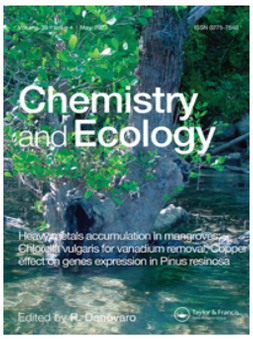

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Ionic adjustments do not alter plankton composition in low salinity *Penaeus vannamei* intensive nursery with synbiotic system

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ABSTRACT

The aim of this study was to test the effect of different ionic adjustments in low salinity water on the composition and temporal variation of plankton from intensive shrimp nurseries with a synbiotic system. For this, a *Penaeus vannamei* nursery (35 days) was carried out with three treatments: T1 – diluted seawater (control; salinity ~2.5 g L^{-1}), T2 – salinity ~2.5 g L^{-1} with K^+ adjustment to approximate the seawater equivalent concentration, and T3 – salinity ~2.5 g L^{-1} with Ca:Mg:K ratio adjusted to 1:3:1. Rice bran processed by probiotic microorganisms was used as an organic carbon source. Phytoplankton and zooplankton were sampled weekly and analysed using standard methods. The temporal variation of phytoplankton and zooplankton composition were more pronounced than differences among treatments indicating that the ionic adjustment had little effect on these communities. During the experimental time, the dominant phyla in phytoplankton were Ochrophyta, Cyanophyta and Chlorophyta, whereas zooplankton's dominant phyla were Ciliophora, Amoebozoa, and Cercozoa. Cyanophyta's relative abundance was lower than traditional biofloc systems, suggesting a higher control of these microorganisms in synbiotic systems. Ionic adjustments have then a low potential to affect plankton, likely because limitation by these ions was not achieved under the ionic manipulations tested.

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1. Introduction

Crustaceans farming in inland regions represented in 2020 a total of 4.4 million tonnes, corresponding to approximately 39% of total world`s crustacean production [1]. This production is often carried out using water from inland brackish water wells or seawater diluted with freshwater [2]. Seawater, the natural habitat of *Penaeus vannamei* shrimp, has high concentrations of various ions, such as sodium (Na^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), and potassium (K^+), which play an important role in the animal's osmoregulatory processes and growth [3,4]. Three of these key ions, Ca^{2+}, Mg^{2+} and K^+, are present in seawater in Ca:Mg:K ratios close to 1:3:1 [5], which, when unbalanced, can influence shrimp growth and survival due to greater energy expenditure by the animal to perform osmotic regulation [6].

In inland regions, groundwater used for shrimp farming may present variable ionic profiles [7] and not necessarily present adequate ionic concentrations and ratios to maintain an osmotically comfortable condition for shrimp growth. Thus, ionic adjustments are required, which can be performed by changing ion concentrations and ratios to the desired salinity in order to simulate a conservative seawater dilution [6,7], which can be carried out through adding mineral fertilisers to the water. The common use of calcium carbonate ($CaCO_3$), potassium chloride (KCl) and magnesium sulfate ($MgSO_4$) often alter water quality variables such as alkalinity, total hardness, and the concentrations of Ca^{2+}, Mg^{2+}, K^+, Cl^-, and SO_4^{2-} [7–9]. This strengthens the water buffer system and reduces pH variation when alkalinity is raised [10]. Changes in water quality then can create favourable conditions for the famed animals' growth and increase organic matter decomposition and recycling of essential inorganic components (e.g. carbon dioxide, ammonia, and phosphate) by the bacterial community activity, providing nutrients necessary for the phytoplankton community development (primary producers), and consequently, zooplankton [5].

This change in phytoplankton and zooplankton productivity can also be affected by the use of the synbiotic system as well, which is carried out through the application of vegetable bran processed by microorganisms [11,12]. This system aims at simulating natural estuarine conditions, providing an increase in the zooplankton and bacterial community, which can act as a supplementary source of nutrients for shrimp and improve water quality conditions [11,12]. De Andrade et al. [13] tested the synbiotic system in *P. vannamei* intensive nursery and found zooplankton concentrations higher than in traditional [5] biofloc systems.

Nitrogen (N) and phosphorus (P) are among the main macroelements that are essential for phytoplankton growth [5]. However, other water quality variables, such as alkalinity, can improve phytoplankton productivity through inorganic carbon supply [e.g. bicarbonate (HCO_3^-)] to the photosynthesis process, increasing their biomass, which is available to be converted into animal biomass through the food chain [10,14]. For example, tanks with low alkalinity (<10 mg L^{-1}) have low gross primary production and abundance of phytoplankton, when compared to tanks with alkalinity close to 30 mg L^{-1} [15].

Ca^{2+} can sequester phosphorus and impose nutrient limitation in some phytoplankton groups. For example, Giri and Boyd [8] and Wu and Boyd [16] found low photosynthetic rates and concentrations of chlorophyll-a in tanks that received the application of $CaCO_3$ and calcium sulfate ($CaSO_4$), respectively, that was attributed to the immobilisation of dissolved P by Ca^{2+}, which precipitates as calcium phosphate ($Ca_3(PO_4)_2$). Mg^{2+} is the central

atom in the chlorophyll molecule and takes part in cell metabolic processes, such as N metabolism and activation of enzymes involved in energy transfer [17–19]. Cl^-, Na^+ and K^+ participate in the exchange and transport of ions through cell membranes [17]. K^+ also acts in the establishment of the membrane potential and in the activation of enzymes [18]. Thus, if the ionic composition of water may influence the composition and productivity of phytoplankton, it can also change the productivity and composition of the zooplankton community in the system.

The lack of evidence about the role of low salinity water ionic adjustment on phytoplankton and zooplankton points to an important gap in the understanding of the plankton microbial community ecology in intensive aquaculture systems. Moreover, studies that have monitored the temporal variation of the planktonic community in low salinity shrimp farming are also scarce. Required ionic adjustments have the potential to alter plankton compositions with further repercussions on the organisms farmed, but so far, no assessment of this potential has been evaluated. Therefore, this work aims to test the effect of different ionic adjustment strategies in low salinity water on the composition and temporal variation of the planktonic community of intensive shrimp nurseries with synbiotic system.

