A Bioaugmentation Agent in Super Intensive Marine Shrimp Farming System with Zero Water Exchange

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Abstract

This study evaluated the feasibility of using Comambio®, commercial product for bioremediation, in super-intensive system of Litopenaeus vannamei with zero water exchange. First, the concentration was determined to bioaugmentation on the settleable solids (SSed) in water containing microbial flakes. The second stage consisted in testing the application frequency in weekly and biweekly basis upon performance of shrimp. Were described physical and chemical parameters of water quality in the treated trial and that without application. The concentration of 0.56 g/L was selected by reducing significantly (p<0.05) the SSed value. The frequency of weekly application promoted the chance of livelihood and increased the final biomass growth of the shrimp by 46.6% of growth rate. 17.0% in the final biomass and 10.23% of livelihood with regard to the control sample. Eventually, there was a dramatic drop in the SSed (63.4%) with Comambio® while the total suspended solids increased with the suspended fixed solids. On the other hand, the biochemical oxygen demand showed the least value, Comambio® and control, 70.2% and 17.4% respectively. Summing up the bioaugmentation agent declined the value of SSed, contributing to the growth and reducing feed conversion as well as the final livelihood biomass of the shrimp cultivation system. The total average gain in weight per fish was higher in the automatic feeding (89.50 g) than in manual (78.50 g). An FE of 20.9% was obtained in the automatic feeding and 18.6% in manual, in relation to their FCRs. A t-test, conducted at 5% significance level, indicated a significant difference in the two feeding methods.

Keywords: Wastewater shrimp; Bioremediation; Pacific white shrimp; Sustainable shrimp; Environmental degradation

Introduction

The increasing demand of food consumption and improving the food security system have led to aquaculture development worldwide. FAO [1] predicts that this industry is likely to boom in the near future due to world population growth. The decline of the fishing industry has demanded underlying challenges to fish farming practices in order to ensure the availability of fish as an important source of protein. On the other hand, the aquaculture industry has responded to a number of environmental challenges such as water shortage and quality, land degradation, high costs of land hiring, environmental impact and diseases that has promoted the development of intensive aquaculture crop oriented [2,3].

The super-intensive culture system with marine shrimp is a response to the need for increased production in places where water and land are limited. Therefore, there is a need to maintain the bio-security in spaces contaminated by diseases, especially in already endangered areas [4-8].

In these systems the microbial degradation of organic waste is responsible for the maintenance of water quality parameters suitable for the cultivation of penaeid shrimp [9,10]. Nevertheless, at high stocking densities, the water column has a limited capacity for self-purification [11]. Bioaugmentation is a bioremediation strategy that consists in the introduction of microorganisms and/or its metabolites in the polluted environment that accelerates the removal of unwanted biodegradation contaminants [12].

The external charge of beneficial microorganisms to the aquaculture cropping system is crucial to increase the capacity for self-purification as well as to improve the water quality, which is suitable for animal growth within the farming system [11]. The usage of commercial bioaugmentation agents containing bacteria of the genus Bacillus sp., Nitrosomonas sp., Nitrobacter sp. and Lactobacillus in intensive cultivation of marine shrimp Penaeus monodon and L. vannamei increased the survival and reduced considerably the concentrations of Vibrio sp., total organic carbon and total nitrogen in waters of cultivation [13,14].

This study evaluated the feasibility of using Comambio®, commercial product for bioremediation treatment of domestic sewage in super intensive culture of L. vannamei in microbial flakes system with zero water exchange.

Materials and Methods

Three subsequent tests were conducted to evaluate the feasibility of applying Comambio® in super intensive cropping systems in the pacific white shrimp with zero water exchange. The first step examined the dosage of Comambio® to be applied in systems with super intensive with microbial flakes on the reduction of settleable solids (SSed) microbial flakes used in marine shrimp cultivation schemes. After setting the concentration to be utilized, the frequency of use of the commercial bioaugmentation agent was evaluated on the production performance in marine shrimp grown in super intensive system with microbial flakes. The last test aimed to characterize the effects of Comambio® on the physical and chemical variables of water quality in super intensive microbial flakes farming system.

