. deviation | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) | Std. deviation |
|---|---|---|---|---|---|---|---|
| 10/17/05 | 0 | 0.029 | 0.004 | 16.315 | 0.296 | 1.406 | 0.147 |
| 10/19/05 | 2 | 0.021 | 0.006 | 16.684 | 0.416 | | |
| 10/21/05 | 4 | 0.011 | 0.006 | 17.729 | 0.586 | | |
| 10/23/05 | 6 | - | - | 17.361 | 0.349 | | |
| 10/25/05 | 8 | - | - | 15.916 | 0.369 | | |
| 10/28/05 | 11 | 0.038 | 0.020 | 17.299 | 0.461 | n.d. | |
| 10/29/05 | 12 | 0.038 | 0.020 | 17.299 | 0.461 | n.d. | |
| 10/31/05 | 14 | - | - | 16.346 | 0.680 | | |
| 11/2/05 | 16 | - | - | 16.346 | 1.049 | | |
| 11/8/05 | 22 | - | - | 16.039 | 0.464 | | |
| 11/10/05 | 24 | 7.413 | 0.310 | 16.346 | 0.349 | | |
| 11/12/05 | 26 | 6.493 | 0.498 | 16.838 | 0.402 | | |
| 11/14/05 | 28 | 6.437 | 0.670 | 17.268 | 0.508 | | |
| 11/16/05 | 30 | 6.361 | 0.328 | 16.500 | 0.784 | | |
| 11/18/05 | 32 | 6.649 | 0.495 | 16.254 | 0.716 | | |
| 11/20/05 | 34 | 6.484 | 0.594 | 16.961 | 0.667 | | |
| 11/22/05 | 36 | 6.059 | 0.277 | 16.715 | 0.628 | | |
| 11/28/05 | 42 | 6.074 | 0.128 | 17.084 | 0.508 | n.d. | |

Table 17. Treatement 2 (Inorganic Carbon)

| Date | Day | TAN (mg/l) | Std. deviation | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) | Std. deviation |
|---|---|---|---|---|---|---|---|
| 10/17/05 | 0 | 0.029 | 0.004 | 15.977 | 0.349 | 1.406 | 0.147 |
| 10/19/05 | 2 | 0.021 | 0.006 | 16.623 | 0.296 | | |
| 10/21/05 | 4 | 0.011 | 0.006 | 17.699 | 0.296 | | |
| 10/23/05 | 6 | - | - | 17.238 | 0.436 | | |
| 10/25/05 | 8 | - | - | 16.069 | 0.532 | | |
| 10/28/05 | 11 | 0.038 | 0.020 | 17.822 | 1.292 | n.d. | |
| 10/29/05 | 12 | 0.038 | 0.020 | 17.299 | 0.461 | n.d. | |
| 10/31/05 | 14 | - | - | 15.316 | 0.587 | | |
| 11/2/05 | 16 | - | - | 15.731 | 0.522 | | |
| 11/8/05 | 22 | - | - | 17.714 | 0.848 | | |
| 11/10/05 | 24 | 7.137 | 0.010 | 15.178 | 0.522 | n.d. | |
| 11/12/05 | 26 | 5.637 | 0.110 | 14.440 | 0.000 | | |
| 11/14/05 | 28 | 4.583 | 0.300 | 15.409 | 0.196 | | |
| 11/16/05 | 30 | 4.696 | 0.540 | 14.763 | 0.456 | | |
| 11/18/05 | 32 | 3.975 | 0.240 | 12.873 | 0.261 | | |
| 11/20/05 | 34 | 2.871 | 0.260 | 12.089 | 0.326 | | |
| 11/22/05 | 36 | 1.145 | 0.440 | 10.429 | 0.065 | | |
| 11/28/05 | 42 | 0.131 | 0.106 | 5.910 | 2.152 | 9.305 | 1.694 |

Table 18. Treatement 2 (Organic Carbon)

| Date | | TAN (mg/l) | Nitrite-N (mg/l) | Nitrate-N (mg/l) |
|---|---|---|---|---|
| 10/29/05 | 12 | 0.038 | 17.299 | n.d. |
| 10/31/05 | 14 | - | 16.469 | |
| 11/2/05 | 16 | - | 15.270 | |
| 11/8/05 | 22 | - | 16.100 | |
| 11/10/05 | 24 | 7.158 | 14.994 | n.d. |
| 11/12/05 | 26 | 6.239 | 14.071 | |
| 11/14/05 | 28 | 4.838 | 16.100 | |
| 11/16/05 | 30 | 4.201 | 14.994 | |
| 11/18/05 | 32 | 0.649 | 17.391 | |
| 11/20/05 | 34 | 0.010 | 17.668 | |
| 11/22/05 | 36 | 0.048 | 15.824 | |
| 11/24/05 | 42 | 0.010 | 3.558 | 11.045 |