2. Materials and methods

This study was carried out at the *Laboratório de Carcinicultura* (LACAR [Shrimp Culture Laboratory]), of the *Departamento de Pesca e Aquicultura* (DEPAq [Fisheries and Aquaculture Department]) of *Universidade Federal Rural de Pernambuco* (UFRPE [Rural Federal University of Pernambuco]). Ionic adjustment strategy, *P. vannamei* growth data, water quality, water ionic profile, C, N and P absolute composition, and C:N:P ratios of the microbial community (MC) and dissolved fraction (DF) data and ions determination method are described in detail in Pimentel et al. [20].

2.1. Design and experimental conditions

A nursery of *P. vannamei* was carried out for 35 days, and 24-days post-larvae (PL) were stocked at a density of 2000 PL m^{-3}, with salinity water $\sim$ 2.5 g L^{-1} (seawater diluted to freshwater) in experimental units with useful volume of 60 L under constant aeration (dissolved oxygen >5 mg L^{-1}) and temperature (29 °C). Three treatments with different ionic adjustments were established, all in triplicate: T1 – diluted seawater (control; salinity $\sim$2.5 g L^{-1}), T2 – salinity $\sim$2.5 g L^{-1} with K^+ adjustment to approximate to the seawater equivalent concentration, and T3 – salinity $\sim$2.5 g L^{-1} with Ca:Mg:K ratio adjusted to 1:3:1.

The system was fertilised with rice bran submitted to a period of fermentation (24 h) and microbial cellular respiration (24 h) by probiotic microorganisms [21]. The synbiotic was composed of 20 g m^{-3} of rice bran (< 200 µm), 2 g m^{-3} of molasses, 4 g m^{-3} of sodium bicarbonate, and 0.5 g m^{-3} of commercial bacterial mix (Kayros Agrícola and Ambiental, Brazil) and chlorinated (and dechlorinated through aeration) water in proportion to 10× the amount of rice bran. During the experimental time, the fertiliser was applied four times a week, and was suspended when the settleable solids exceeded 5 ml L^{-1}.

To assist in the nitrification process and the development of the microbial community of the system, an artificial substrate composed of mollusk *Anomalocardia brasiliana* shells was added to the experimental units. A biological activator was added to the system, and

no water exchange was made during the experimental time, except for replacing the loss of water by evaporation with freshwater addition.

2.2. Ionic adjustment

In the T1 treatment, the seawater was diluted with freshwater to a salinity of 2.5 g L^{-1} and no ionic adjustment was performed in this treatment. The choice of mineral fertilisers for the ionic adjustment was based on a previous analysis of the ionic profile of the water and its specific need for ions. Thus, in the T2 treatment, K^+ supplementation was made according to the estimated seawater concentration [7]. This adjustment was made on day 0 of the experimental time with the application of potassium chloride (KCl). In the T3 treatment, the Ca:Mg:K ratio was adjusted to 1:3:1 [5]. In this treatment, adjustments were made on days 0 and 17 of the experimental time with the application of calcium carbonate ($CaCO_3$) and magnesium sulfate ($MgSO_4$). Ionic adjustment strategies used are described in detail in Pimentel et al. [20].

2.3. Water quality variables

During the experimental time, dissolved oxygen (DO, mg L^{-1}; Yellow Springs multiparameter, model 556), temperature (°C; Yellow Springs multiparameter, model 556), pH (pH-689), salinity (g L^{-1}; salinity metre AZ, model 8371), and settleable solids (SS, mL L^{-1}) [22] were analysed.

Weekly, water samples from the tank were collected and fractionated into microbial community (MC; >1.6 µm) and dissolved fraction (DF; < 1.6 µm) by filtration [23]. From the MC fraction, the total particulate carbon (C – MC), total particulate nitrogen (N – MC) (carbon and nitrogen analyzer TOC-V, Shimadzu), and total particulate phosphorus (P – MC) [24,25] were determined. From the DF it was determined the total dissolved carbon (C – DF), total dissolved nitrogen (N – DF) (carbon and nitrogen analyzer TOC-V, Shimadzu), total dissolved phosphorus (P – DF) [24,25], total alkalinity [26], total ammonia nitrogen (TAN) [26], nitrite [27], and orthophosphate [26]. Total phosphorus (TP) was determined in the unfiltered samples [24].

C:P, C:N and N:P ratios in the MC and DF fractions were calculated in mmol g^{-1}:mmol g^{-1} and µM:µM, respectively.

2.4. Phytoplankton and zooplankton community

Phytoplankton and zooplankton were sampled in the subsurface of the water on 0, 7, 14, 21, 28 and 35 days of culture using 500 mL beakers. The homogeneity of the water was assured by the constant aeration of the tanks, which is a mandatory requirement for intensive systems.

The phytoplankton samples were filtered sequentially through cylindrical filters with 250, 120, 50 and 35 µm mesh size, respectively, to reduce large suspended solids. Then, the sample was filtered with a 15 µm mesh for phytoplankton retention, thus capturing the nano- and microphytoplankton fraction [19]. Samples were stored in 5 mL cryogenic tubes and fixed with buffered formalin at a final concentration of 4%. A Sedgewick-Rafter chamber and binocular optical microscope (Coleman N-120) with a magnification of 400×

[28] were used for identification of phytoplankton organisms at the possible lowest taxonomic level. Phytoplankton was expressed in cells per millilitre (cells mL^{-1}) according to Hötzel and Croome [29].

