








# Chitosan Foam–Based Filter: Maintenance of Water Quality for Nile Tilapia Cultivation

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**Abstract** The objectives of this research were to evaluate the maintenance of water quality when using chitosan foam filters in water recirculation systems during the cultivation of Nile tilapia and to verify the zootechnical performance and the hepatic and branchial histopathological changes, in comparison with the use of biological filter with bioballs. Two Nile tilapia

cultivation trials were carried out (trial 1: 35-L tanks; trial 2: 130-L tanks), using six individual water recirculation systems, consisting of a culture tank, decantation tank, submerged pump for recirculation, aeration and external filter. The physical and chemical parameters related to water quality were evaluated every 48 h. At the end of each trial, the fish biometrics was performed to obtain final weight, feed conversion, and survival. In trial 2, the histopathological analysis of the hepatopancreas and gills was performed and the organ index was calculated. The daily averages of the physical and chemical parameters of the water quality, the zootechnical performance of the fish, and the organ indexes of the treatments of trials 1 and 2 were compared by the *t* test ( $p \leq 5\%$ ). There were no significant differences in final weight, feed conversion, survival, or organ indexes between treatments ( $p > 0.05$ ) in relation to the two trials. It was observed that the use of the filter with chitosan foam in the water recirculation systems resulted in lower or equal concentrations of total ammonia, nitrate, and dissolved orthophosphate, and maintained the same or higher alkalinity than in tanks with bioball biological filter. It is recommended to change the chitosan foam every 30 days of cultivation. The use of water recirculation systems with treatment using filters filled with chitosan foam during the cultivation of Nile tilapia is equally effective or better than the use of a biological filter with bioballs.

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## 1 Introduction

Aquaculture has become an effective alternative to supply the demand for food for the growing population of the planet. At the same time, aquaculture farmers must seek sustainable production due to the limitation on the use and availability of fresh water and the greater emphasis on the conservation and the use of water resources (Silva et al. 2013a; Bregnballe 2015; Vilbergsson et al. 2016).

Recirculation systems for aquaculture are designed to treat and recirculate farm water to minimize water use, manage waste, and recycle nutrients. They are built from subsystems designed to control water quality parameters (Vilbergsson et al. 2016). The necessary components for a recirculation system are the culture tanks, decanters, filters and/or biofilters, aeration/oxygenation system, pumps and drainage and return pipes (Silva et al. 2013a).

Recirculation is a traditional technology, based on the use of mechanical and biological filters. The wastewater that comes out of the recirculation process is usually directed to a mechanical filter, where the feces, and the organic matter, such as feed remains, are separated before entering the biological filter. The organic material accumulated in the mechanical filter can be spread on the soil as a fertilizer in agriculture (Bregnballe 2015).

Biological filters are used to reduce the concentration of ammonia by oxidation to nitrate, by the action of nitrifying bacteria (Pedreira et al. 2016). A minimum period of conditioning of the biofilter for 14 days is necessary to guarantee a stable nitrification process before being used in aquaculture production (Suantika et al. 2016). In biological filters, various types of materials can be used as a substrate for the formation of bacterial biofilms, such as shell, granite gravel, quartz gravel, porcelain (Pedreira et al. 2016) or the most varied types of commercially available media (Elliott et al. 2017).

The adsorption and coagulation processes are being used with activated carbon, zirconia, clay minerals, polyaluminum chloride, among other products, to further improve water quality and reduce the concentrations of other aquaculture pollutants simultaneously. However, they may not be effective for aquaculture wastewater treatment due to their low treatment capacity, high costs, toxic waste, and high selectivity for specific pollutants. Thus, it is necessary to use non-toxic, inexpensive, and multifunctional filtering

materials to improve the quality of cultivation water and aquaculture effluent (Chung et al. 2005).

Chitosan is a non-toxic and polycationic biopolymer, and in aquaculture effluents, it can perform the functions of adsorption, coagulation, charge neutralization, inter-particle bridge, bacteriostatic disinfection, or a combination of these. It is worth emphasizing that if the chitosan remains in water, it is safe for aquatic organisms and consumers (Chung et al. 2005). The existence of amino and hydroxyl groups in their molecules allows adsorption interactions between chitosan and various types of environmental pollutants. However, until the present date, the industrial use of chitosan as an adsorbent has been limited due to the disadvantage of swelling in aqueous solutions (Kyzas and Bikiaris 2015) and leaving the water turbid (Patil et al. 2013), and in powder form it is not structured on a support that allows its easy application and recovery.

The use of chitosan foam in filters for water treatment in aquaculture cultivation systems is facilitated due to the fact that the material is not a powder, but structured and malleable. The dosage of 0.10 (% m v<sup>-1</sup>) of chitosan foam in the filter was indicated for efficient adsorption of ammonia, nitrite, and orthophosphate, and turbidity, and its use in the recovery of aquaculture wastewater is promising (Zadineo et al. 2018).

The objective of this work was to verify the maintenance of water quality by the use of chitosan foam in filters in water recirculation systems during Nile tilapia cultivation, compared to the use of biological filters with bioballs. Possible hepatic and branchial histopathological changes were also verified, and the effects on final weight, survival, and feed conversion resulting from the two cultivation systems were verified.

## 2 Material and Methods

The experiment was conducted at the Fish Production and Reproduction Laboratory (LAPERP) and the Water Quality and Limnology Laboratory (LaQaL) of the Federal University of Paraná - Palotina Sector. The experimental procedures were conducted according to the Ethical Principles of Animal Experimentation adopted by the National Council for the Control of Animal Experimentation (CONCEA), according to protocol no. 10/2018 approved by the Ethics Committee on the Use of Animals of the Palotina Sector of UFPR (CEUA/Palotina).

Two cultivation trials of Nile tilapia fingerlings were carried out in two volume scales of the culture tanks (trial 1: 35-L tanks; trial 2: 130-L tanks).

Six individual water recirculation systems for cultivation (experimental units) were used in trials 1 and 2, composed of a culture tank (35 or 130 L, respectively), a decanting tank (5 L or 21 L, respectively), a submerged pump for recirculation (400 or 1000 L h<sup>-1</sup>, respectively), aeration and external filter. In both trials, the water that came out of the culture tank was directed to the decanting tank and it was pumped through the filter, and then returned to the culture tank. The structure was allocated within a closed room, with a photoperiod of 12 h of light to 12 h of darkness.

Of these six systems of each assay, three contained filters filled with chitosan foam, and in the other three, the filters were filled with bioballs, which is a material that has a large contact area for bacterial adherence, commonly used as and considered a biological filter. In both assays, the bioballs used in the biological filter were already mature, that is, colonized by nitrifying bacteria, because they were obtained from a biofilter of a fish recirculation system from the experimental laboratory system. The experimental design used was completely randomized with two treatments and three replicates. In each trial, all the media of the filter materials had the same useful volume and were manufactured as described in Zadinelo et al. (2018).

In both tank volume scales, the following physical and chemical parameters of water quality were monitored every 48 h: total ammonia, nitrite, nitrate, dissolved orthophosphate, alkalinity, hardness, pH, electrical conductivity, temperature, and dissolved oxygen.

Total ammonia was determined by the indophenol method (Koroleff 1976) and nitrite by the Griess reaction method (Baumgarten 1996). Dissolved orthophosphate was obtained by the ascorbic acid method, and nitrate was obtained by ultraviolet spectrophotometric screening method (APHA 2005). The determinations were made in a Spectrophotometer Belpotonics SP2000UV®. Total alkalinity and total hardness were determined by titration, following the methodology proposed by Macêdo (2003). The pH was measured in Kasvi AI 03449® pH meter and the electrical conductivity in a Tecnonon NT-CVM® conductivity meter. Dissolved oxygen and temperature were measured in an Alfakit AT160® oximeter.

In trial 1, i.e., 35-L tanks, the experiment was divided into two phases (phase I and phase II). Phase I

comprised the period between day 1 and day 31, and phase II comprised the period between day 31 and day 75. Between these two phases, there was the replacement of foam in the filter. The filters had a column of 23.5 cm height by 6 cm in diameter, with a volume of 664 cm<sup>3</sup>. The biological filters were filled with twenty bioballs of 3.2 cm in diameter. Water exchange was not performed, but each tank was refilled with approximately 0.5 L (1.43% of the total volume) per day, due to evaporation losses.

In phase I, the filters were filled with 0.05% (m v<sup>-1</sup> in g mL<sup>-1</sup>), chitosan foam, less than recommended by Zadinelo et al. (2018), because the tanks were filled with clean water and were free of pollutants. The tanks were filled with water from the supply network, dechlorinated with sodium thiosulfate, and remained with water recirculation through the filters and aeration for 24 h, before the fish were housed. One hundred twenty Nile tilapia fingerlings were sexually reversed with an average weight of 0.86 ± 0.03 g, and in each tank (experimental unit) 20 fish were housed.

The fish were fed at 9:00 am and 6:00 pm, with extruded commercial feed, with 38% crude protein and 1 mm in diameter. The same amount of food (3% of biomass) was supplied daily to fish in all culture tanks. The weight of the food consumed daily in each tank was recorded to calculate the feed conversion. The experiment was conducted for 30 days. After this period, the fish were fasted for 24 h, and were subsequently captured and anesthetized for a short time using 75 mg L<sup>-1</sup> of eugenol (Vidal et al. 2008), and then, biometrics was performed to obtain the final weight of the fish.

For phase II, the foam filters used in phase I were replaced by new filters filled with 0.10% (m v<sup>-1</sup> in g mL<sup>-1</sup>) of chitosan foam. The new filters were placed in recirculation tanks with clean and dechlorinated water, with aeration for 24 h before being used in the experimental tanks. After the biometry of the first phase, sixty fingerlings of uniform size were selected: 30 fingerlings from the tanks with filter filled with chitosan foam and 30 fingerlings from the tanks with biological filter.

