



# Fostering bacterial growth in BFT aquaculture tanks by early Nile tilapia stocking

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**ABSTRACT.** The current study aimed at fostering bacterial growth in BFT aquaculture tanks by early Nile tilapia stocking. Control tanks had no tilapia but received daily applications of dry molasses (NT+) or had tilapia but no C:N ratio adjustment (T-). Experimental tanks had tilapia and received daily application of molasses to adjust the C:N ratio of water to 15:1 (T+). The development of bioflocs in NT+ was insignificant as demonstrated by low levels of settleable solids (SS) and total suspended solids (TSS). Total ammonia nitrogen (TAN) was significantly higher in NT+ than in T+. In the C:N-ratio adjusted tanks, the presence of fish shortened the control of TAN in several days. As nitrite declined in T+, it increased in NT+. The final concentrations of TSS in T+, T- and NT+ were  $236 \pm 29 \text{ mg L}^{-1}$ ,  $100 \pm 32 \text{ mg L}^{-1}$ , and  $40 \pm 22 \text{ mg L}^{-1}$ , respectively ( $p < 0.05$ ). It can be concluded that stable nitrifying and heterotrophic bacterial communities could be attained in BFT aquaculture tanks, before the end of the 4<sup>th</sup> week of culture, if the bacterial growth is fostered by early Nile tilapia stocking.

**Keywords:** biofloc maturation; ammonia; nitrite; total suspended solids; water quality.

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## Introduction

In green-water tanks, absorption by phytoplankton is an important sink of ammonia from water (Ray & Lotz, 2014). The rate of ammonia absorption by phytoplankton, however, may vary significantly because microalgae metabolism is highly influenced by environmental factors, especially aquatic luminosity (Ebeling, Timmons, & Bisogni, 2006). Therefore, green-water tanks are chemically unstable over time, with relevant circadian variations in total ammonia nitrogen (TAN),  $O_2$ , and pH (Hargreaves, 2006). Contrarily, bacterial absorption is the main sink of ammonia in BFT (bioflocs) tanks (Xu, Morris, & Samocha, 2016). They are chemically more stable than green-water tanks because heterotrophic bacteria are less sensitive to luminosity than phytoplankton.

A complex microbiota develops inside outdoor BFT tanks mainly composed of heterotrophic and autotrophic bacteria, and microalgae (Xu et al., 2016). In indoor BFT tanks, on the other hand, the main microbiological groups are bacteria, existing little or even no phytoplankton. Regardless of the BFT system, whether outdoor or indoor, the role of heterotrophic and nitrifying bacteria on ammonia and nitrite uptake is very important (Azim & Little, 2008). Differently, bacterial nitrification is the main sink of ammonia in clear-water recirculating aquaculture system (RAS) tanks. However, the capacity of bioflocs to remove ammonia is several times greater than for nitrifiers (Schveitzer et al., 2013). The C:N ratio of water is adjusted upward to promote the growth of heterotrophic bacteria (Avnimelech, 1999; Ebeling et al., 2006), which prevail over the nitrifying bacterial community.

The development of bioflocs in aquaculture tanks demands some time until a steady state of maturity is reached (Xu et al., 2016). Frequently, a reliable BFT system is only attained 30 days after the first applications of organic carbon in water (Bakar et al., 2015). Consequently, dangerously high peaks of TAN and nitrite usually occur in the first weeks of the BFT culture (Bakshi, Najdegerami, Manaffar, Tukmechi, & Farah, 2018; Ren, Li, Dong, Tian, & Xue, 2019; Luo et al., 2014). Ren et al. (2019) observed peaks of TAN over the first two culture weeks, and high nitrite up to the end of the 7<sup>th</sup> week.

Some strategies have been proposed to shorten the delay period required for bioflocs establishment or maturation in BFT tanks. The most employed method on that regard is the inoculation of a certain volume of mature bioflocs some time before the initial stocking of fish or shrimp (Krummenauer et al., 2012; Serra,

Gaona, Furtado, Poersch, & Wasielesky, 2015; Gaona, Poersch, Krummenauer, Foes, & Wasielesky, 2011). Malpartida-Pasco et al. (2018) obtained early control of TAN in Nile tilapia BFT tanks, which previously received mature bioflocs at 45% of the tank volume. Similarly, Krummenauer, Samocha, Poersch, and Wasielesky Jr (2014) observed that the use of bioflocs-rich water as a starting inoculum speed up bioflocs development in *Litopenaeus vannamei* culture tanks. Nevertheless, it is not also uncommon to observe TAN and nitrite spikes even in tanks that were previously inoculated with mature bioflocs (Souza, Cardozo, Wasielesky Jr, & Abreu, 2019).