The zooplankton samples were filtered sequentially through cylindrical filters with 250 and 120 µm mesh size, respectively, to reduce the large suspended solids in the sample. Then, the sample was filtered with a 50 µm mesh for zooplankton retention, thus capturing the microzooplankton fraction [19]. The samples were stored in 5 mL cryogenic tubes and fixed with buffered formalin at a final concentration of 4%. A Sedgewick-Rafter chamber and binocular optical microscope (Coleman N-120) with a magnification of 400× [28] were used for identification at the possible lowest taxonomic level. Zooplankton concentration was expressed in organisms per millilitre (orgs mL^{-1}) according to APHA [26].

Absolute and relative (%) abundances were calculated for each taxon. Taxonomic groups corresponding to more than 50% of the total number of organisms in the sample were considered dominant [30].

2.5. Data analysis

To analyse the differences in phytoplankton and zooplankton total density among treatments for each sampled day of the experimental time, a one-way analysis of variance (ANOVA) was applied. For that, the data were tested for normality with the Shapiro–Wilk test and homoscedasticity with the Levene test, which was performed with the car package [31]. When ANOVA was significant ($p < 0.05$), the Tukey test was applied to detect differences among treatments.

To inspect the grouping of phytoplankton and zooplankton community samples, an exploratory cluster analysis using Bray Curtis index was performed using the *vegdist* function of the vegan package [32]. The clustering was performed using the algorithm UPGMA (unweighted pair group method with arithmetic mean). The raw abundance data was $\log_{10}(x+1)$ transformed.

Differences in phytoplankton and zooplankton composition and abundance among treatments (analysed separately for days 0, 7, 14, 21, 28 and 35) and sampling times were tested through analysis of similarity (ANOSIM) followed by a pairwise test with Bonferroni correction for multiple testing. Prior to the ANOSIM, the statistical temporal dependence among the sampled periods was analysed using an autocorrelation test, with the ccf function of the stats package [36]. This test was performed using the first axis of a principal component analysis (PCA) run from phytoplankton and zooplankton abundance matrix [33]. The data were Hellinger transformed prior to analysis to reduce the effect of variable abundances [34]. The PCA was performed using the vegan package [32]. To analyse the contribution of the most influential species of phytoplankton and zooplankton for the differences among weeks, a similarity percentage (SIMPER) was carried out.

A redundancy analysis (RDA) was built using the *rda* function of the vegan package [32]. The RDA was performed to explore the relationship between the water quality variables and the composition of phytoplankton and zooplankton communities. Phytoplankton and zooplankton abundance data were transformed using Hellinger transformation prior to analysis [34]. The main explanatory variables were selected with variance inflation factors (VIF), with variables with VIF >20 (strong collinearity) being excluded

from the model [35]. The significance of the RDA model was tested using ANOVA function with 1000 permutations and the r^2 and adjusted r^2 were computed using the RsquareAdj function.

ANOVA and its post hoc, Bray Curtis index, PCA, and RDA were carried out using R statistical software [36]. ANOSIM and SIMPER were carried out using Past 4.03, 2020 [37].

3. Results

3.1. Phytoplankton community

No significant differences in the total phytoplankton density among the treatments were observed at 0, 7, 14, and 35 days ($p > 0.05$). On day 21, T2 and T3 treatments had higher cells concentrations than the T1 treatment ($p = 0.018$; Figure 1(a)). On day 28 of the experimental time, T1 and T3 had higher cells concentrations than T2 treatment ($p = 0.021$; Figure 1(a)). The total phytoplankton density increased from a mean of 1,854.09 ± 208.85 cells mL^{-1} on day 0 to a mean of 4,444.00 ± 1,700.80 cells mL^{-1} at the end of the trial on day 35 across all treatments (Figure 1(a)).

Throughout the experiment, a total of 10 phytoplankton genus were identified, 1 from the phylum Ochrophyta (*Nannochloropsis*), 3 from Cyanophyta (*Aphanocapsa, Oscillatoria*, and *Geitlerinema*), 2 from Chlorophyta (*Closteriopsis* and *Mychonastes*), 2 from Bacillariophyta (*Cyclotella* and *Diatoma*), and 2 from Euglenophyta (*Euglena*, and *Phacus*) (Figure 2). The dominant phyla during the experimental time were Ochrophyta, Cyanophyta and Chlorophyta (Figure 2(a)), while the dominant genera were *Nannochloropsis*, *Aphanocapsa*, *Oscillatoria*, *Geitlerinema* and *Closteriopsis* (Figure 2(b)). The patterns of phytoplankton relative abundance were similar among treatments along the experimental time.

ANOSIM did not show significant differences in phytoplankton composition among treatments for days 0, 7, 14, 21, 28 and 35 of the experimental time. Significant differences were observed in phytoplankton composition along the experimental time ($p < 0.01$). Days 7, 21 and 28 showed a significantly different composition from day 0. Days 21 and 28 had significantly different composition when compared to day 7 (Table 1). Day 21 had a different composition from day 14, and day 28 had a different composition when compared to day 21 (Table 1).

Table 1. Summary of analysis of similarity (ANOSIM) among time for the phytoplankton community in a *Penaeus vannamei* nursery at low salinity under different ionic adjustment strategies for 35 days. Significant differences are displayed in bold (*p*-values).The first line is the global test and the remaining *p*-values are the post hoc pairwise comparisons among days.

	R		0.65	*p*-value		0.0001
Days	0	7	14	21	28	35
0	–	**0.042**	0.313	**0.010**	**0.004**	0.162
7		–	1	**0.003**	**0.039**	0.099
14			–	**0.003**	0.069	0.187
21				–	**0.001**	0.100
28					–	0.408
35						–

Note: T1 – diluted seawater (control; salinity ~2.5 g L^{-1}), T2 – salinity ~2.5 g L^{-1} with K^+ adjustment to close to the seawater equivalent concentration, and T3 – salinity ~2.5 g L^{-1} with Ca:Mg:K ratio adjusted to 1:3:1.