The average weight of the selected fish was 5.95 ± 0.60 g, and these were distributed in the tanks of their respective treatments (10 fish per tank). The same food management as in phase I was used; however, the fish were fed with extruded commercial feed, with 32% crude protein and 3 mm in diameter. The same food management as in phase I was used; however, the fish were fed with extruded commercial feed, with 32%

crude protein and 3 mm in diameter. Phase II of trial I was conducted for 44 days. At the end of this period, the fish were fasted for 24 h; after they were captured and anesthetized for a short time using  $75 \text{ mg L}^{-1}$  of eugenol (Vidal et al. 2008); then, a biometry was performed again to use the final weight.

Trial 2 (in 130-L tanks) was conducted in a single phase. Of the six recirculation systems used, three tanks contained filters filled with chitosan foam at a dosage of  $0.10\%$  ( $\text{m v}^{-1}$  in  $\text{g mL}^{-1}$ ) and in the other three tanks there were biological filters filled with 87 bioballs of 3.2 cm in diameter. The filters used had a column of 41.5 cm in height and 10 cm in diameter, with a volume of  $3258 \text{ cm}^3$ . Sixty Nile tilapia fingerlings were used sexually reversed with an average weight of  $29.8 \pm 4.5 \text{ g}$ , where 10 fish were housed in each tank (experimental unit). The fish were fed at 9:00 am and 6:00 pm, with extruded commercial feed with 28% crude protein and 5 mm in diameter. The same amount of food (3% of biomass) was supplied daily to fish in all culture tanks. The weight of the daily food consumed in each tank was recorded to calculate the feed conversion.

During the entire experimental period, water exchange was not performed but each tank was refilled with approximately 0.5 L (1.43% of the total volume) per day, due to evaporation losses. After 30 experimental days, the chitosan foam filters were replaced by new filters, filled with a dosage of 0.10% (mass per volume of solution:  $\text{m v}^{-1}$  in  $\text{g mL}^{-1}$ ). The experiment was conducted for 60 days. After this period, the fish were fasted for 24 h, then captured and anesthetized for a short time using  $75 \text{ mg L}^{-1}$  of eugenol (Vidal et al. 2008) and then biometrics was performed to obtain the final weight of the fish.

In trial 2, three fish were selected per experimental unit for the collection and histological analysis of the organs to assess possible hepatic and branchial histopathological changes in Nile tilapia after cultivation using the biological filter with bioballs and filter with chitosan foam. The selected fish were euthanized with  $286.55 \text{ mg L}^{-1}$  of eugenol for 600 s (Vidal et al. 2008) or until the cessation of the opercular movement was observed. Two segments of the right and left gills (medial portion of the second branchial arch) and a sample of hepatopancreas per fish were collected.

The organ samples were fixed in Davis solution, accommodated on cassettes and dehydrated in an increasing series of alcohols (70%, 80%, 90%, and absolute), diaphonized in xylol (three baths) and

impregnated in histological paraffin at  $56^\circ\text{C}$ , both lasting 40 min. After inclusion, 5- $\mu\text{m}$ -thick cuts were made in a microtome (Leica, model RM2245). The samples were stained with periodic acid–Schiff (PAS) and/or hematoxylin and eosin (HE). The organ images were captured with a  $2048 \times 15636$  megapixel digital camera (Leica, model DFC295) attached to a microscope (Leica, model DM 1000) and visualized by the Leica Application Suite V3 software (Leica, model LAS V3.8).

The changes observed in the organs were evaluated according to the Organ Index (*Iorg*). For each organ investigated, pathological changes are classified into five reaction patterns: circulatory disorders, regressive changes, progressive changes, inflammation, and tumor. In each reaction pattern, a factor of importance (*w*) was assigned to each change according to the degree of reversibility, where “1” = easily reversible changes; “2” = moderate changes, reversible with the end of exposure; and “3” = irreversible changes, presenting partial or total loss of organ function. The score value (*a*) was applied according to the degree of distribution and intensity of the organ damage, where “0” = absence; “1–2” = mild occurrence; “3–4” = moderate occurrence; and “5–6” = accentuated occurrence (Bernet et al. 1999). From the above classifications, the Organ Index (*Iorg*) was calculated:

$$I_{org} = \sum alt(a.w)$$

where:

- “*Iorg*” Organ index;
- “*alt*” alteration;
- “*a*” score value;
- “*w*” importance factor.

*Iorg* is the index that represents the degree of organ damage, and a high value of the index represents a high degree of organ damage.

## 2.1 Statistical Analysis

The daily averages of the physical and chemical parameters referring to water quality (total ammonia, nitrite, nitrate, dissolved orthophosphate, alkalinity, hardness, electrical conductivity, and turbidity), fish zootechnical performance (final weight, feed conversion, and survival), and organ indexes (*Iorg*) of the treatments of trials 1 and 2 were submitted to verify the normality of data

distribution and homogeneity of variances. After confirming these assumptions, the data were compared using the *t* test, at the level of 5% probability, in the Statistica 7.0 program (StatSoft Inc. 2004).

### 3 Results and Discussion

#### 3.1 Zootechnical Performance

In trials 1 and 2, no significant differences were observed ( $p > 0.05$ ) for the parameters of total weight, feed conversion, and survival at the end of both phases (I and II), between treatments (tanks with foam filter of chitosan and tanks with biological filter with bioballs) (Table 1).

The results of both assays indicate that the use of chitosan foam in filters in water recirculation systems does not affect the performance of Nile tilapia fingerlings when compared to the use of recirculation systems with bioball biological filters.

#### 3.2 Dissolved Oxygen

In trial 1, during the phase I period, the average dissolved oxygen in the water of the tanks containing the chitosan foam filter was  $7.66 \pm 1.12 \text{ mg L}^{-1}$  and in the tanks containing biological filters, the mean was  $7.91 \pm 0.83 \text{ mg L}^{-1}$ . In phase II, the average dissolved oxygen

in the tanks containing the chitosan foam filter was  $4.39 \pm 0.27 \text{ mg L}^{-1}$  and in the tanks containing biological filters, the mean was  $4.41 \pm 0.56 \text{ mg L}^{-1}$ .

In trial 2, in the water of the tanks with chitosan foam filter, the average dissolved oxygen in the first 31 days of cultivation was  $6.71 \pm 0.42 \text{ mg L}^{-1}$  while in the water of tanks with biological filter of bioballs, the result for the average dissolved oxygen was  $6.77 \pm 0.52 \text{ mg L}^{-1}$ . In the last 30 days, the result for this parameter was  $5.80 \pm 0.66 \text{ mg L}^{-1}$  in the water of tanks with a chitosan foam filter, and  $5.85 \pm 0.68 \text{ mg L}^{-1}$  in the water of tanks with biological filters of bioballs.

In both trials, a decrease in dissolved oxygen was observed during the experimental period, corroborating Marengoni et al. (2013), who verified that, during intensive cultivation of Nile tilapia juveniles in a recirculation system, dissolved oxygen levels decreased and approached  $2 \text{ mg L}^{-1}$  at 84 days of cultivation. These results are considered critical for intensive tilapia culture, reaching the limit of the system's support capacity.

#### 3.3 Temperature

In trial 1, the mean water temperature during phase I in both treatments ( $p > 0.05$ ) was  $26 \pm 2 \text{ }^\circ\text{C}$ , and during phase II, it was  $25 \pm 1 \text{ }^\circ\text{C}$ . In trial 2, the average temperature of the cultivation water was  $25 \pm 0.67 \text{ }^\circ\text{C}$  for both treatments.

The water temperature between 25 and 30  $^\circ\text{C}$  is more suitable for the cultivation of Nile tilapia fingerlings to obtain optimal growth performance and survival rate (El-Sherif and El-Feky 2009). In both trials, the water temperature remained within the thermal comfort zone for optimum performance of tilapia fingerlings. However, it is worth mentioning that there was no water heating system in the culture tanks with a water recirculation system used for the trials with both filters (chitosan foam and bioballs), and the temperature variations were due to the external environment.

#### 3.4 pH

The pH of the water used to start trial 1 (in phase I) was 6.00. During the phase I experimental period, in tanks with biological filters with bioballs, the mean pH was  $5.85 \pm 0.76$ , and in tanks with a chitosan foam filter, the mean values were  $6.41 \pm 0.52$ . In phase II, in tanks with a biological filter with bioballs, the mean pH was  $6.77 \pm 0.31$ , and in tanks with a chitosan foam filter, the mean values were  $6.56 \pm 0.42$ .

**Table 1** Zootechnical performance of Nile tilapia fingerlings from trials 1 and 2

Treatments	Final weight (g)	Survival (%)	Food conversion
Trial 1 (35-L tanks)			
Phase I			
Filter with chitosan foam	$5.45 \pm 0.89$	$95 \pm 5.00$	$0.87 \pm 0.24$
Biological filter with bioballs	$5.86 \pm 0.24$	$85 \pm 15.00$	$0.89 \pm 0.21$
Phase II			
Filter with chitosan foam	$33.95 \pm 2.47$	$100 \pm 0$	$0.85 \pm 0.06$
Biological filter with bioballs	$33.99 \pm 2.17$	$97 \pm 5.77$	$0.87 \pm 0.05$
Trial 2 (130-L tanks)			
Filter with chitosan foam	$75.9 \pm 27.1$	$97 \pm 5.77$	$1.47 \pm 0.26$
Biological filter with bioballs	$70.5 \pm 34.6$	$80 \pm 26.45$	$1.85 \pm 0.82$

In trial 2, the mean pH was  $8.43 \pm 0.25$  in the water of the tanks with chitosan foam filter and  $8.23 \pm 0.36$  in the water of the tanks with a biological filter with bioballs.