One alternative to establish bacteria in indoor aquaculture BFT tanks without much delay was the one proposed by Tierney and Ray (2018). Those researchers reared Nile tilapia, *Oreochromis niloticus* juveniles for 23 days in *Litopenaeus vannamei* BFT culture tanks before shrimp stocking. Tilapia is a suitable fish to be used as a bacterial fosterer due to its sturdiness, euryhaline condition and availability (Wang & Lu, 2016). Based mainly on TAN results, however, Tierney and Ray (2018) stated that “the bioflocs treatment may not have benefited as much from the bacterial-establishment period as the other treatments (clear-water and hybrid)”. Therefore, some research still needs to be carried out to obtain stable nitrifying and heterotrophic bacterial communities in BFT aquaculture tanks before the end of the 4<sup>th</sup> week of culture. The present work aimed at fostering the bacterial growth in BFT aquaculture tanks by early Nile tilapia stocking. The degree of bacterial growth was evaluated by monitoring selected water quality variables.

## Material and methods

The experimental system consisted of fifteen 100-L round polyethylene tanks provided with constant aeration from a 2.5 hp blower and filled with freshwater. No water exchange was carried out over the entire experimental period. New water was added only to compensate for evaporation and restore the initial level. There were two controls and one experimental group, each one with five repetitions (Table 1). Control tanks had no tilapia but received daily applications of dry molasses (NT+) or had tilapia but no C:N ratio adjustment (T-). Experimental tanks had tilapia and received daily application of molasses. The C:N ratio of water was adjusted to 15:1 following the procedures of Avnimelech (1999).

**Table 1.** Experimental design.

Early tilapia stocking	C:N ratio adjustment	
	No	Yes
No	-	NT+ <sup>1</sup>
Yes	T- <sup>1</sup>	T+

<sup>1</sup>Control tanks.

Tanks with Nile tilapia were stocked with 15 fish per tank (initial body weight =  $3.0 \pm 0.08$  g). Fish were daily fed a commercial powdered diet for tropical fish (45% CP, Aquamix PL-0, Integral Mix, Brazil), at feeding rates prescribed by the laboratory's table. Fortnightly, daily allowances of artificial diet and dry molasses were corrected after fish weighing. Tanks without fish (NT+) received the same daily amounts of diet and molasses as T+. When necessary, sodium bicarbonate was applied in water to maintain its total alkalinity at 60 mg L<sup>-1</sup> or above.

Water quality variables were monitored as follows: pH (pH-meter mPA210, Tecnoyon, Piracicaba, São Paulo State, Brazil) and dissolved oxygen (YSI O<sub>2</sub>-meter model 55, Yellow Springs, OH, USA), daily at 0900; total ammonia nitrogen (TAN, indophenol method) and nitrite (sulfanilamide method), three times a week; total alkalinity (titration with a standard H<sub>2</sub>SO<sub>4</sub> solution), weekly. These variables were determined according to Clesceri, Greenberg, and Eaton (1998). Furthermore, the concentrations of total suspended solids (TSS) and settleable solids (SS) were determined twice and three times a week, respectively, according to Boyd and Tucker (1992).

Water quality results were analyzed by the Student's t-test and one-way ANOVA for completely randomized experiments. Pairs of means were compared by Tukey's test when significant differences were detected ( $p < 0.05$ ). Statistical analyses were carried out with the aid of the SigmaPlot for Windows software (version 12.0, Systat Software, Inc.).

## Results and discussion

In all tanks, O<sub>2</sub> in water was lower at the end of the experimental period ( $p < 0.05$ ). No significant differences were detected for O<sub>2</sub> between the tanks with and without fish (Table 2).

Tanks without fish but with applications of dry molasses (NT+) had higher final pH and total alkalinity (TA) compared with their respective initial values. In tanks with fish but no adjustment of the C:N ratio (T-), values of pH and TA of water were reduced over time. Finally, only TA increased over time in tanks with fish and applications of molasses (T+); their pH remained nearly constant (Table 2).