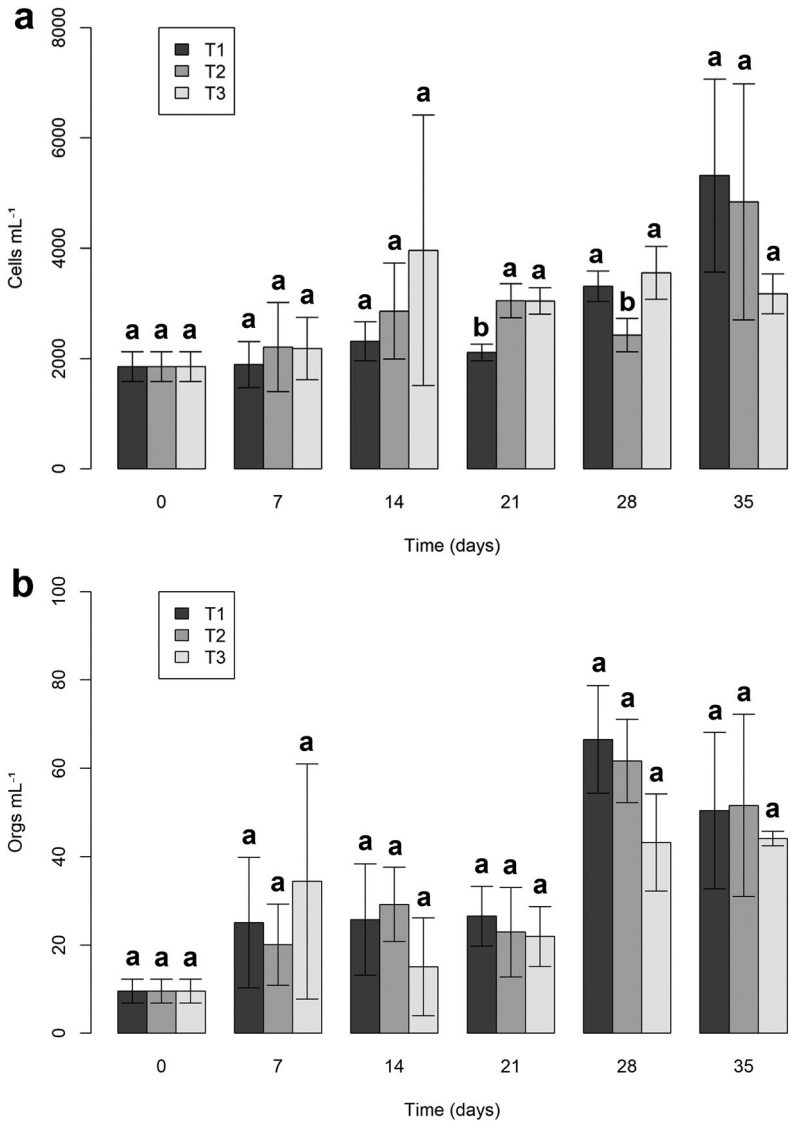

Figure 1. Variations in phytoplankton (a) and zooplankton (b) density during a *Penaeus vannamei* nursery at low salinity under different ionic adjustment strategies for 35 days. T1 – diluted seawater (control; salinity ~2.5 g L^{-1}), T2 – salinity ~2.5 g L^{-1} with K^+ adjustment to close to the seawater equivalent concentration and T3 – salinity ~2.5 g L^{-1} with Ca:Mg:K ratio adjusted to 1:3:1. Different small letters above the treatment bars indicate differences among treatments for each day.

The cluster analysis revealed a clustering by time with two different groups: one group with samples of all treatments from days 0, 7, and 14 plus treatment T1 from day 21, and a second group with samples of T2 and T3 treatments from day 21 plus all treatments from days 28 and 35 (Figure 3(a)). The SIMPER analysis showed that the main phyla that contributed to the phytoplankton similarity throughout the experimental time were, in decreasing order: Ochrophyta (52.82%), Cyanophyta (40.87%), Euglenophyta (4.08%), Chlorophyta (2.21%), and Bacillariophyta (0.02%). The main genus that contributed to

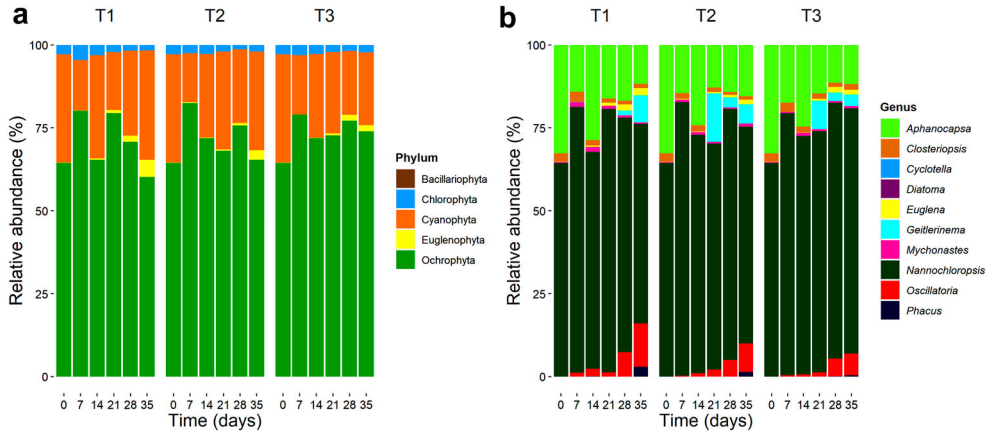

Figure 2. Relative abundance of the phytoplankton community at phylum (a) and genus (b) level in the *Penaeus vannamei* nursery at low salinity under different ionic adjustment strategies for 35 days. T1 – diluted seawater (control; salinity ~2.5 g L^{-1}), T2 – salinity ~2.5 g L^{-1} with K^+ adjustment to close to the seawater equivalent concentration and T3 – salinity ~2.5 g L^{-1} with Ca:Mg:K ratio adjusted to 1:3:1.