Rebouças et al. (2016) indicate that the pH range of the water supported for Nile tilapia cultivation ranges from 5.5 to 9.0. However, according to Kubitzka (2011), for optimal tilapia growth, the pH range should be maintained between 6.00 and 8.50. The pH values of the culture water of both treatments were within the range tolerated by the species.

### 3.5 Total Ammonia

In phase I of trial 1, between the 15th and 27th days, an increase in total ammonia was detected in the water of tanks containing biological filter, which reached  $0.098 \text{ mg L}^{-1}$ , while in the water of the tanks with chitosan foam filter, the total ammonia reached values up to  $0.025 \text{ mg L}^{-1}$  (9th day) (Fig. 1).

During phase II (trial 1), in the water of the tanks containing the chitosan foam filter, the following results were detected:  $0.023$ ,  $0.017$ , and  $0.013 \text{ mg L}^{-1}$  of total ammonia on the 31st, 35th, and 37th days, respectively, and ammonia was no longer detected until the 65th day; from then on, increasing levels of ammonia were detected due to the saturation of the filter material, which reached  $0.43 \text{ mg L}^{-1}$  on the 74th day. In the water of the tanks containing a biological filter,  $0.88 \text{ mg L}^{-1}$  of total ammonia was detected on the 37th day, which reached concentrations of  $2.40 \text{ mg L}^{-1}$  on the 65th day (Fig. 1).

In trial 1, significant differences were observed in the concentrations of total ammonia between treatments ( $p < 0.05$ ), on days 27, 31, 33, 39–67, and 73. The use of the chitosan foam filter in the culture tanks was more efficient, and the daily concentrations of total ammonia remained significantly lower or equal throughout the experimental period.

In trial 2, in tanks with a biological filter and bioballs, the maximum total ammonia concentrations were detected on days 7 ( $0.20 \text{ mg L}^{-1}$ ), 17 ( $0.28 \text{ mg L}^{-1}$ ), 33 ( $0.39 \text{ mg L}^{-1}$ ), 37 ( $0.45 \text{ mg L}^{-1}$ ), and 61 ( $0.60 \text{ mg L}^{-1}$ ), while in chitosan foam filter tanks, the maximum concentrations detected were on days 7 ( $0.17 \text{ mg L}^{-1}$ ), 25 ( $0.24 \text{ mg L}^{-1}$ ), and 35 ( $0.17 \text{ mg L}^{-1}$ ). From then on, they remained below  $0.10 \text{ mg L}^{-1}$ . However, significant differences were observed in the concentrations of total ammonia between treatments ( $p < 0.05$ ), only on days 3, 59, and 61 (Fig. 2).

It was observed that, from the 56th day on, the levels of nitrogen compounds increase in closed water recirculation systems during the cultivation of Nile tilapia juveniles, and the efficiency of the biological filter decreases. This result influences the water quality of the system (Marengoni et al. 2013). A similar result was found in this study in trial 1 and 2, in which, from the 61st day on, ammonia concentrations increased in tanks with biological filter and, from the 69th day on, an exponential increase in total ammonia was measured in tanks using the chitosan foam filter, due to foam saturation.

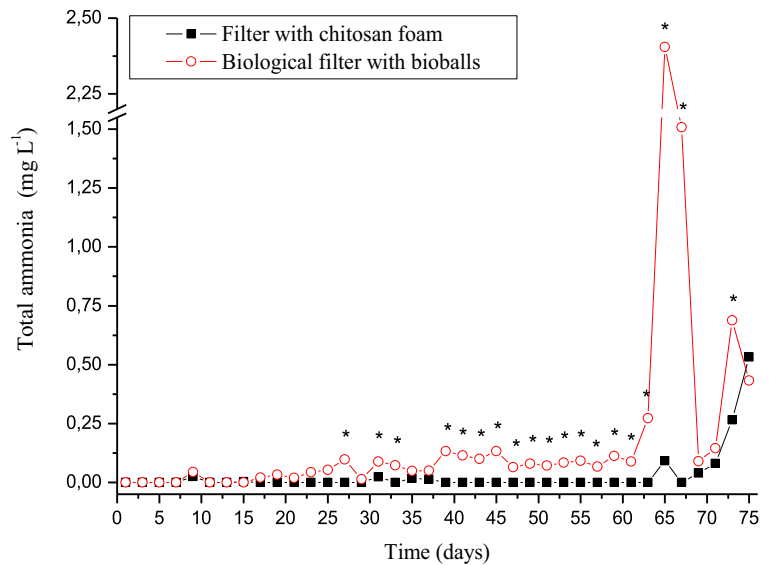
The median lethal concentration at 96 h post-exposure ( $LC_{50-96h}$ ) of non-ionized ammonia ( $NH_3$ ) for Nile tilapia ( $12.6 \pm 2.9 \text{ g}$ ) is  $0.98 \text{ mg L}^{-1}$ . Biochemical, physiological, histological, and behavioral changes caused by sublethal levels induce growth suppression and immunological impairment. The signs of intoxication in sublethal concentrations are hyperactivity and loss of balance, and in more severe cases, the signs are decreased swimming activity, lethargy, darkened staining, and mortality (Evans et al. 2006).

During the entire experimental period, considering the values of total ammonia, pH, and temperature, in both treatments and trials, the  $LC_{50-96h}$  of non-ionized ammonia of  $0.098 \text{ mg L}^{-1}$  defined by Evans et al. (2006) was not exceeded. The maximum calculated values of non-ionized ammonia in trial 1 were  $0.002 \text{ mg L}^{-1}$  on the 75th day ( $24 \text{ }^\circ\text{C}$ , pH 7.0) in tanks with a chitosan foam filter and  $0.014 \text{ mg L}^{-1}$  on the 75th day ( $26 \text{ }^\circ\text{C}$ , pH 7.0) in the tanks with biological filters. In trial 2, the concentrations of non-ionized ammonia calculated were  $0.038 \text{ mg L}^{-1}$  on the 25th day ( $26 \text{ }^\circ\text{C}$ , pH 8.4) in tanks with chitosan foam filter and  $0.072 \text{ mg L}^{-1}$  on the 37th day ( $26 \text{ }^\circ\text{C}$ , pH 8.4) in tanks with biological filter.

### 3.6 Nitrite

In trial 1, in phase I, the daily average concentration of nitrite was higher ( $p < 0.05$ ) in the water of the tanks that contained the filter with chitosan foam. The results reached a maximum value of  $0.90 \text{ mg L}^{-1}$  on the 11th day. After this period, there was a decrease and stability occurred on the 25th day, with  $0.075 \text{ mg L}^{-1}$ , equaling ( $p > 0.05$ ) the concentrations within the tanks that contained biological filters (Fig. 3). This fact may have occurred due to the foam dosage used in the filter ( $0.05\% \text{ m v}^{-1}$ ) at the beginning of the experimental period, for the adsorption

**Fig. 1** Average concentrations of total ammonia of the tanks with the use of chitosan foam filter and tanks using the biological filter with bioballs, during the 74-day experimental period from trial 1. Asterisks indicate significant difference between treatments on each day by *t* test ( $p \leq 0.05$ )

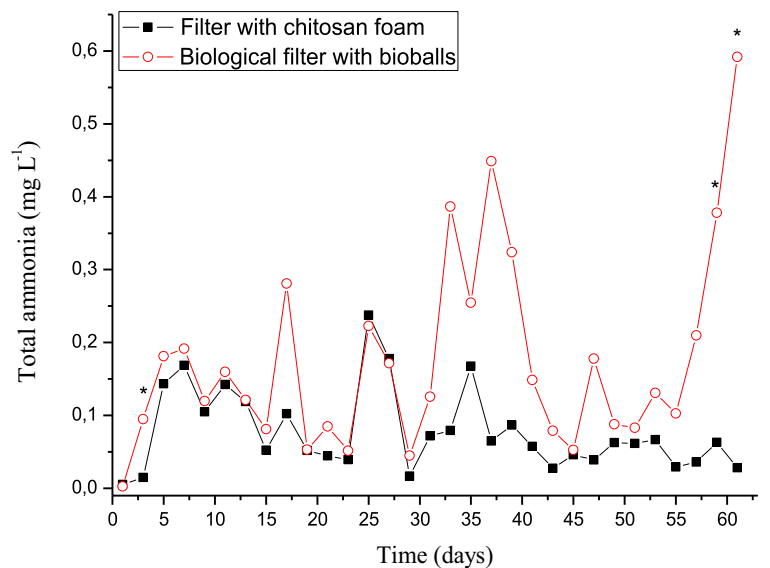


phenomenon, being lower than the recommended one ( $0.05\% \text{ m v}^{-1}$ ) by Zadinelo et al. (2018). These authors also point out that the higher the dosage, the greater the nitrite adsorption capacity. Another fact that may have contributed is the absence of an established bacterial community in tanks with a filter with chitosan foam, unlike the biological filter, where mature filters (> 14 days) were used (Suantika et al. 2016).