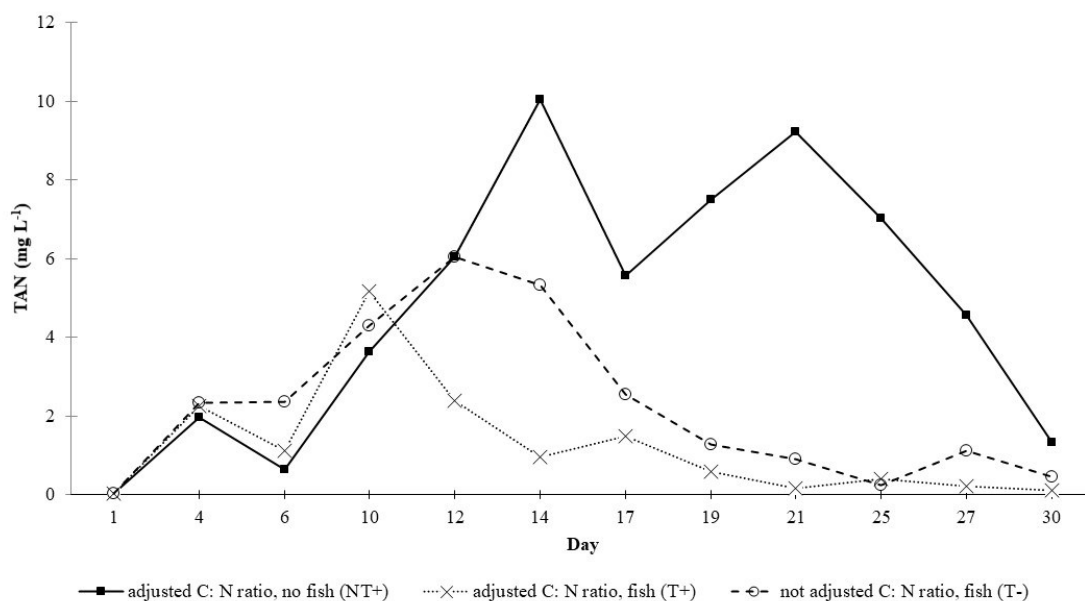
**Table 2.** pH, dissolved O<sub>2</sub> (mg L<sup>-1</sup>) and total alkalinity (TA; mg L<sup>-1</sup> CaCO<sub>3</sub>) of experimental indoor tanks stocked or not with Nile tilapia fingerlings, and whose C:N ratio was adjusted or not to 15:1 (mean ± S.D.; n = 5).

Variable	Time	C:N ratio adjustment	Early tilapia stocking	
			No	Yes
O <sub>2</sub>	Start	No	-	7.51 ± 0.01*
		Yes	7.53 ± 0.05* <sup>2</sup>	7.53 ± 0.06*
	End <sup>1</sup>	No	-	6.53 ± 0.10
		Yes	6.23 ± 0.33	6.47 ± 0.13
pH	Start	No	-	7.99 ± 0.11*
		Yes	7.96 ± 0.08*	7.94 ± 0.11
	End	No	-	7.09 ± 0.09 Ab <sup>3</sup>
		Yes	8.25 ± 0.09 a	8.03 ± 0.11 Bb
TA	Start	No	-	82.8 ± 1.1*
		Yes	84.0 ± 1.8*	83.0 ± 0.9*
	End	No	-	60.9 ± 2.8 Ab
		Yes	178.1 ± 6.5 a	120.3 ± 9.3 Bb

<sup>1</sup>After 30 days; <sup>2</sup>For the same variable and treatment, between different times, means with an asterisk are significantly different by paired *t*-test ( $p < 0.05$ ). For example, the difference between 7.53 ± 0.05 mg O<sub>2</sub> L<sup>-1</sup> and 6.23 ± 0.33 mg O<sub>2</sub> L<sup>-1</sup> is significant at 5%; <sup>3</sup>For the same variable and time, means with distinct lowercase and uppercase letters in the row and column, respectively, are significantly different by Student's *t*-test ( $p > 0.05$ ). Absence of letters indicates non-significant difference at 5%.