## 7.4 Appendix D. Effects of commercial probiotic on shrimp

Table 19 and 20. Analysis of TAN, nitrite-N and nitrate-N concentration (mg/l) mean values and standard deviation of the control and probiotic treatments.

Table 19. Control

| Date | Day | TAN (mg/l) | Std. deviation | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) |
|---|---|---|---|---|---|---|
| 10/23/05 | 0 | 0.011 | 0.000 | 0.607 | 0.000 | |
| 10/24/05 | 1 | 0.041 | 0.024 | 0.792 | 0.612 | |
| 10/29/05 | 6 | 0.618 | 0.138 | 0.000 | 0.315 | |
| 11/1/05 | 9 | 0.184 | 0.035 | 0.492 | 0.295 | |
| 11/2/05 | 10 | 0.011 | 0.000 | 0.607 | 0.000 | |
| 11/5/05 | 13 | 0.410 | 0.095 | 0.100 | 0.442 | |
| 11/8/05 | 16 | 2.454 | 0.601 | 0.538 | 0.175 | |
| 11/11/05 | 19 | 6.500 | 1.902 | 1.045 | 0.157 | |
| 11/12/05 | 20 | 0.011 | 0.000 | 0.607 | 0.000 | |
| 11/16/05 | 24 | 2.365 | 0.055 | 0.654 | 0.479 | |
| 11/19/05 | 27 | 2.478 | 0.096 | 1.045 | 0.243 | |
| 11/23/05 | 31 | 5.287 | 0.623 | 1.483 | 0.684 | n.d. |

Table 20. Probiotic treatment

| Date | Day | TAN (mg/l) | Std. deviation | Nitrite-N (mg/l) | Std. deviation | Nitrate-N (mg/l) | Std. deviation |
|---|---|---|---|---|---|---|---|
| 10/23/05 | 0 | 0.011 | 0.000 | 0.607 | 0.000 | | |
| 10/24/05 | 1 | 0.114 | 0.026 | 0.515 | 0.310 | | |
| 10/29/05 | 6 | 0.776 | 0.038 | 0.000 | 0.106 | | |
| 11/1/05 | 9 | 0.191 | 0.009 | 0.400 | 0.422 | | |
| 11/2/05 | 10 | 0.011 | 0.000 | 0.607 | 0.000 | | |
| 11/5/05 | 13 | 0.554 | 0.107 | 0.077 | 0.305 | | |
| 11/8/05 | 16 | 2.730 | 0.448 | 0.584 | 0.204 | | |
| 11/11/05 | 19 | 5.570 | 0.903 | 1.022 | 0.220 | | |
| 11/12/05 | 20 | 0.011 | 0.000 | 0.607 | 0.000 | | |
| 11/16/05 | 24 | 3.108 | 0.053 | 0.308 | 0.422 | | |
| 11/19/05 | 27 | 3.073 | 0.848 | 1.437 | 0.271 | | |
| 11/23/05 | 31 | 4.693 | 3.094 | 2.867 | 2.925 | 0.460 | 0.43 |

## 7.5 Appendix E. Un-ionized Ammonia Calculator (Seawater) spreadsheet.

Table 21. This spreadsheet computes the concentration of un-ionized ammonia, in seawater, as a function of total ammonia, pH, temperature and salinity. It assumes that total ammonia is reported as nitrogen, therefore computed un-ionized ammonia will also be reported as nitrogen.