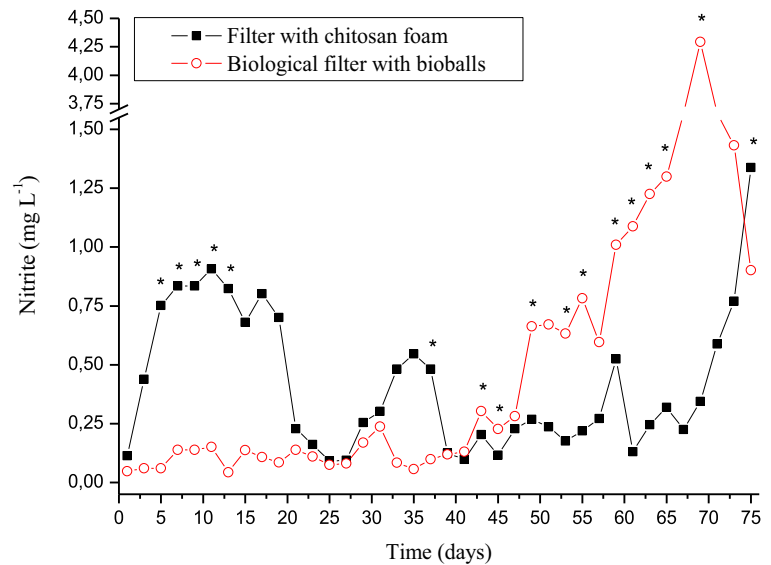
In phase II, the average nitrite concentrations were higher ( $p < 0.05$ ) in the water of the tanks that contained the filter with chitosan foam, between the 31st and 39th

days. After this period, the concentrations remained lower ( $p < 0.05$ ) than in the water of the tanks with biological filters (Fig. 3). Significant differences were observed between treatments ( $p < 0.05$ ) on days 5–13 and 37, when the use of the biological filter with bioballs maintained lower concentrations of nitrite, and on days 43–69, the filter with chitosan foam was more efficient. On the 74th day, nitrite concentrations were significantly higher ( $p < 0.05$ ) in the water of the tanks that contained the filter with chitosan foam, due to the saturation of the adsorbent material.

**Fig. 2** Average concentrations of total ammonia of the tanks with the use of chitosan foam filter and tanks using the biological filter with bioballs, during the 61-day experimental period from trial 2. Asterisks indicate significant difference between treatments on each day by *t* test ( $p \leq 0.05$ )



**Fig. 3** Average concentrations of nitrite of the tanks with the use of chitosan foam filter and tanks using the biological filter with bioballs, during the 74-day experimental period from trial 1. Asterisks indicate significant difference between treatments on each day by *t* test ( $p \leq 0.05$ )



In trial 2, increasing concentrations of nitrite were detected at the beginning of the experimental period in both treatments, which reached maximum values on the 27th day, with an average of  $2.85 \text{ mg L}^{-1}$  in the water of tanks with bioball biological filters, and  $1.80 \text{ mg L}^{-1}$  in the water of tanks with a filter with chitosan foam. After this period, the concentrations remained below  $1.1 \text{ mg L}^{-1}$  in the water of the tanks with chitosan foam filters, and in the tanks with bioball biological filter, below  $1.97 \text{ mg L}^{-1}$ . Significant differences were observed between treatments on days 5, 7, 13, 43, 49, and 51 (Fig. 4).

The toxicity of nitrite in Nile tilapia (*Oreochromis niloticus*) can be inhibited by adding chloride to water, using calcium chloride or sodium chloride. The median lethal concentration of 96 h of nitrite-N was  $81 \text{ mg L}^{-1}$  in tilapia with an average weight of  $4.40 \pm 1.50 \text{ g}$ , and  $8 \text{ mg L}^{-1}$  for tilapia with an average weight of  $90.7 \pm 16.43 \text{ g}$  (Atwood et al. 2001). Both in trial 1 (phases I and II) and in trial 2, nitrite concentrations remained adequate for cultivation in both treatments. Chitosan foam can contribute to the reduction of nitrite toxicity to fish, since it contains chlorides in its composition (Zadinelo et al. 2018), and it is equally effective for the biological filter with bioballs in maintaining nitrite concentrations.

### 3.7 Nitrate

In phase I, in both treatments, the average concentrations of nitrate in the water remained between 0 and

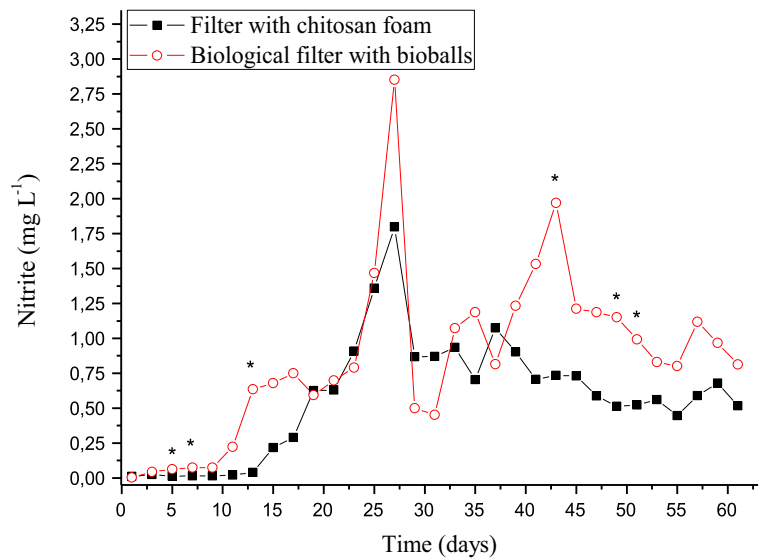
$15 \text{ mg L}^{-1}$ ; however, in phase II, the concentrations in the water of the tanks with biological filter with bioballs from the 67th day were increasing and reached  $46.91 \text{ mg L}^{-1}$  on the 73rd day. In contrast, the average concentrations of nitrate in the filter with chitosan foam reached the maximum level of  $28.01 \text{ mg L}^{-1}$  on the 69th day (Fig. 5). Significant differences between treatments ( $p < 0.05$ ) in days 3–9, and 55, 57, 73, and 74 were detected, where the use of the filter with chitosan foam kept lower nitrate concentrations. Significant differences between treatments ( $p < 0.05$ ) in days 3–9, and 55, 57, 73, and 74 were detected, where the use of the filter with chitosan foam kept lower nitrate concentrations.

In trial 2, the average nitrate concentrations in the water of the tanks with bioball biological filter will remain between  $0.10$  and  $69.20 \text{ mg L}^{-1}$  (maximum observed in 47th day), while in the water of the tanks with chitosan foam filter, the concentrations remained between  $0.05$  and  $11.17 \text{ mg L}^{-1}$  (maximum observed in 53th day). Significant differences were observed between treatments on days 3, 17, and 47 (Fig. 6).

Nitrate is relatively non-toxic to tilapia; however, prolonged exposure to high concentrations of nitrate can decrease the immune response and cause mortality (El-Sayed 2006). In the present study, nitrate concentrations did not exceed that recommended by Monsees et al. (2016), which is up to  $500 \text{ mg L}^{-1}$  nitrate-N in the cultivation of juvenile Nile tilapia, to ensure the health and growth of the fish.



**Fig. 4** Average concentrations of nitrite of the tanks with the use of chitosan foam filter and tanks using the biological filter with bioballs, during the 61-day experimental period from trial 2. Asterisks indicate significant difference between treatments on each day by *t* test ( $p \leq 0.05$ )

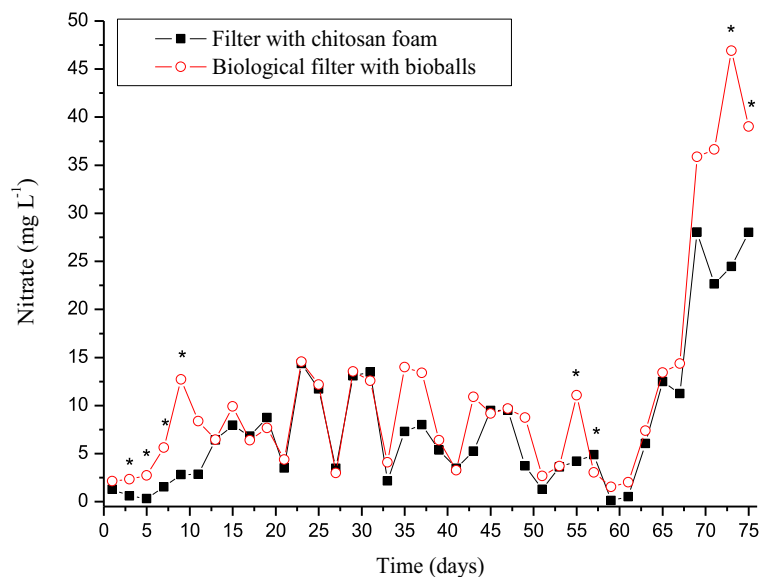


In the cultivation of tilapia in a recirculation system with zero water discharge and without removal of organic matter, it is observed that the sludge is digested biologically and the nitrate is reduced to gaseous nitrogen (El-Sayed 2006). In the present study, there was no removal of water or organic matter during the experimental period. Based on the results of both trials, it was possible to verify that the use of chitosan foam in the filters resulted in lower or equal levels of nitrate, when compared to the use of the biological filter with bioballs.