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The test used Comambio® which has in its formulation the following microorganisms: the Bacillus cereus, B. amylophilica and B. subtilis at concentrations of 6.17 × 10⁵ CFU/mL; whereas the concentration of 9.00 × 10⁸ CFU/mL, the microorganisms are the following: Geotrichum sp., Aspergillus niger, Penicillium sp., Mycelia sterile and Trichoderma koningii; for B. brevis and Corynebacterium sp. the concentration was 1.00 × 10⁹ CFU/mL. The Trades Comambio® seawater until there were used without microbial flakes, at 28°C of heated titanium Scam group. Aeration and temperature were kept constant at 27°C with Water without application of the product was used for the control and four replicates. Plastic bottles of 1.5 L (conical cylinder) were used.

Experiment 1

The experiment was randomly designed with two treatments and six replicates, with application of Comambio® and without (control). There were 12 experimental units of the circular fiberglass with 1.1 m² and 900 L capacity each. The water used to supply the experimental units was taken from a tank of super-intensive shrimp cultivation with microbial flakes already established assuring that all the tanks were under same conditions of water quality at the beginning of the experiment.

Water temperature and dissolved oxygen were kept constant with the aid of titanium heaters Scam® 1000 W controlled by thermostats and sufficient aeration in the quest to keep the suspended particulate matter. On the other hand, the concentration of oxygen dissolved in appropriate values for the growth of the animals were monitored using YSI 55 digital oximeter three times daily.

Shrimp with an average weight of 20.62 ± 0.39 g were kept in these units until they reached final weight of 24 g. The density in the stand was 120 shrimp/m³ and the experiment lasted 30 days. Commercial feed was provided with 35 % Eptima Guabi® (35% Protein). The feeding rate was 1.7% of the biomass in each experimental unit, given in equal portions of three times a day, following recommendations [17]. At the end of the experiment, growth, survival, biomass and food conversation was assessed for each treatment. Ssed were determined by gravimetric method using volumetric Inoff cones as described by AMERICAN PUBLIC HEALTH ASSOCIATION [16].

In the Experiment 1 alkalinity and hydrated lime was added to all tanks when its value was less than 120 mg/L according to the amounts proposed by Ebeling et al. [18]. The concentration of toxic nitrogen compounds (ammonia and nitrite) in Experiment 2, as well as orthophosphate was monitored with the methods described in AMERICAN PUBLIC HEALTH ASSOCIATION [16]. The solids serial like a TSS, SSF and SSF was determined weekly the according to APHA [19] by method 2540D and 2540E. The temperature was measured using YSI 55 field thermometer, dissolved oxygen (O₂) using YSI 55 digital Oximeter in Experiment 1 and 2. These data were evaluated daily in the morning and evening. In Experiment 2 pH was measured using YSI 30 digital pH meter, for transparency, Secchi disk were used. All measurement was done daily. BOD and TOC were determined at the beginning and the end of the experiment using the methods proposed by AMERICAN PUBLIC HEALTH ASSOCIATION [16] - 5210B and [16] - 52310D, respectively.

Bacteriological analysis

The determination of concentrations of total heterotrophic bacteria and water Vibrioaceae was held at the beginning and end of the experiment 1 and 2 of the project. Water samples were seeded on petri plates with agar Thiosulfate-Citrate-Bile-Sucrose and Marine Agar and incubated at 35°C for 24 h before the count according to Madigan et al. [20]. Each water sample was analyzed in triplicate.

Statistical analysis

Data were analyzed using the program STATISTIC® version 7.0.
Concentration of the Comambio® was defined by simple regression significance at 1% and then analysis of variance was performed by ANOVA test followed by comparison of means by Tukey test at 5% significance between the values obtained.

The data resulting of the Experiment I were assessed for normality, and once detected, Bartlet test was applied to the investigation of variances homoscedasticity. Data were transformed to log (x + 1) and subjected to analysis of variance (α = 5%) where was not homogeneity. The Student’s t-test was used to investigate the significant differences (α = 5%) for the data obtained Experiment 2 [21]. Additionally, the confidence interval was calculated in the means resulting from the total counts of heterotrophic bacteria and vibrios in samples with levels of significance of 5%.