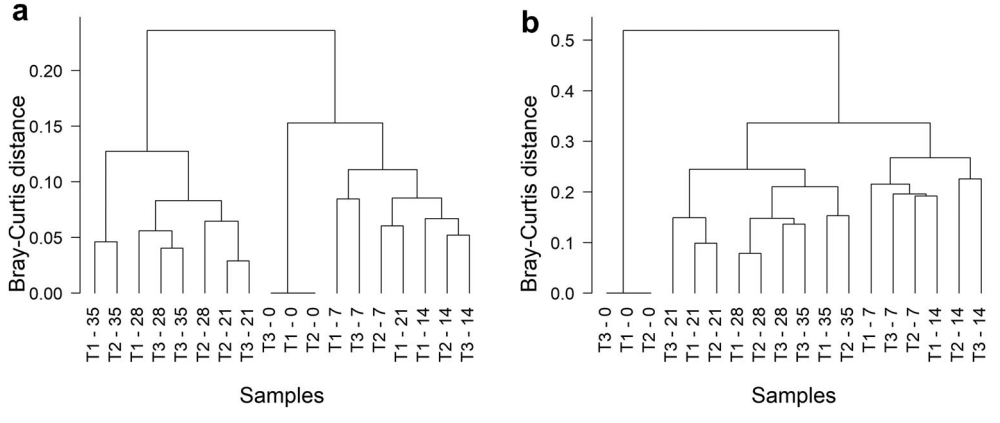

Figure 3. Bray Curtis similarity cluster analysis of the phytoplankton (a) and zooplankton (b) community during a *Penaeus vannamei* nursery at low salinity under different ionic adjustment strategies for 35 days. T1 – diluted seawater (control; salinity ~2.5 g L^{-1}), T2 – salinity ~2.5 g L^{-1} with K^+ adjustment to close to the seawater equivalent concentration and T3 – salinity ~2.5 g L^{-1} with Ca:Mg:K ratio adjusted to 1:3:1.

the phytoplankton similarity throughout the experimental times were *Nannochloropsis* (52.82%), *Aphanocapsa* (17.03%), *Oscillatoria* (12.73%) and *Geitlerinema* (11.11%) (Table 2). The other genus contributed 6.31% to the phytoplankton similarity (Table 2).

The first and second canonical axes of the RDA explained 47.74% (p-value = 0.001) and 7.44% (p-value = 0.360) of the total inertia, respectively. The r^2 and adjusted r^2 of the RDA were 0.62 and 0.46, respectively. The global p-value of the RDA was equal to 0.001. The selected RDA model included TAN, nitrite, orthophosphate, TSS, temperature, salinity, SS, DO, pH, C – DF, N – DF, N:P – DF and alkalinity as explanatory variables of the

Table 2. Summary of the analysis of percentage of similarity (SIMPER) of the phytoplankton community genus in a *Penaeus vannamei* nursery at low salinity under different ionic adjustment strategies for 35 days.

	Average dissimilarity (cells mL^{-1})	Contribution (%)	Cumulative contribution (%)	Mean 0 (cells mL^{-1})	Mean 7 (cells mL^{-1})	Mean 14 (cells mL^{-1})	Mean 21 (cells mL^{-1})	Mean 28 (cells mL^{-1})	Mean 35 (cells mL^{-1})
Nannochloropsis	12.81	52.82	52.82	1,200.00	1,670.00	2,080.00	1,990.00	2,310.00	2,800.00
Aphanocapsa	4.13	17.03	69.85	603.00	338.00	846.00	392.00	437.00	560.00
Oscillatoria	3.08	12.73	82.58	0.00	12.50	33.20	44.00	186.00	504.00
Geitlerinema	2.69	11.11	93.69	0.00	0.00	0.00	237.00	69.60	316.00
Euglena	0.57	2.36	96.05	2.01	2.35	5.89	16.80	47.20	79.70
Phacus	0.41	1.72	97.77	0.00	0.00	0.71	0.00	0.58	93.10
Closteriopsis	0.29	1.18	98.95	46.30	53.00	52.10	38.60	33.40	59.00
Mychonastes	0.25	1.03	99.98	4.78	15.90	32.20	17.60	17.30	28.50
Cyclotella	0.004	0.015	99.99	0.27	0.31	0.00	0.00	0.00	0.00
Diatoma	0.002	0.01	100.00	0.00	0.23	0.14	0.00	0.00	0.00

Note: T1 – diluted seawater (control; salinity ~2.5 g L^{-1}), T2 – salinity ~2.5 g L^{-1} with K^+ adjustment to close to the seawater equivalent concentration, and T3 – salinity ~2.5 g L^{-1} with Ca:Mg:K ratio adjusted to 1:3:1.