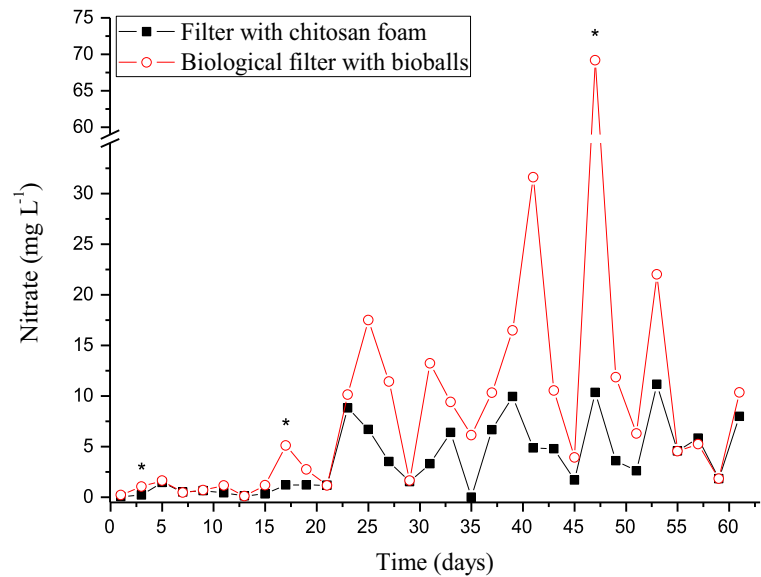
### 3.8 Dissolved Orthophosphate

In trial 1, during phase I, the dissolved orthophosphate was detected in higher concentrations in the water of the tanks that contained biological filters, from the first to the 27th experimental day ( $p < 0.05$ ) and reached maximum levels of  $2.55 \text{ mg L}^{-1}$  on the 23rd day. However, in the water of the tanks that contained the filter with chitosan foam, the maximum amount of orthophosphate dissolved on the same day was detected, where only  $1.95 \text{ mg L}^{-1}$  was detected (Fig. 7). In phase II, orthophosphate was detected in higher concentrations in

**Fig. 5** Average concentrations of nitrate of the tanks with the use of chitosan foam filter and tanks using the biological filter with bioballs, during the 74-day experimental period from trial 1. Asterisks indicate significant difference between treatments on each day by *t* test ( $p \leq 0.05$ )



**Fig. 6** Average concentrations of nitrate of the tanks with the use of chitosan foam filter and tanks using the biological filter with bioballs, during the 61-day experimental period from trial 2. Asterisks indicate significant difference between treatments on each day by *t* test ( $p \leq 0.05$ )



water in tanks that contained biological filters from the 33rd to the 75th experimental day ( $p < 0.05$ ) and reached maximum levels of  $9.97 \text{ mg L}^{-1}$  on the 71st day. However, in the water of the tanks that contained the filter with chitosan foam, a maximum concentration of dissolved orthophosphate of  $6.40 \text{ mg L}^{-1}$  was detected (on the 73rd day (Fig. 7).

In trial 2, significant differences between treatments were observed only on the 25th day, when average concentrations of  $17.50 \text{ mg L}^{-1}$  were measured in the water of tanks with biological filter with bioballs and  $6.69 \text{ mg L}^{-1}$  in the water of tanks with filter with chitosan foam (Fig. 8). After the 30th day of cultivation, the tendency of increasing growth in the concentrations of orthophosphate dissolved in water in both treatments was obtained.

Dissolved orthophosphate is equivalent to soluble reactive inorganic phosphorus. Phosphorus does not seem to be a serious problem with regard to the risk of poisoning, since lethal concentrations are high and they are far from being found in cultivation waters. A safety level for juvenile tilapia exposure is up to  $100 \text{ mg L}^{-1}$  of phosphorus, which represents 10% of the lethal concentration in 24 h. Although high concentrations of phosphorus have no effect on fish survival, the cumulative effect is directly related to the overgrowth of the phytoplankton community and the growth potential of cyanobacteria that produce toxic secondary metabolites that cause disease and even mortality (Cagol et al. 2016).

As in this work, Marengoni et al. (2013) observed that the levels of total phosphorus increase according to the days of cultivation in a recirculation system. This may have been caused by the release of phosphate compounds from the decomposition of organic matter from leftover feed and from the metabolic excretion of fish.

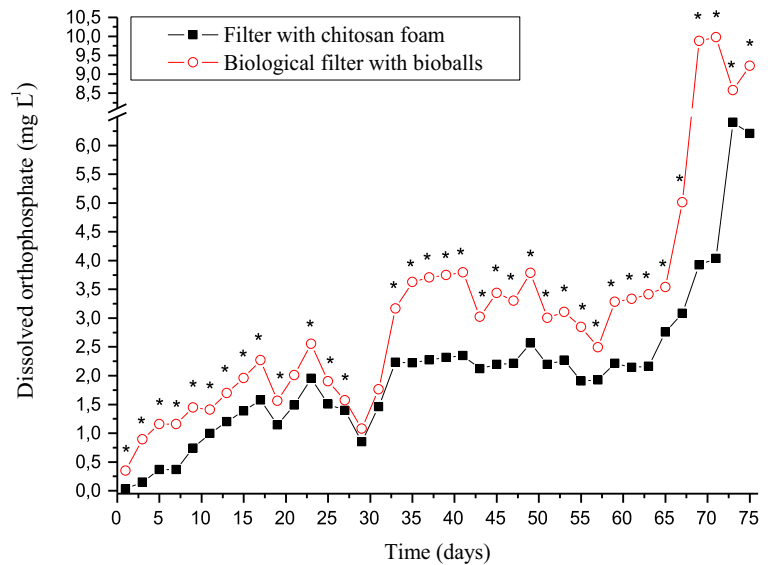
Chitosan foam has a greater affinity for dissolved orthophosphate, followed by nitrite and total ammonia; however, it is able to carry out the adsorption of these pollutants together (Zadinelo et al. 2018). The use of the filter with chitosan foam in the culture tanks kept the average concentrations of dissolved orthophosphate lower or equal throughout the experimental period, when compared to the biological filter with bioballs.

### 3.9 Total Alkalinity

The total alkalinity was higher throughout the experimental period in both phases in the water of the tanks with chitosan foam filters and varied between 45 and  $145 \text{ mg L}^{-1} \text{ CaCO}_3$ . In tanks with bioball biological filters, the alkalinity varied between 9 and  $82 \text{ mg L}^{-1} \text{ CaCO}_3$  (Fig. 9). There were no significant differences between treatments ( $p > 0.05$ ) on days 23, 27, 29, and 51–74.

In trial 2, the total alkalinity was higher during the entire experimental period in both phases in the water of the tanks with chitosan foam filters and varied between 30 and  $147 \text{ mg L}^{-1} \text{ CaCO}_3$ . In the water of tanks with a biological filter with bioballs, the alkalinity varied

**Fig. 7** Average concentrations of dissolved orthophosphate of the tanks with the use of chitosan foam filter and tanks using the biological filter with bioballs, during the 74-day experimental period from trial 1. Asterisks indicate significant difference between treatments on each day by *t* test ( $p \leq 0.05$ )



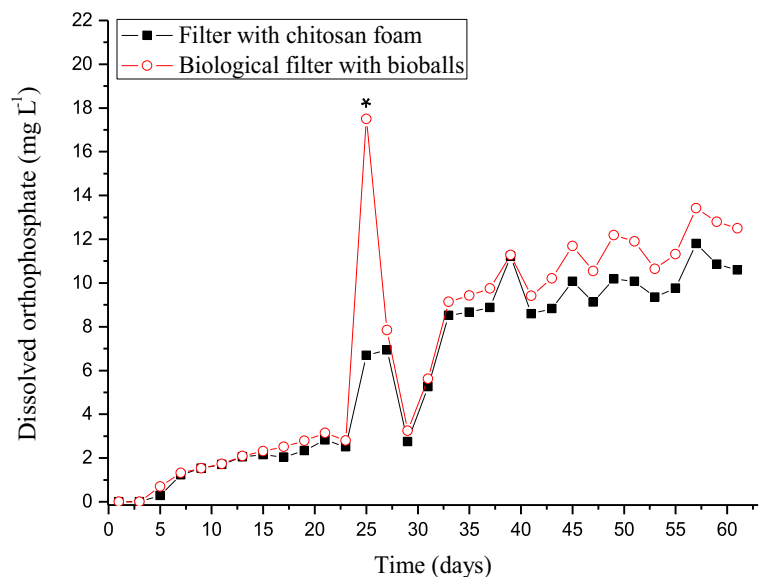
between 30 and 127 mg L<sup>-1</sup> CaCO<sub>3</sub> and significant differences were observed between treatments on days 11, 15, 17, 19, 21, and 29 (Fig. 10).

The alkalinity in the water of fish farming ponds for human consumption must be 60 mg L<sup>-1</sup> or higher (Boyd et al. 2016). The formation of hydrogen ions occurs during the conversion of ammonia to nitrate by the action of nitrifying bacteria, and in the buffering process of these ions, there is consumption of alkalinity (in the form of calcium carbonate); consequently, there is a reduction in both alkalinity and pH. In a semi-

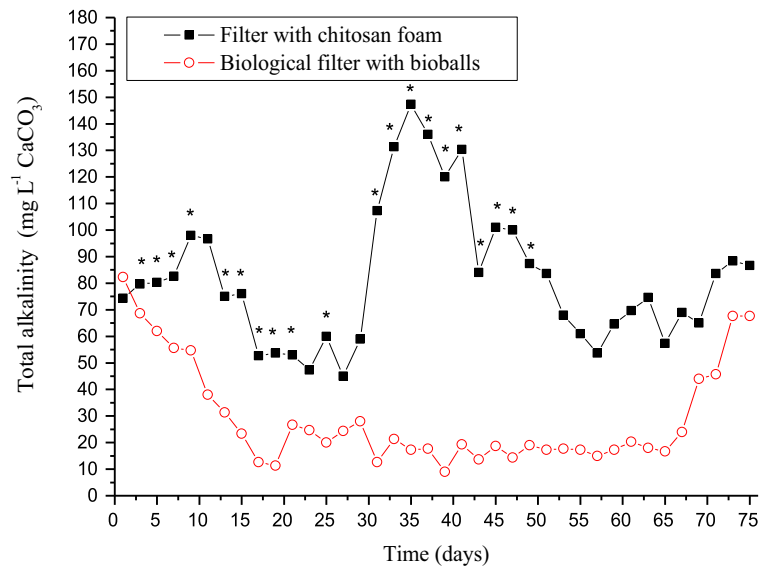
commercial cultivation system for Atlantic salmon, with water recirculation, it was found that the increase in alkalinity leads to lower concentrations of total ammoniacal nitrogen, but does not affect the removal of nitrite or CO<sub>2</sub> (Summerfelt et al. 2015).