**Results**

**Comambio® features**

The data in sea water “crystal”, and 3.2 g/L salinity showed pH 8.2, alkalinity 126 mg/L CaCO₃, 574.5 mg/L TSS, 495.0 mg/L SSF, 79.5 mg/L SSF; 2.0 BOD, 2.0 mg/L COD, TOC 1.8 mg/L.

Significant linear relationship was observed (p<0.01) between the concentration of Comambio® SSed in water and super intensive shrimp cultivation, Figure 1, described by the equation $Y = -3.3439x + 8.9882$, $R^2 = 0.889$. The increased concentration of Comambio® increased the volume reduction SSed. There was also found that from 0.56 to 0.89 mg/L SSV; 2.0 BOD, 2.0 mg/L COD, TOC 1.8 mg/L.

The concentrations of total heterotrophic bacteria in the control group at the end of the experiment.

**Microbiological analysis**

The concentrations of total heterotrophic bacteria in the control

**Zootechnical**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Control</th>
<th>Weekly</th>
<th>Biweekly</th>
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<tbody>
<tr>
<td>Livelihood rate (%)</td>
<td>72.5 ± 5.54a</td>
<td>71.65 ± 4.56a</td>
<td>71.86 ± 6.57a</td>
</tr>
<tr>
<td>Final biomass (g)</td>
<td>308.41 ± 38.37a</td>
<td>361.01 ± 15.95a</td>
<td>351.34 ± 30.06a</td>
</tr>
<tr>
<td>Feed conversion</td>
<td>1.99 ± 0.62a</td>
<td>1.27 ± 0.11a</td>
<td>1.36 ± 0.26a</td>
</tr>
<tr>
<td>Weekly weight gain (g)</td>
<td>1.07 ± 0.02a</td>
<td>1.52 ± 0.14b</td>
<td>1.57 ± 0.29b</td>
</tr>
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</table>

**Physical and chemical variables in super intensive farming system with microbial flakes**

During the experimental phase in Experiment 2, the temperature was maintained in the range of 27.90 ± 0.21 to 28.50 ± 031°C and minimum DO values were 5.80 and maximum of 6.30 mg/L in both the control and Comambio® in the treatment. There were no significant differences for the variables $O_2$, pH, salinity, transparency and SSed as can be seen in Table 3. Significant difference (p<0.05) was observed in the water alkalinity amongst the treatment from the second week of cultivation. Alkalinity was higher in bioaugmentation agent treatment (160.01 ± 60.70 mg/L CaCO₃) and 102.00 ± 6.50 mg/L for the control group at the end of the experiment.

The concentration of $NH_4$-N in Experiment 2 in water cultivation
Table 2: Biological indexes (mean ± standard deviation) in the cultivation of super intensive Litopenaeus vannamei, with application to microbial flakes bioremediation (Comambio®) and without bioremediation (CONTROL).

<table>
<thead>
<tr>
<th>Performance</th>
<th>Treatments</th>
<th>Control</th>
<th>Comambio®</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final weight (g)</td>
<td></td>
<td>20.64 ± 0.34</td>
<td>20.64 ± 0.34</td>
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<tr>
<td>Final weight (g)</td>
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<td>24.44 ± 1.03</td>
<td>24.81 ± 0.63</td>
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<tr>
<td>Weekly weight gain (g)</td>
<td></td>
<td>1.09 ± 0.22</td>
<td>1.07 ± 0.23</td>
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<tr>
<td>Final biomass (g)</td>
<td></td>
<td>20,288.93 ± 170.4*</td>
<td>22,588.58 ± 54.36</td>
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<tr>
<td>Rate of livelihood (%)</td>
<td></td>
<td>82.24 ± 3.98*</td>
<td>92.72 ± 2.96</td>
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Label: (*) statistical differences (p<0.05).

Discussion

The data from Comambio® in sea water “crystal” suggest an increased amount of total suspended and fixed solids which may be harmful to use. The increase in alkalinity of the water due to use of the product (8.2 mg/L CaCO₃) could be discussed with reference to the material of microorganisms in the product which were not supplied and approved by the manufacturer.