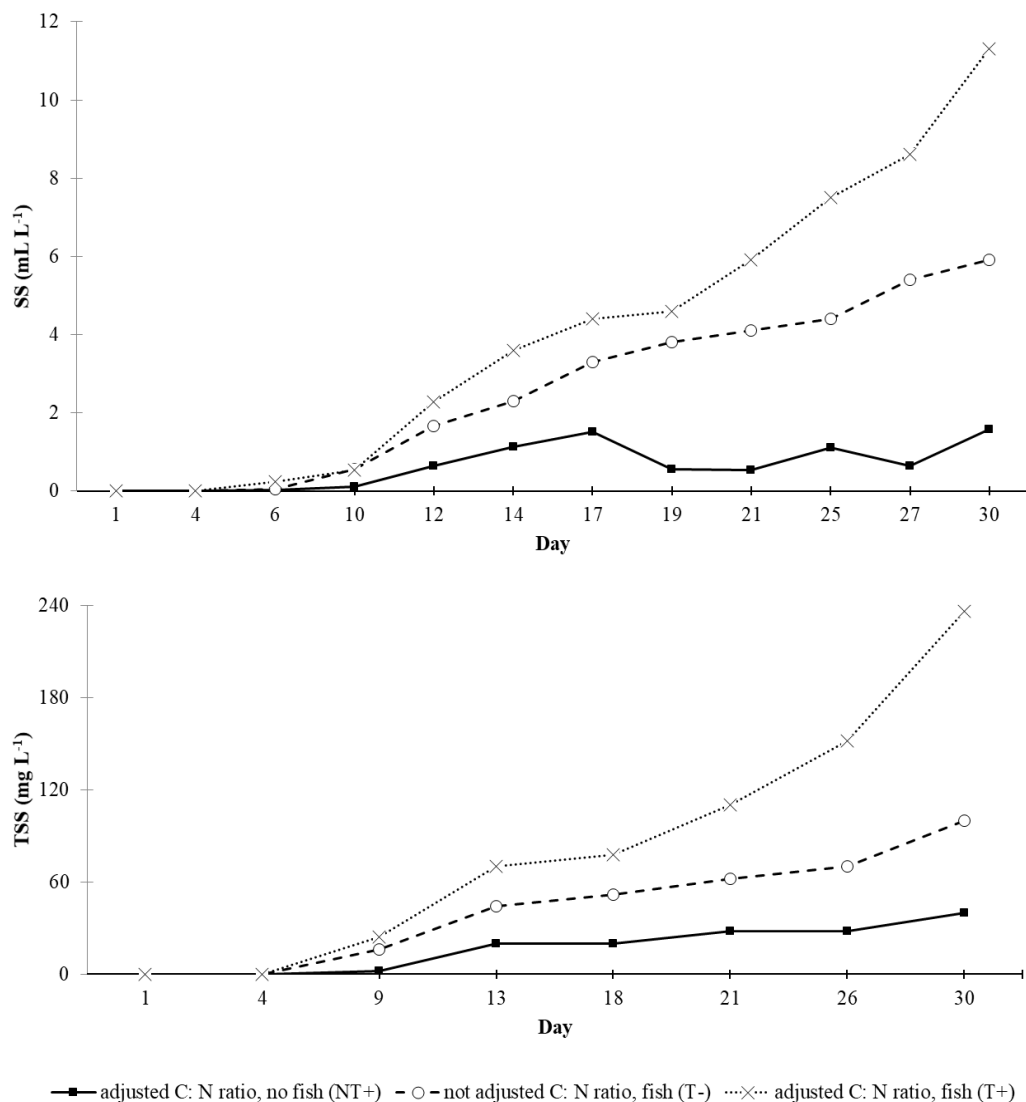
Rezende et al. (2018) have also observed increases in TA after applications of dry molasses in culture tanks. Commercial dry molasses is a blend of sugarcane molasses, limestone and some additives. Therefore, the application of commercial dry molasses in water increases simultaneously its C:N ratio and TA. While the upward adjustment of the C:N ratio of water is especially important for heterotrophic bacteria, the increase in TA is particularly beneficial for autotrophic bacteria. On the other hand, applications of dry molasses increase the BOD of the water. Pérez-Fuentes, Hernández-Vergara, Pérez-Rostro, and Fogel (2016) observed a O<sub>2</sub> decline from 3.2 to 1.5 mg L<sup>-1</sup> soon after the application of dry molasses to water. Hence, only well-aerated aquaculture tanks should receive regular applications of dry molasses.

In all tanks, TAN declined after an initial increase but at different patterns (Figure 1).



**Figure 1.** Concentration of total ammonia nitrogen (TAN) in water of indoor tanks stocked or not with Nile tilapia fingerlings and whose C:N ratio was adjusted or not to 15:1 (n = 5). A. In each time, the differences between means are significant by Student's *t*-test ( $p < 0.05$ ), except at days 1, 4, 6, and 10; B. In each time, differences between means are significant by Student's *t*-test ( $p < 0.05$ ), except at days 1, 4, 10, and 25.

NT+ tanks had increasing levels of TAN up to the 14<sup>th</sup> day, when it was reached a concentration of  $10 \pm 2$  mg L<sup>-1</sup>. Next, TAN decreased progressively in NT+ tanks, which exhibited  $1.34 \pm 0.3$  mg L<sup>-1</sup> at the end. Biofloc development in NT+ tanks was insignificant as demonstrated by their low levels of settleable solids (SS) and total suspended solids (TSS; Figure 2).