In this study, in both treatments, the alkalinity was lower than that recommended in some experimental days. However, both for the well-being of fish and to maintain water quality parameters, it is interesting to use chitosan foam in filters, which maintains alkalinity at higher levels than the biological filter.

**Fig. 8** Average concentrations of dissolved orthophosphate of the tanks with the use of chitosan foam filter and tanks using the biological filter with bioballs, during the 61-day experimental period from trial 2. Asterisks indicate significant difference between treatments on each day by *t* test ( $p \leq 0.05$ )



**Fig. 9** Average concentrations of total alkalinity of the tanks with the use of chitosan foam filter and tanks using the biological filter with bioballs, during the 74-day experimental period from trial 1. Asterisks indicate significant difference between treatments on each day by *t* test ( $p \leq 0.05$ )



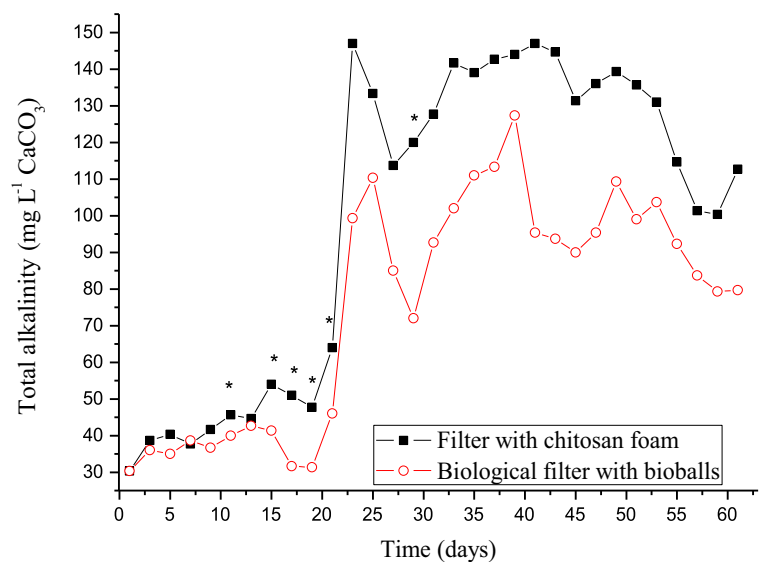
### 3.10 Total Hardness

The total hardness of the water showed an increasing trend throughout the experimental period in both phases, for both treatments, but the values were higher in the tanks with bioball biological filters, which reached 264 mg L<sup>-1</sup> CaCO<sub>3</sub> on the 59th day. In the water of the tanks with a filter with chitosan foam, the maximum concentration of 213 mg L<sup>-1</sup> CaCO<sub>3</sub> was reached on the 71st day (Fig. 11). Significant differences were observed between treatments ( $p < 0.05$ ) on days 21, 27, and 33–69, where the highest concentrations of total

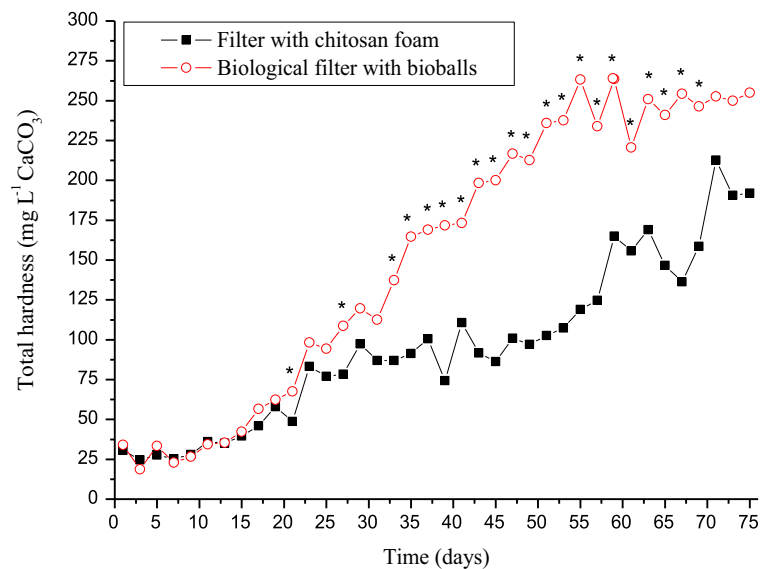
hardness were in the water of tanks with bioballs biological filters.

In trial 2, the total hardness in water showed an increasing trend throughout the experimental period in both phases, for both treatments, but the values were higher in the tanks with biological filters with bioballs and reached 154 mg L<sup>-1</sup> CaCO<sub>3</sub> on the 59th day. The maximum concentration reached 137 mg L<sup>-1</sup> CaCO<sub>3</sub> on the 59th day, in tanks with chitosan foam filters (Fig. 12). Significant differences were observed between treatments on days 13, 17, 19, 23, 25, and 29 (Fig. 12).

**Fig. 10** Average concentrations of total alkalinity of the tanks with the use of chitosan foam filter and tanks using the biological filter with bioballs, during the 61-day experimental period from trial 2. Asterisks indicate significant difference between treatments on each day by *t* test ( $p \leq 0.05$ )



**Fig. 11** Average concentrations of total hardness of the tanks with the use of chitosan foam filter and tanks using the biological filter with bioballs, during the 74-day experimental period from trial 1. Asterisks indicate significant difference between treatments on each day by *t* test ( $p \leq 0.05$ )



The water hardness of freshwater ponds should be  $40 \text{ mg L}^{-1}$  or more (Boyd et al. 2016). In trial 1, from the 15th day, and in trial 2, from the 31st day, the total hardness concentrations were suitable for cultivation in both treatments.

### 3.11 Electrical Conductivity

The electrical conductivity of the water showed a predominantly increasing trend during the two experimental phases. Maximum values of  $2447 \text{ } \mu\text{S cm}^{-1}$  were measured in tanks with filter with chitosan foam and  $3220 \text{ } \mu\text{S cm}^{-1}$  in tanks with bioball biological filter on the 74th day (Fig. 13). The electrical conductivity was significantly lower ( $p < 0.05$ ) in the water of the tanks with the use of the chitosan foam filter on days 36–41 and 49–74.

In trial 2, the electrical conductivity of the water showed a predominantly increasing trend during the two experimental phases. Maximum values of  $490 \text{ } \mu\text{S cm}^{-1}$  were measured in tanks with filter with chitosan foam and  $709 \text{ } \mu\text{S cm}^{-1}$  in tanks with bioball biological filter on the 74th day. Significant differences were observed between treatments on days 27–41, and 45–55 (Fig. 14).

The electrical conductivity indicates the number of ions in the water. The higher the ionic concentration, the greater the water's capacity to conduct electricity. High conductivity values show high rates of decomposition, and a consequent increase in the availability of nutrients

in the aquatic environment, which help to verify the incidence of water pollution (Moreira et al. 2001).

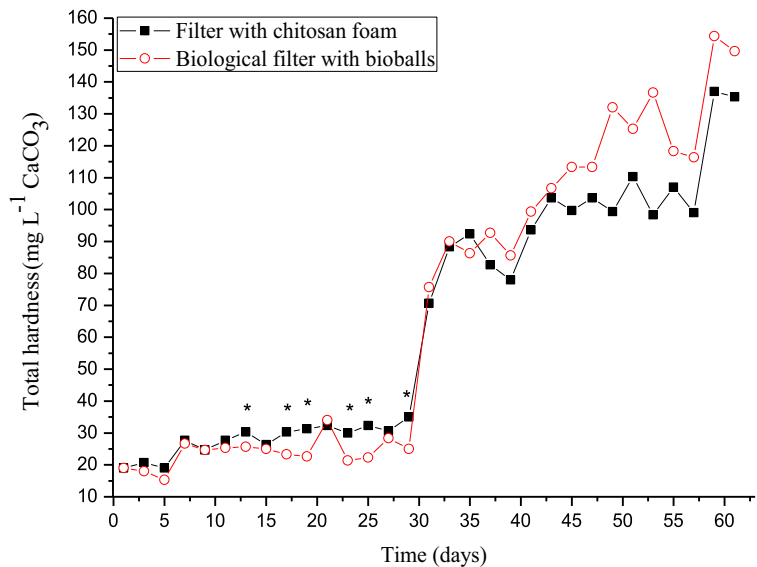
The increase in conductivity levels may be associated with the increase of organic matter in the water from the excretion of fish and the rest of uneaten feed, and contributes to the accumulation of ions in the cultivation environment (Silva et al. 2013b). The chitosan foam seems to have the ability to adsorb part of the polluting ions dissolved in the water, since it resulted in lower conductivity values.

### 3.12 Turbidity

In trial 2, from the 1st to the 51st day, the turbidity of the water was greater in the tanks with chitosan foam filters, and from then on, the turbidity was greater in the water of the tanks with bioball biological filters. The maximum values of turbidity observed were  $135.6 \text{ NTU}$ , on the 41st day, in the water of the tanks with chitosan foam filters, and  $80.6 \text{ NTU}$ , on the 57th day, in the water of the tanks with bioball biological filters. Significant differences were observed between treatments on days 41 and 43 (Fig. 15).