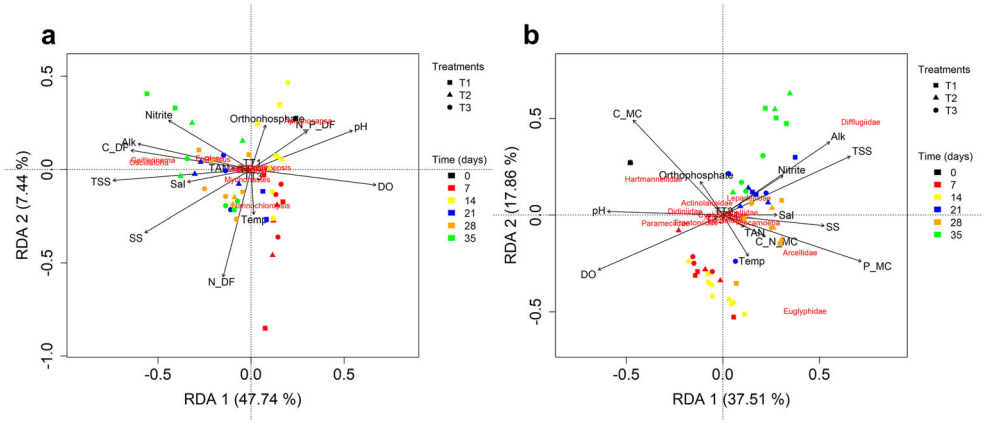

Figure 4. Redundancy analysis (RDA) of phytoplankton (a) and zooplankton (b) community and water quality variables in a *Penaeus vannamei* nursery at low salinity under different ionic adjustment strategies for 35 days. T1 – diluted seawater (control; salinity ~2.5 g L^{-1}), T2 – salinity ~2.5 g L^{-1} with K^+ adjustment to close to the seawater equivalent concentration, and T3 – salinity ~2.5 g L^{-1} with Ca:Mg:K ratio adjusted to 1:3:1. Alk: Alkalinity; DO: Dissolved oxygen; Sal: Salinity; TAN: Total ammonia nitrogen; Temp: Temperature; C_DF: carbon content of dissolved fraction (C – DF); TSS: Total suspended solids; SS: Settleable solids; N_DF: nitrogen content of dissolved fraction (N – DF), N_P_DF: N:P ratio of dissolved fraction (N:P – DF); C_MC: carbon content of floc microbial community (C – MC); P_MC: phosphorus content of floc microbial community (P – MC); C_N_MC: C:N ratio of floc microbial community (C:N – MC).

phytoplankton community composition (Figure 4(a)). Higher abundances of *Oscillatoria* and *Geitlerinema* were found in the latter stages of the experiment (days 21–35), when higher concentrations of nitrite, carbon in dissolved fraction, alkalinity, TSS, SS and salinity occurred (Figure 4(a)). Higher abundances of *Aphanocapsa* were found at the first days and in the middle of the experiment (0 and 14), when pH, orthophosphate, and N:P ratio in dissolved fraction were higher (Figure 4(a)).

3.2. Zooplankton community

No significant differences in zooplankton density among the treatments were observed for the days analysed during the experimental time. The mean total density across treatments varied from 9.54 ± 2.11 orgs mL^{-1} on day 0 to 48.70 ± 14.07 orgs mL^{-1} on day 35 (Figure 1(b)).

At the beginning of the experimental period (day 0) 5 families were identified, 3 from the phylum Ciliophora (Parameciidae, Frontoniidae and Didiniidae), 1 from Amoebozoa (Hartmannellidae), and 1 from Nematoda (Actinolaimidae) (Figure 5). During the experimental time, the main dominant phyla observed were Ciliophora, Amoebozoa and Cercozoa (Figure 5(a)). On day 35, 11 families were identified, 3 from the phylum Ciliophora [Parameciidae, Didiniidae and Vorticellidae (exclusively in treatment T3)], 4 from Amoebozoa (Hartmannellidae, Thecamoeba, Difflugiidae and Arcellidae), 1 from Rotifera (Lepadellidae), 1 from Cercozoa (Euglyphidae), 1 from Nematoda (Actinolaimidae) and, exclusively in the T3 treatment, the family Lecanidae, from the phylum Rotifera (Figure 5).

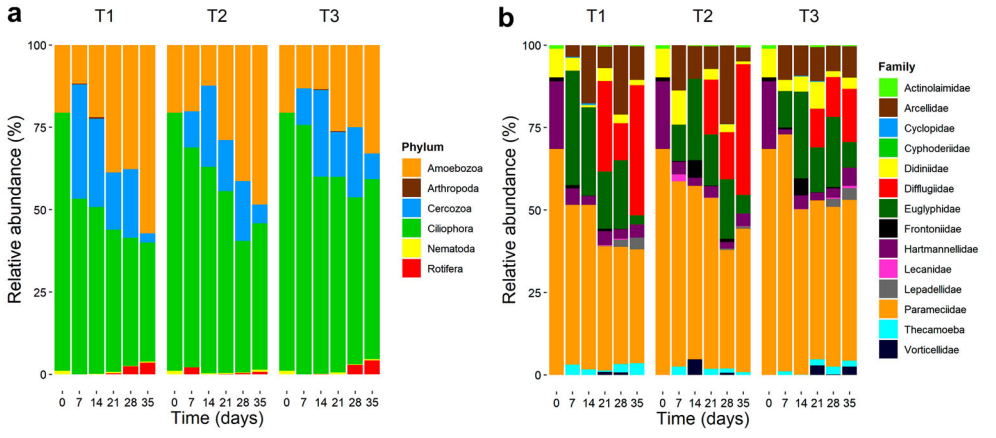

Figure 5. Relative abundance of the zooplankton community at phylum (a) and family (b) level in the *Penaeus vannamei* nursery at low salinity under different ionic adjustment strategies for 35 days. T1 – diluted seawater (control; salinity ~2.5 g L^{-1}), T2 – salinity ~2.5 g L^{-1} with K^+ adjustment to close to the seawater equivalent concentration, and T3 – salinity ~2.5 g L^{-1} with Ca:Mg:K ratio adjusted to 1:3:1.

ANOSIM did not show significant differences in zooplankton among treatments for days 0, 7, 14, 21, 28 and 35 of the experimental time. Significant differences in composition were observed in zooplankton among days of experimental time, with all weeks differing except for days 7 and 14 and days 7 and 21 (Table 3).

The cluster analysis revealed a clustering by time with three different groups formation: one group with samples from day 0, a second group with samples from days 21, 28 and 35, and a third group with samples from days 7 and 14 (Figure 3(b)). The SIMPER analysis showed that the main families that contributed to the zooplankton similarity throughout the experimental times were, in decreasing order: Parameciidae (28.81%), Difflugiidae (23.79%), Euglyphidae (18.71%), and Arcellidae (13.76%). Families Hartmannellidae, Didiniidae, Thecamoeba, Lepadellidae, Frontoniidae and Vorticellidae together contributed 14.09% to the similarity during the experimental time (Table 4). Lecanidae, Actinolaimidae, Cyclopidae and Cyphoderiidae contributed less than 1% to the similarity over the experimental period (Table 4).