The increased concentration of bioaugmentation agent in water containing microbial flakes resulted in a higher proportion of microorganisms in the product, which probably must have caused higher microbial activity in the system resulting in greater decline of Ssed. According to Jiao et al. [22] the high concentration of microorganisms through the bioaaugmentation agent is vital in the process of bioaugmentation agent to maintain the necessary concentration of beneficial microorganisms in the system that is intended to remedy. The author claims that this happens because many bacteria have a gene expression mechanism for coordinating; a process called ‘quorum-sensing’ that regulates the responses and processes of production of phenotypes such as enzymes production, toxins and biofilm formation [23].

The influence of bioaugmentation agents on growth and survival of cultured shrimp has been reported by other authors [13, 24]. The use of Comambio®, showed no harmful effects to the raised animals since they showed positive growth and no physiological change was noticed after the application continued. Its incorporation in the farming system showed improvement in biological indexes of the animals under super intensive condition. Despite the fact that the bioaugmentation agent was not tested in super intensive cultivation, Janeo et al. [24] observed a similar result in intensive system of Penaeus monodon after applying bioaugmentation agent containing Nitrobacter and Bacillus (approximately 3 × 10⁹ CFU/g) and lipases and proteases applied in doses de100 g/ha, 150 g/ha, 200 g/ha and 300 g/ha. The impact of bio-remediation in survival of cultured shrimp verified this trial might be related to its contribution into composition microbial composition of the gastrointestinal tract and the shrimp cultivation environment.

In spite of the fact that the study has not used bacteria indigenous to the cultivation of prawns, Comambio® presents in its composition Bacillus spp. Bacteria of the genus Bacillus were able to reduce the amount of pathogens present in water cultivation, as well as lower concentrations of nutrients in the water, thus acting as bioaugmentation agent and as bio-control [25].

The other microorganisms also found in products used as Aspergillus niger and Trichoderma sp. are known for their ability to secrete digestive enzymes like amylases, proteases and cellulases for the environment [26], which may have contributed to increased the growth and survival of shrimp cultivation. Janeo et al. [24], reported results in increased growth in P. monodon by applying a bioaugmentation agent in water cultivation that had bacteria of the genus Nitrobacter and Bacillus well as lipases and proteases.

Bioremediation has been reported as a solution to control pathogenic bacteria in aquaculture crops [24,27]. In this study, the effect
Control weeks Comambio® Weeks