Water turbidity in fish ponds is associated with the presence of suspended particles (Sipaúba-Tavares 1995). The accumulation of organic matter increases the turbidity of the water, increases the biochemical oxygen demand, and causes a reduction in the levels of dissolved oxygen (Sandre et al. 2009).

**Fig. 12** Average concentrations of total hardness of the tanks with the use of chitosan foam filter and tanks using the biological filter with bioballs, during the 61-day experimental period from trial 2. Asterisks indicate significant difference between treatments on each day by *t* test ( $p \leq 0.05$ )



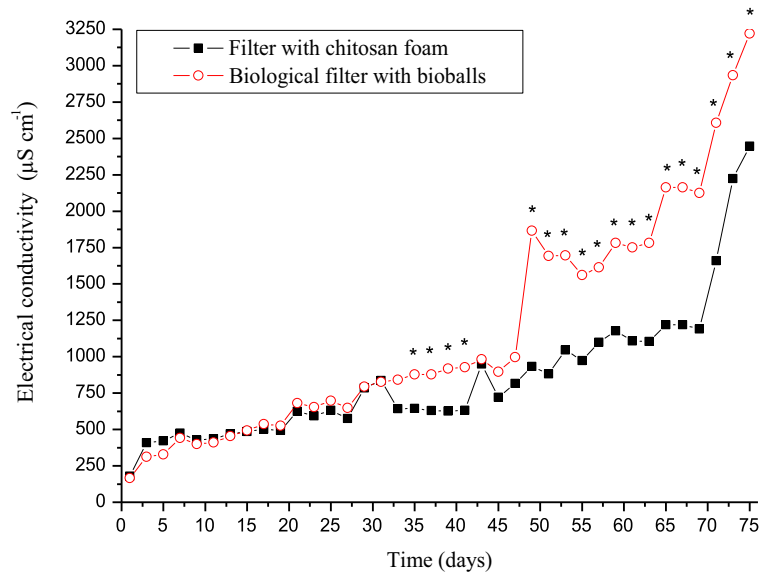
At the end of both experimental periods, it was observed that the foam placed inside the filter had partially degraded, what was already expected, since the chitosan foam is biodegradable (Zadinelo et al. 2018).

Chitosan foam can act as a pollutant adsorbent (Zadinelo et al. 2018), but it can also be a substrate for fixing nitrifying bacteria, and play the role of biofiltration, as according to Lertsutthiwong et al. (2013), chitosan is a promising biopolymer that can be used as an alternative biofilter for fixing nitrite oxidizing bacteria, capable of removing up to  $0.82 \pm 0.05$  mg-N/(g day). Such data corroborate with those of Chung

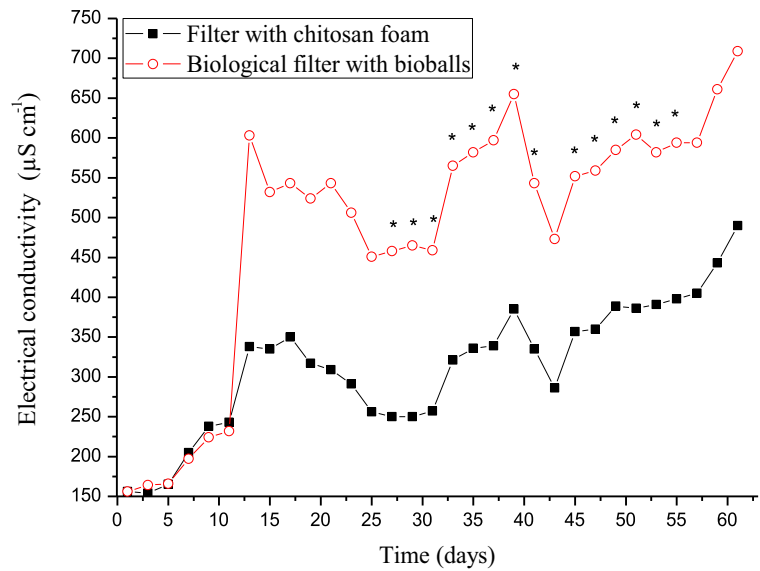
(2006), who also states that chitosan does not affect nitrifying bacteria, but presents selective bacterial removal; therefore, it is effective against *Edwardsiella ictaluri*.

According to the results of total ammonia, nitrite, and dissolved orthophosphate during the experimental period of trials 1 and 2, it was observed that the foam replacement at 30 days of cultivation resulted in improvement and maintenance of water quality. However, when there was no change at 60 days, the beginning of the deterioration of water quality was observed; that is, the foam became saturated in 30 days.

**Fig. 13** Average concentrations of electrical conductivity of the tanks with the use of chitosan foam filter and tanks using the biological filter with bioballs, during the 74-day experimental period from trial 1. Asterisks indicate significant difference between treatments on each day by *t* test ( $p \leq 0.05$ )



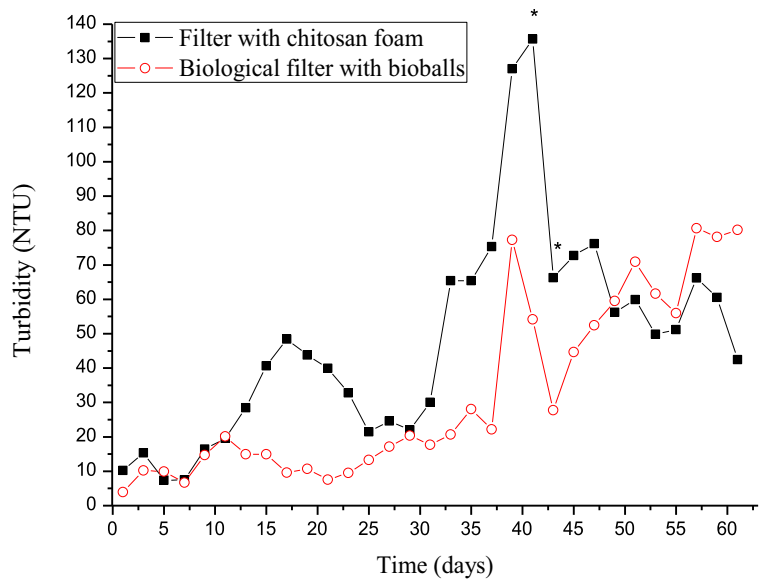
**Fig. 14** Average concentrations of electrical conductivity of the tanks with the use of chitosan foam filter and tanks using the biological filter with bioballs, during the 61-day experimental period from trial 2. Asterisks indicate significant difference between treatments on each day by *t* test ( $p \leq 0.05$ )



Based on the results of water quality and zootechnical performance in both phases, it is concluded that the use of water recirculation systems with treatment using filters filled with chitosan foam during the cultivation of Nile tilapia is equally efficient or better than using a biological filter with bioballs.

The use of filters filled with chitosan foam allows the physical, chemical, and biological treatments of aquaculture effluents, since chitosan foam can serve as a support for nitrifying bacteria and eliminate pathogens, in addition to exhibiting excellent adsorptive capabilities (physical and chemical) pollutants from aquaculture.

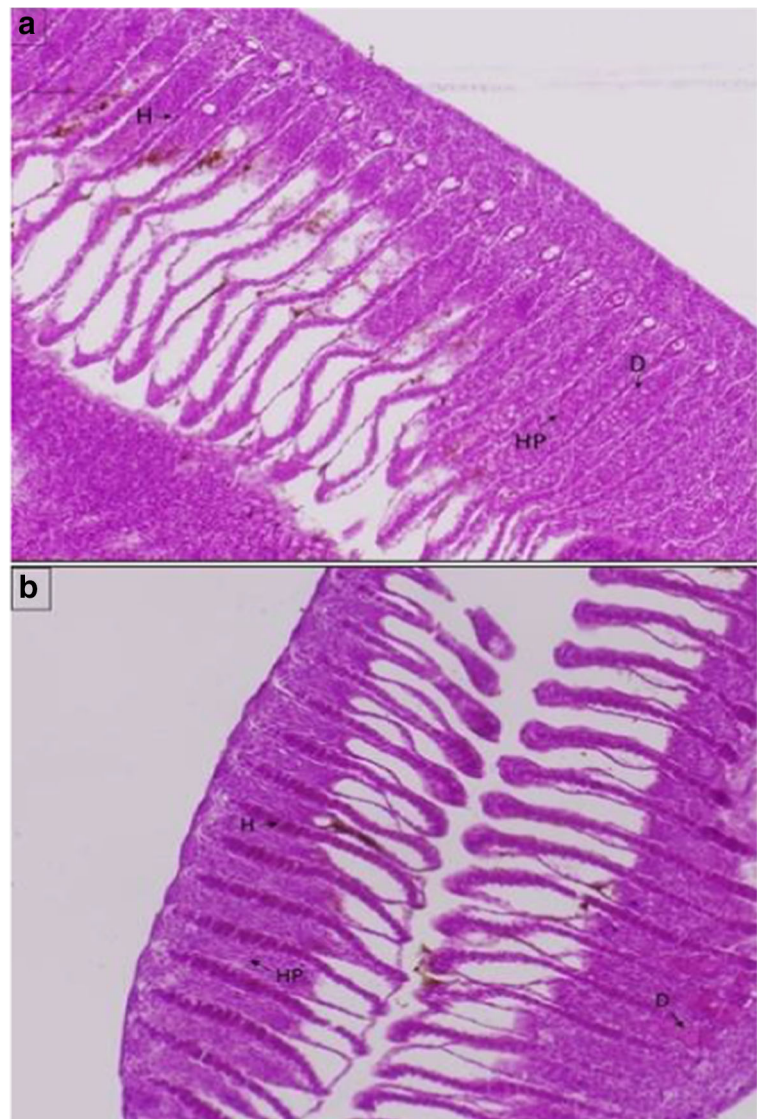
**Fig. 15** Average concentrations of turbidity of the tanks with the use of chitosan foam filter and tanks using the biological filter with bioballs, during the 61-day experimental period from trial 2. Asterisks indicate significant difference between treatments on each day by *t* test ( $p \leq 0.05$ )



### 3.13 Hepatic and Branchial Histopathology

The same histopathological changes (Fig. 16) were observed in the Nile tilapia gills of both treatments (tanks with chitosan foam and tanks with bioball biological filters). There was mild hyperemia for the pattern of reaction of circulatory disorders. However, for the reaction pattern of regressive changes in the epithelium, structural changes in plasma and deposit, of mild occurrence, there were no changes obtained in the supporting tissue. There was moderate hypertrophy and hyperplasia for the reaction pattern of progressive changes in the

**Fig. 16** Histopathology of Nile tilapia fingerlings gills, grown in tanks with chitosan foam filters (A—a 10× increase) and in tanks with bioball biological filters (B—a 10× increase). H, hyperemia; HP, hyperplasia; D, deposit/plasma/cells with hypertrophy



epithelium, but there were no changes in the supporting tissue. Margin and infiltration were observed for the inflammation reaction pattern, but no changes in the tumor reaction patterns were observed.