Table 3. Summary of the results of analysis of similarity (ANOSIM) among time for the zooplankton community in a *Penaeus vannamei* nursery at low salinity under different ionic adjustment strategies for 35 days. Significant differences are displayed in bold (*p*-values).The first line is the global test and the remaining *p*-values are the post hoc pairwise comparisons among days

	R	0.62		*p*-value	0.0001	
Days	0	7	14	21	28	35
0	–	**0.022**	**0.004**	**0.001**	**0.003**	**0.003**
7		–	1	0.070	**0.001**	**0.003**
14			–	**0.016**	**0.001**	**0.001**
21				–	**0.003**	**0.001**
28					–	**0.001**
35						–

Note: T1 – diluted seawater (control; salinity ~2.5 g L^{-1}), T2 – salinity ~2.5 g L^{-1} with K^+ adjustment to close to the seawater equivalent concentration, and T3 – salinity ~2.5 g L^{-1} with Ca:Mg:K ratio adjusted to 1:3:1.

Table 4. Summary of the results of the analysis of percentage of similarity (SIMPER) of the zooplankton community families in a *Penaeus vannamei* nursery at low salinity under different ionic adjustment strategies for 35 days.

	Average dissimilarity (orgs mL^{-1})	Contribution (%)	Cumulative contribution (%)	Mean 0 (orgs mL^{-1})	Mean 7 (orgs mL^{-1})	Mean 14 (orgs mL^{-1})	Mean 21 (orgs mL^{-1})	Mean 28 (orgs mL^{-1})	Mean 35 (orgs mL^{-1})
Parameciidae	13.36	28.81	28.81	6.51	15.50	11.20	10.10	21.70	19.80
Difflugiidae	11.04	23.79	52.60	0.00	0.00	0.00	4.87	7.33	16.70
Euglyphidae	8.68	18.71	71.31	0.00	5.74	6.39	3.88	11.50	2.30
Arcellidae	6.38	13.76	85.07	0.00	1.99	3.03	1.99	10.90	3.83
Hartmannellidae	1.65	3.56	88.63	2.11	0.74	0.61	0.81	1.41	2.05
Didiniidae	1.31	2.83	91.46	0.70	1.39	0.14	1.24	1.42	0.95
Thecamoeba	1.16	2.50	93.96	0.00	0.77	0.22	0.31	1.16	1.11
Lepadellidae	0.85	1.84	95.80	0.00	0.00	0.00	0.02	0.88	1.24
Frontoniidae	0.84	1.80	97.61	0.12	0.14	1.07	0.09	0.28	0.00
Vorticellidae	0.72	1.56	99.16	0.00	0.00	0.56	0.31	0.36	0.37
Lecanidae	0.21	0.46	99.62	0.00	0.18	0.00	0.02	0.09	0.10
Actinolaimidae	0.13	0.29	99.91	0.10	0.00	0.04	0.10	0.07	0.18
Cyclopidae	0.04	0.09	99.99	0.00	0.02	0.04	0.02	0.00	0.01
Cyphoderiidae	0.001	0.003	100.00	0.00	0.00	0.00	0.00	0.00	0.00

Note: T1 – diluted seawater (control; salinity ~2.5 g L^{-1}), T2 – salinity ~2.5 g L^{-1} with K^+ adjustment to close to the seawater equivalent concentration, and T3 – salinity ~2.5 g L^{-1} with Ca:Mg:K ratio adjusted to 1:3:1.

In the RDA, the first and second canonical axes explained 37.51% (p-value = 0.001) and 17.86% (p-value = 0.001) of the total inertia, respectively. The r^2 and adjusted r^2 of the RDA were 0.62 and 0.47, respectively. The global p-value of the RDA was equal to 0.001. The selected RDA model included TAN, nitrite, orthophosphate, TSS, temperature, salinity, SS, DO, pH, C – MC, C:N – MC and alkalinity as explanatory variables of the zooplankton community composition (Figure 4(b)). Higher abundances of Difflugiidae were associated with higher concentrations of alkalinity, TSS, and nitrite, and lower concentrations of DO (Figure 4(b)). Higher densities of Hartmannellidae were associated with higher concentrations of C – MC and orthophosphate and lower concentrations of TAN, C:N – MC, and P – MC plus lower temperatures (Figure 4(b)). The highest concentrations of organisms from the Arcellidae and Euglyphidae families are associated with higher concentrations of P – MC, C:N – MC, and TAN, in addition to higher temperatures and lower concentrations of C – MC and orthophosphate (Figure 4(b)).

4. Discussion

4.1. Differences among treatments

The ionic adjustment strategies used in this study did not present any significant effect on phytoplankton and zooplankton composition, as the most significant differences were temporal, rather than among treatments. Hence, our results show that the ionic adjustments, besides not affecting the shrimp development and productivity [20], do not influence phytoplankton and zooplankton communities' composition either. This is an important finding because it indicates that marine shrimp production using low salinity with ionic supplementation is also ecologically viable.

The different treatments adopted in our experiment presented low potential to affect plankton. It is estimated that the major ions concentration in the phytoplankton biomass is 220 mg Kg^{-1} of Ca^{2+}, 90 mg Kg^{-1} of Mg^{2+} and 190 mg Kg^{-1} of K^{+} [5], with a Ca:Mg:K ratio of 1.16:0.4:1 (mass:mass:mass). Thus, in the T3 treatment of this study, we had 7.5 times more Mg^{2+} available in the water than in the phytoplankton biomass. This would make K^{+} and Ca^{2+} probably more prone to become limiting ions in our system.

From a stoichiometric point of view, the same ratios can be obtained from different numerators and denominators, if their proportions remain the same [38]. In this way, an environment with the same ratios among major ions may be truly limited due to denominator exhaustion or probably not limited if the major ions concentration is excessively high [38]. This can be stated, because throughout the experiment there was a constant input of ions such as Ca^{2+} and K^{+} through the feed that was offered to the shrimps. The feed contained a minimum percentage of Ca^{2+} of 1.2% and 0.8% of K^{+} (minimum percentage guaranteed by the manufacturer), which could have alleviated any possible limitation by these ions.