<table>
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<th>Chemical and physical Characteristics</th>
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<tr>
<td>DO (mg/L)</td>
<td>5.9 ± 0.28 5.9 ± 0.08 5.8 ± 0.09 5.8 ± 0.09 5.8 ± 0.08</td>
<td>5.9 ± 0.28 5.8 ± 0.16 5.8 ± 0.14 5.80 ± 0.13</td>
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<tr>
<td>Temperature (°C)</td>
<td>27.09 ± 0.32 27.9 ± 0.27 27.9 ± 0.23 27.9 ± 0.22 27.9 ± 0.21</td>
<td>27.9 ± 0.32 28.1 ± 0.30 28.0 ± 0.31 27.90 ± 0.30</td>
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<td>pH</td>
<td>7.8 ± 0.08 7.8 ± 0.05 7.8 ± 0.06 7.8 ± 0.08 7.8 ± 0.09</td>
<td>7.8 ± 0.12 7.8 ± 0.10 7.8 ± 0.10 7.80 ± 0.01</td>
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<td>Salinity (g/L)</td>
<td>3.20 ± 0.12 3.31 ± 0.12 3.26 ± 0.36 3.25 ± 0.47 3.22 ± 0.50</td>
<td>3.32 ± 0.09 3.25 ± 0.21 3.23 ± 0.27 3.21 ± 0.30</td>
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<td>NH 4-N (mg/L)</td>
<td>0.04 ± 0.03 0.12 ± 0.04 0.14 ± 0.04 0.10 ± 0.05 0.10 ± 0.05</td>
<td>0.13 ± 0.07 0.14 ± 0.05 0.11 ± 0.05 0.12 ± 0.05</td>
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<tr>
<td>NO 2-N (mg/L)</td>
<td>0.09 ± 0.02 0.12 ± 0.03 0.11 ± 0.06 * 0.07 ± 0.01 0.04 ± 0.01</td>
<td>0.18 ± 0.05 0.16 ± 0.08 0.10 ± 0.05 0.06 ± 0.02</td>
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<tr>
<td>NO 3-N (mg/L)</td>
<td>8.87 ± 1.24 12.3 ± 0.68 16.50 ± 2.90 17.9 ± 0.22 17.9 ± 0.21</td>
<td>14.3 ± 2.10 11.3 ± 1.69 22.7 ± 3.55 23.4 ± 4.60</td>
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<td>PO 4-P (mg/L)</td>
<td>3.40 ± 0.20 3.71 ± 0.20 3.27 ± 0.26 3.38 ± 3.12 3.66 ± 0.23</td>
<td>3.70 ± 0.16 3.26 ± 0.26 3.26 ± 0.15 3.34 ± 0.35</td>
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<td>CaCO 3 mg/L</td>
<td>12.0 ± 0.10 96 ± 3.80 106 ± 7.90 115 ± 3.50 102 ± 6.50</td>
<td>147 ± 28.5 168 ± 48.70 160 ± 60.70</td>
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<td>Transparency (cm)</td>
<td>15.10 ± 0.05 14.40 ± 1.20 13.7 ± 0.90 13.10 ± 1.40 12.5 ± 1.00</td>
<td>11.2 ± 0.0 10.8 ± 1.20 10.1 ± 1.40 9.70 ± 1.30</td>
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<td>Turbidity (NTU)</td>
<td>104.30 ± 24.80 104.30 ± 24.80 125.7 ± 36.10 140.19 ± 44.10</td>
<td>161.9 ± 45.10* 174.8 ± 49.50* 179.9 ± 37.6 217.2 ± 55.40 277.03 ± 64.30</td>
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<td>SST (mg/L)</td>
<td>501.07 ± 32.67 569.80 ± 91.60* 689.8 ± 44.40* 642.0 ± 63.00* 644.5 ± 63.70*</td>
<td>854.3 ± 68.70* 994.8 ± 142.70 1223.5 ± 380.20 1558.80 ± 553.20</td>
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<td>SSF (mg/L)</td>
<td>289.01 ± 32.67 321.30 ± 65.90* 386.6 ± 30.50* 402.1 ± 245.40* 428.5 ± 39.20*</td>
<td>566.0 ± 79.91 670.6 ± 142.10 809.3 ± 344.20 1120.8 ± 376.50</td>
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<tr>
<td>SSV (mg/L)</td>
<td>204.33 ± 35.75 248.5 ± 34.70 303.2 ± 17.40 344.0 ± 44.50 416.0 ± 33.50</td>
<td>288.3 ± 69.30 324.0 ± 42.80 323.6 ± 111.40 432.2 ± 76.20</td>
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<tr>
<td>SSed (mL/L)</td>
<td>8.41 ± 0.07 9.3 ± 1.10 11.4 ± 1.10 13.9 ± 2.40 16.6 ± 3.10</td>
<td>8.4 ± 1.10* 6.9 ± 1.30* 10.9 ± 3.10* 13.80 ± 2.60</td>
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Label: (*) statistical differences between treatments in the same week cultivation (p<0.05).

Table 3: Chemical and physical characteristics of water (mean ± standard deviation) during the experimental cultivation of super intensive marine shrimp, Litopenaeus vannamei with bioflocs with application of microbial bioremediation (Comambio®) and without bioremediation (Control). The data on weeks zero (0) correspond to analysis performed in the two treatments at the beginning of the experiment.

The entry of bioremediation in Comambio® treatment may have contributed to the increase in alkalinity as the need to use lime during cultivation was lower than the control material probably due to the viability of microorganisms of the product (not supplied by the manufacturer) that perhaps contained materials that interfere with the alkalinity.

The reduction of alkalinity in controls might be related to the occurrence of nitrification since nitrate increase was observed in the culture [5].