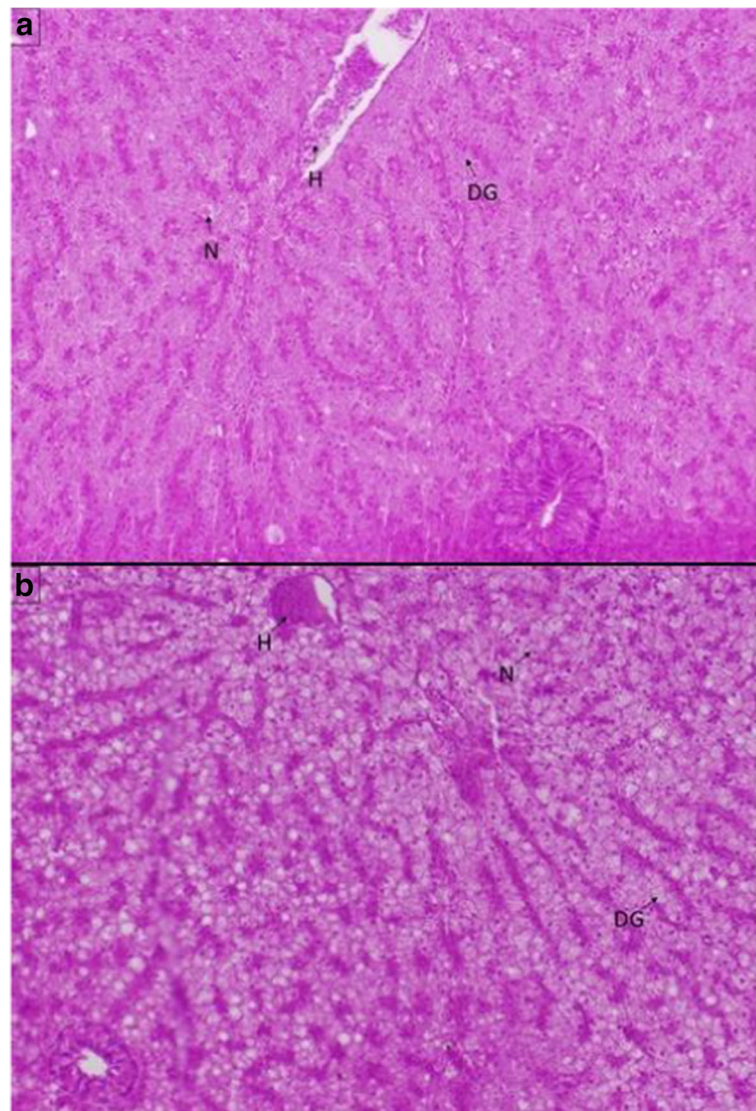
Reis et al. (2009) observed that the continuous decline in temperature, pH, and dissolved oxygen concentration in the water of Nile tilapia intensive culture tanks causes a progressive increase in morphological changes in the branchial epithelium (interlamellar hyperplasia, lamellar fusion, epithelial detachment, and telangiectasias), due to less favorable conditions. In this work, in both treatments (tanks with chitosan foam filters and tanks with bioball biological filters) and trials (1 and 2), a decline in the concentration of dissolved oxygen was

observed throughout the experimental period, which may have resulted in changes in the gills.

Garcia-Santos et al. (2007) verified the histopathological effects of cadmium ( $25 \text{ mg L}^{-1}$  of  $\text{CdCl}_2$ ) on the gills of Nile tilapia by optical microscopy and found that fish exposed to cadmium showed alterations such as fusion in the lamellae due to hyperplasia and epithelial hypertrophy, rupture of the system of pillar cells, aneurysms, and necrosis. Such histological changes function as defense mechanisms because they reduce the vulnerable surface area of the gill and/or increase the diffusion barrier to the pollutant, making it difficult for the pollutant to access the blood, and therefore, the performance of gas exchanges is impaired. In this work, in both treatments and trials, the



**Fig. 17** Histopathology of hepatopancreas of Nile tilapia fingerlings grown in tanks with chitosan foam filters (A—a 10× increase) and in tanks with bioball biological filters (B—a 20× increase). FD, fatty degeneration/hypertrophic cells; H, hyperemia; N, necrosis



alterations verified may have been due to the defense mechanism against the compounds that accumulated in the cultivation water (because no water is exchanged), by the diet, among others.

The same histopathological changes (Fig. 17) were seen in the Nile tilapia hepatopancreas of both

treatments (tanks with chitosan foam and tanks with bioball biological filters). For the pattern of reaction of circulatory disorders, mild hyperemia was observed. In the reaction pattern of regressive changes in liver tissue, structural changes, plasma changes, deposits, nuclear changes, and mildly occurring

**Table 2** Organ indexes (Iorg) of gills and hepatopancreas of Nile tilapia fingerlings after cultivation in tanks containing chitosan foam filter and in tanks containing biological filter with bioballs

Treatments	Gills organ index (Iorg) ( $n = 9$ )	Hepatopancreas organ index (Iorg) ( $n = 9$ )
Filter with chitosan foam	$9.33 \pm 0.66$	$6.66 \pm 1.20$
Biological filter with bioballs	$9.66 \pm 0.33$	$7.00 \pm 1.66$

necrosis were observed; in hepatic interstitial tissue, mild structural changes were noted, and in the bile duct, no changes were noted. There were no changes in the reaction pattern of progressive changes in liver tissue, in hepatic interstitial tissue, or in the bile duct. Margin and infiltration of mildly occurring leukocytes were observed in the inflammation reaction pattern; however, there were no changes in the reaction patterns of tumor presence.

In this work, leukocyte infiltration was observed, which, according to Bernet et al. (1999), it has functions related to the neutralization and destruction of the aggressor source; therefore, the leukocyte infiltration cleanses the tissue, removes the aggressor agent and dead cells, and induces the recovery of the injured tissue.

Marchand et al. (2012) analyzed histopathological changes in livers of two indicator species, *Clarias gariepinus* and *Oreochromis mossambicus*, resulting from hypertrophic waters, and they found focal necrosis, nuclear changes (hypertrophy and pycnosis), and infiltration of inflammatory cells in hepatocytes, in addition to vacuolar changes. Liebel et al. (2013) used specimens of *Astyanax fasciatus* and *Oreochromis niloticus* to assess the anthropogenic impact on water quality in two lakes. Two main lesions were found in the liver of both species, the presence of leukocyte infiltration and areas of necrosis. In the present study, the changes observed in both treatments and trials may have been the result of accumulated pollutants in the cultivation water (due to the lack of water exchange), or other factors, such as accumulation of microorganisms in the cultivation water or by the diet provided (Rocha et al. 2010).

There were no significant differences between treatments ( $p > 0.05$ ) between the organ indexes (Iorg) of the gills and hepatopancreas of Nile tilapia fingerlings after cultivation in tanks that contained a filter with chitosan foam and in tanks that contained the biological filter with bioballs (Table 2).

Inadequate water quality parameters can be identified as the main reason for the appearance of histopathological changes. The application of the Bernet index for organs (Iorg), associated with image analysis, allows a more accurate assessment of the morphological effects caused by pollutants (Liebel et al. 2013).

Marchand et al. (2012) found values that are in agreement with the one obtained in the present study (Table 2) for *Oreochromis mossambicus*, from hypertrophic waters Iorg index of the liver of  $12.89 \pm 5.83$ , and of the gills of  $10.89 \pm 3.95$ . Rašković et al.

(2013) also found similar values to this work in the common carp organs, coming from ponds with water supply from a stream and from a well. For gills, the mean indexes were 13 and 7, and for livers 8 and 6, for fish from ponds with water from a stream and a well, respectively.

#### 4 Conclusion

The use of chitosan foam as a filter element in water recirculation systems in aquaculture maintains an adequate water quality that is equivalent to the use of biological filters with bioballs. In addition, the use of chitosan foam does not affect zootechnical performance and neither the hepatic nor branchial histology of Nile tilapia fingerlings. When using chitosan foam, it is recommended to change the foam every 30 days of cultivation to ensure the health and well-being of the fish.

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#### Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

**Ethics Approval** The experimental procedures were conducted according to the Ethical Principles of Animal Experimentation adopted by the National Council for the Control of Animal Experimentation (CONCEA), according to protocol no. 10/2018 approved by the Ethics Committee on the Use of Animals of the Palotina Sector of UFPR (CEUA/Palotina).

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