4.2. Temporal patterns of phytoplankton and zooplankton variation

In this study, during the experimental time, the main groups that contributed to phytoplankton similarity were the microalgae Ochrophyta (species: *Nannochloropsis*) and Cyanophyta (species: *Aphanocapsa*, *Oscillatoria*, and *Geitlerinema*). In intensive marine shrimp

farming systems, phytoplanktonic dominance by the Cyanophyta group is often reported [39–41]. As an example, in a biofloc system, Campos et al. [40], evaluated phytoplankton community in an integrated culture of shrimp *P. vannamei* and the macroalgae *Gracilaria birdiae* and found *Oscillatoria* as one of the species that most contributed to the similarity among treatments. The dominance by this group of nitrogen-fixing microorganisms is favoured by low N:P ratios [42], since in intensive aquaculture systems there is an accumulation of phosphorus throughout the culture period [43], reducing the N:P ratios. However, the relative abundance of Cyanophyta found in this study is lower when compared to other intensive shrimp farming systems, such as the biofloc system [40]. This suggests that the use of the synbiotic system may provide a greater control of these microorganisms in the system.

In this study, a relationship was observed among the high abundance of *Oscillatoria* and *Geitlerinema* species with high concentrations of carbon in the dissolved fraction (C – DF), alkalinity, TSS and with low N:P ratios. As they are photoautotrophic organisms, Cyanophytes use inorganic sources such as carbon dioxide (CO_2) and bicarbonate (HCO_3^-) from the alkaline reserve as carbon source [17]. Still, Yusoff et al. [44] analysing phytoplankton succession in intensive marine shrimp culture ponds found that *Oscillatoria* sp. growth significantly increased when a combination of nitrogen, phosphorus and carbon was added to the pond water. Our results show that these microorganisms are favoured in the final stages of culture (in this study, from day 21; Figure 2(b)), as there is a higher concentration of TSS and nutrients such as carbon and phosphorus (reducing the N:P) [20].

On the other hand, the high abundance of Cyanophyta of the species *Aphanocapsa* is related to high N:P ratios in dissolved fraction. This can be explained by the fact that it is a potentially non-nitrogen fixing species [45]. In all treatments, the highest abundance of this species was recorded at the beginning (day 0) and at day 14 of the experimental time (Figure 2(b)), when, probably, the concentration of dissolved phosphorus was lower and, therefore, the N:P ratio was higher than in the last few weeks of culture, when one of the lowest abundances for this species was recorded (Figure 2(b)).

Regarding the zooplankton community, a temporal variation in the composition was observed through ANOSIM. This variation is confirmed by the cluster analysis, which showed three groups formation: day 0; days 7 and 14, and days 21, 28 and 35. During the experimental time, dominance by groups of the phylum Ciliophora and Amoebozoa was also reported in intensive marine shrimp farming systems using biofloc and synbiotic systems. For example, Reis et al. [46] tested the effect of different photoperiods on *P. vannamei* growth (500 shrimp m^{-3}) and the composition of the microbial community in a biofloc system and found a greater dominance by ciliated and flagellate protozoans in treatments with greater exposure to light. Hosain et al. [47] investigated the effect of different salinities on the growth of *Macrobrachium rosenbergii* post larvae and on the planktonic community in a biofloc system and found a dominance by ciliated protozoa in treatments with salinity 5, 10 and 15 g L^{-1}. The dominance by protozoan microorganisms is due to the presence of the nutrient carbon in the system from fertilisation and in the organic matter form, resulting in an increase in bacterial biomass, thus favouring ciliates [48]. Nagano and Decamp [49] determined the ciliates ingestion rate by *P. vannamei* larvae and found that the larvae ingested amounts greater than 4,000 ciliates shrimp $^{-1}$ d^{-1}, surviving and developing during the experiments. In fact, our data showed a high

abundance of protozoa from Arcellidae and Euglyphidae families with high carbon:nitrogen ratios in the microbial community (C:N – MC) and phosphorus in the microbial community (P – MC), while the high abundance of the Difflugiidae family was related to high TSS. This indicates that the group of Protozoa can behave preying on microorganisms that are present in the biofloc community, such as bacteria and flagellated protozoans [46,48], showing the microbial loop effect in the system.

In the present study, this effect can be confirmed in *P. vannamei* intensive nurseries with synbiotic system using oligohaline water, since the highest concentrations of organisms of higher trophic level, such as rotifers, were recorded in the last weeks of the experimental time (Figure 5(a)). These microorganisms have a 'grazing' behaviour on microalgae, protozoa, and microbial flocs, exerting top-down control and completely influencing trophic interactions in the system [19]. This effect was also observed by Ray et al. [50] who evaluated the microbial community in an intensive shrimp farming system using biofloc technology.

The absence or low concentrations of larger organisms such as copepods and cladocerans in the system may have occurred due to the sample pre-filtration to retain larger microbial flocs, also retaining these microorganisms, and causing them to not be quantified. Even though a dominance of small size plankton is expected in microbial-dominated aquaculture systems [13,41,46,51], future studies targeting the whole planktonic communities should be carried out, as different patterns may arise.

5. Conclusion

Strategies of adjusting K^+ concentration to be equivalent to seawater and adjusting Ca:Mg:K ratio to 1:3:1 had no effect on the phytoplankton and zooplankton community in a *Penaeus vannamei* intensive nursery in low salinity water with a synbiotic system. Since the major ions supplementation in the water did not alter the composition of the planktonic community of the system, temporal changes in plankton composition may have occurred due to the creation of a culture medium rich in nutrients such as carbon, nitrogen, and phosphorus, providing conditions for the rapid growth of the microorganisms.

The organic fertilisation with rice bran processed by probiotic microorganisms may be an explanation for the temporal variation observed in the planktonic community composition. This strategy provides microbial community development in intensive marine shrimp farming systems, improving water quality and animal growth.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Ethical approval

The research undertaken complies with the current animal welfare laws in Brazil.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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