User information spreadsheet

| No. | Temp (˚C) | Measured pH | Total Ammonia Nitrogen (mg/l) | Salinity (ppt) |
|---|---|---|---|---|
| 1 | 27.00 | 7.80 | 7.00 | 16 |
| 2 | 28.00 | 7.50 | 15.00 | 29 |

Computed spreadsheet

| No. | Working pH | Ionic Strength (M) | pKa (infinite dilution) | pKa (SW) | Mole Fraction | Un-ionized Ammonia-N (mg/l) | Un-ionized Ammonia-N (ug/l) |
|---|---|---|---|---|---|---|---|
| 1 | 7.80 | 0.324 | 9.185 | 9.23 | .03559 | .24914 | 249.14 |
| 2 | 7.50 | 0.595 | 9.155 | 9.24 | .01778 | .26668 | 266.68 |

Working pH = Measured pH;

Ionic Strength (M) = 19.9273 x Salinity (ppt) / (1000 - 1.005109 x Salinity (ppt));

pKa (infinite dilution) = 0.0901821 + 2729.92 / (Temp. (˚C) + 273.15);

pKa (SW) = pKa (infinite dilution) + (0.1552 - 0.000314 x Temp. (˚C)) x Ionic Strength (M);

Mole Fraction = 1/(1+10^(pKa (SW)- Working pH);

Un-ionized Ammonia-N (mg/l) = Mole Fraction x Total Ammonia Nitrogen (mg/l);

Un-ionized Ammonia-N (µg/l) = Un-ionized Ammonia-N (mg/l) x 1000.

## 7.6 Appendix F. Standard Calibration Curve

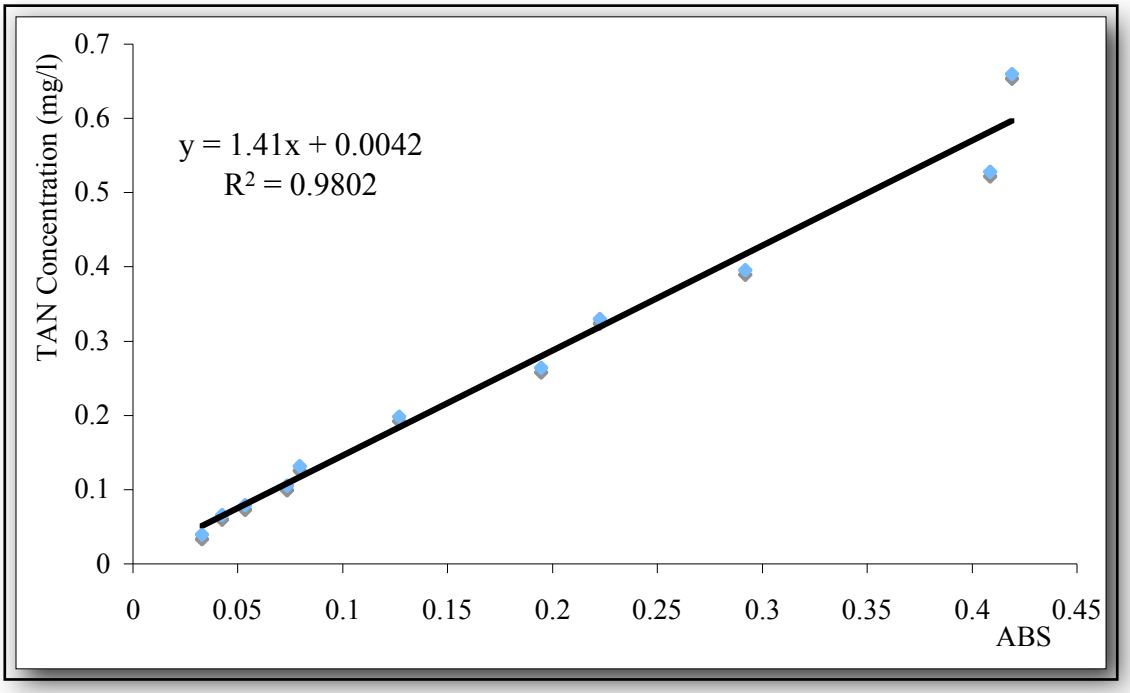


Figure I. Total Ammonia Nitrogen concentration (mg/l) standard calibration curve; Trendline, calibration curve equation and square R value.