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was not observed the effect of bioaugmentation agent in concentrations of bacteria of the genus *Vibrio* since no selection was performed to investigate the inhibitory capacity of pathogens in the product and choice of strains. On the other hand, the method of counting bacteria based on applied microbiology underestimates the quantitative and qualitative measurement of populations of heterotrophic bacteria and may interfere with the interpretation of results of bioremediation by restricting interpretation of biotic and abiotic factors [28]. These findings could be interpreted using methods such as microbial ecology, total extraction of DNA, bloom microscopy among others.
The reduction of Ssed during cultivation immediately after the addition of Comambio® suggests the existence of specific microorganisms in the product relative to the substrate cultivation system since the settling characteristics of sludge in microbial systems are directly affected by microbial composition that exists in the water [29].

The microbiological composition of Comambio® contributed to the reduction of settleable solids found in cultivation system. Similar results were reported by Primavera, Lavilla-Pitogo, Ladji and Dela Peña [30], whose observed a reduction of sludge formed in nurseries producing intensive cultivation of P. monodon that used commercial products containing bacteria Nitrosomonas, Sulfur Bacteria, Bacillus sp. and/or enzymes (proteases, celluloses) for quality control in concentrations defined according to the specifications of each product used by the producer.

Although by the end of the growing concentration of SSF did not interfere on the performance of farmed shrimp, there was not available scientific research describing shrimp growth at higher concentrations than those of SSF with values referenced below 1000 g/L [31].

The content of VSS and TOC concentration did not differ statistically between treatments. This suggests that there must have had no reduction of the amount of organic matter with the addition of bioaugmentation agent. However, Wang and He [14] reported reduction of TOC in the sediment ponds intensive farming of L. vannamei after the application of a commercial bioaugmentation agent containing the 10 × 10 CFU/g from Bacillus sp. Nitrosonomas sp. Nitrabacter sp. and Lactobacillus.

However, the data point to lower the BOD value in the treated group at the end of the trial. This reduction of BOD as a result of application of the product Comambio® may be an indication of the reduction in the fraction of organic matter. Similar results have been reported by Jiao et al. [12], who reported an increase in the reduction of BOD treatment system with application of wastewater bacterial strains as well as the research conducted by Wang and He [14] also observed a decline of BOD and COD in cultured shrimp with application of products containing bacteria the genus Bacillus sp. Nitrosonomas sp. Nitrabacter sp. and Lactobacillus.

Higher values in the concentration of nitrate in the first week may indicate the mineralization of organic matter immediately after the addition product bioremediation, while the concentration of dissolved mineral matter in the form of orthophosphate and nitrate did not differ between treatments [32]. McIntosh [33] also observed no difference in nitrate concentration after application of probiotic in shrimp farming. However, Kuhn, Drahos, Marsh and Flick Jr. [34] claim reduction of ammonia and nitrate as a result of product application bioremediation in tanks of intensive cultivation of marine shrimp.

The dynamics of organic matter and mineralization processes are still poorly understood in super intensive farming system [5,35]. The application of the product may have caused increased microbial biomass in growing through the degradation of organic matter helping to change the quality and diversity of the microbiota [36].

As a recommendation to the understanding of the relations of microorganisms into the super intensive system with bioflocs in relation to microbial bioaugmentation product, it is of extreme importance to use molecular tools for microbial identification, and their relationship with the growth performance as well as physical and chemical parameters of water for cultivation. Another mechanism that should be taken into consideration in future studies is the design of clarifiers to remove suspended material and solids because according to these results, this can be used in conjunction with bioaugmentation products that does not require huge volumes for effluents treatment since they reduced the volume of filtered solid, suggesting an increased rate of sedimentation which was not measured in this test.

It is concluded that the bioaugmentation can be applied in the cultivation of shrimp with zero water exchange, given that its use had immediate effect in reducing the volume of settleable solids. Furthermore, the effects of continued use of bioremediation on the characteristics of the microbial community in the water for cultivation should be studied taking into account that its weekly application has contributed significantly to the growth of shrimp and consequently has increased the final biomass.

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