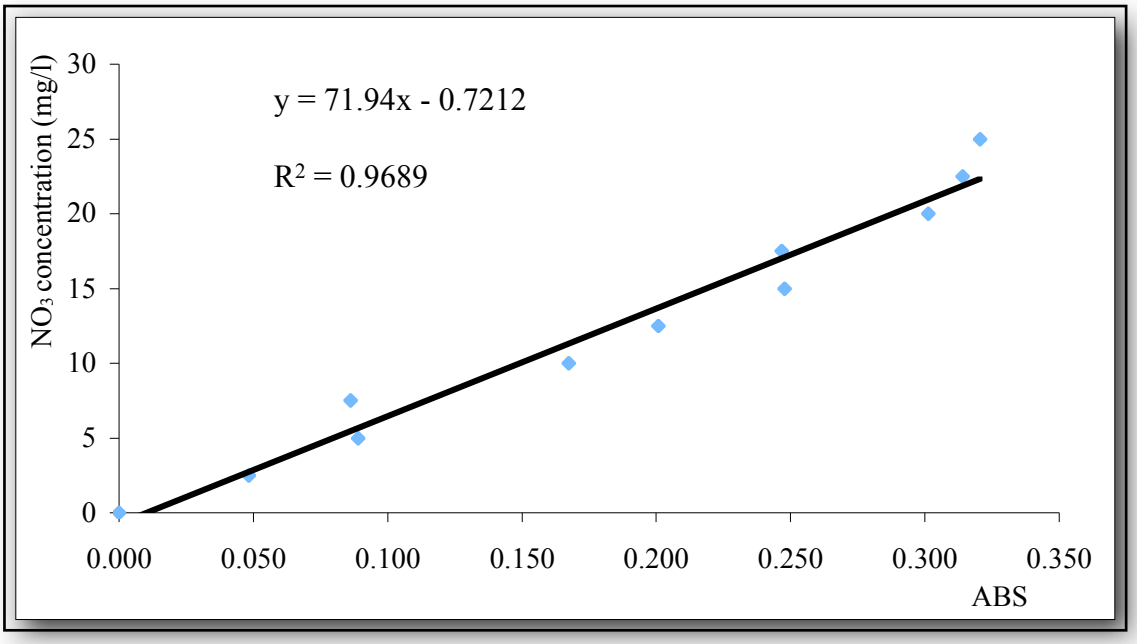


Figure II. Nitrite-nitrogen concentration (mg/l) standard calibration curve; Trendline, calibration curve equation and square R value.

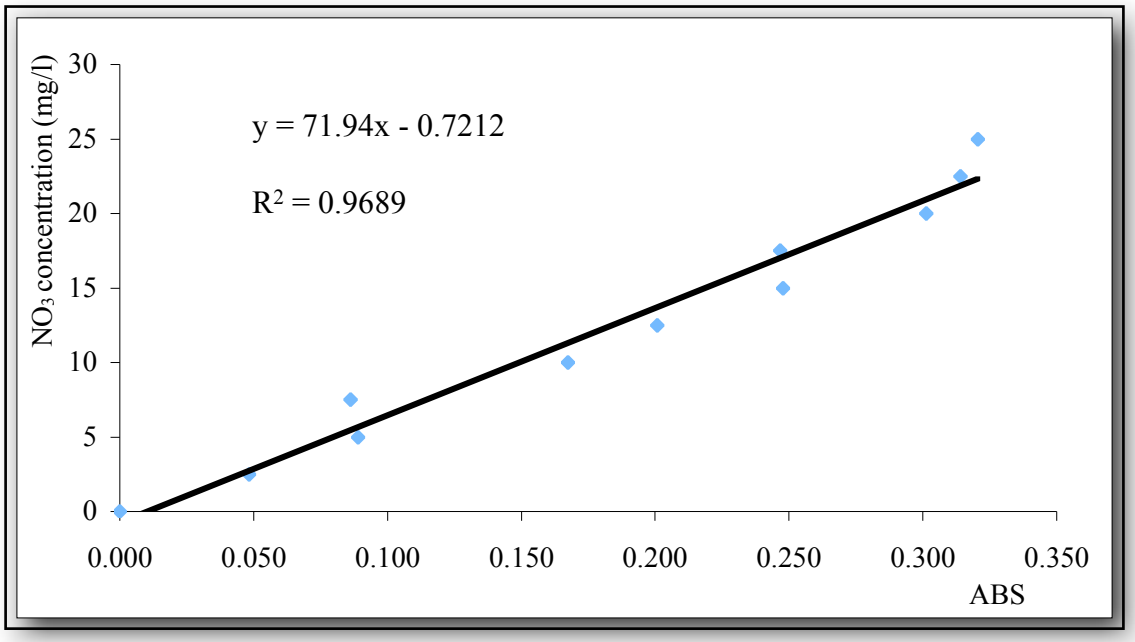


Figure III. Nitrate-nitrogen concentration (mg/l) standard calibration curve; Trendline, calibration curve equation and square R value.