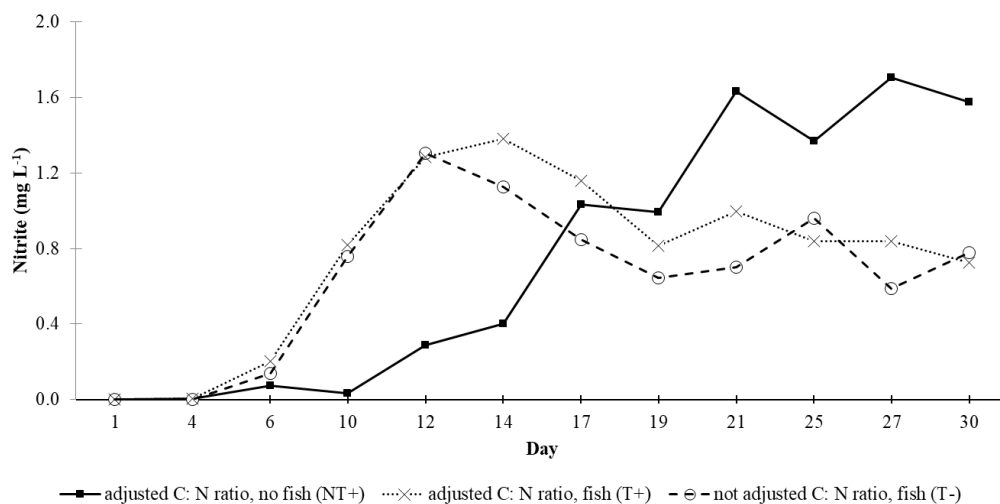


**Figure 2.** Concentrations of settleable solids (SS) and total suspended solids (TSS) in water of indoor tanks stocked or not with Nile tilapia fingerlings and whose C:N ratio was adjusted or not to 15: 1 (n = 5). In each time, differences between the SS means are significant by Tukey's test ( $p < 0.05$ ), except at days 1, 4, 6, and 10. In each time, differences between the TSS means are significant by Tukey's test ( $p < 0.05$ ), except at days 1 and 4.

Therefore, reduction of TAN in NT+ tanks was probably due to the nitrification and volatilization processes. In tanks with fish that received dry molasses (T+), the highest concentration of TAN was observed on the 10<sup>th</sup> experimental day ( $5.16 \pm 1.07$  mg L<sup>-1</sup>). After that, TAN was significantly higher in NT+ tanks than in T+, between days 12 and 30 (Figure 1). Besides, the peak of TAN was higher in NT+ tanks when compared with T+. Therefore, the early tilapia stocking has clearly contributed to reduce the concentrations of TAN concentrations in water. Some of the fish fecal microbiota released in water has probably promoted faster biofloc development in T+ tanks. Accordingly, the best management to foster bacterial growth in BFT aquaculture tanks would be the promotion of the natural bacterial inoculation carried out by fish, such as tilapia, and not the artificial inoculation made from regular applications of phytoplankton (green) or biofloc-rich (brown) waters. While artificial inoculations would be less frequent, the natural bacterial inoculation by fish would be carried out daily, in fact, hourly. Usually, BFT aquaculture tanks are inoculated with biofloc-rich water obtained from older BFT tanks aiming at expediting biofloc maturation (Krummenauer et al., 2014; Serra et al., 2015; Malpartido-Pasco, Carvalho Filho, Espirito Santo, & Vinatea, 2018).

In the C:N-ratio adjusted tanks, the presence of fish shortened the ammonia control in several days. While high peaks of TAN were observed in no-fish tanks even after 3 weeks of molasses applications, the last spike of TAN in tanks stocked with tilapia occurred at day 10. From that day on, TAN concentrations in water remained very low in T+ tanks. Therefore, early tilapia stocking, starting some weeks before the onset of the culture period, has the potential to increase the development of bioflocs in water. Mature bioflocs would be attained in BFT aquaculture tanks if a previous introduction of tilapia is carried out 3 – 4 weeks before the fish or shrimp stockings.

At the 2<sup>nd</sup> experimental week, the concentrations of nitrite were higher in T+ than in NT+ tanks (Figure 3;  $p < 0.05$ ).



**Figure 3.** Concentration of nitrite ( $\text{NO}_2^-$ ) in water of indoor tanks stocked or not with Nile tilapia fingerlings and whose C:N ratio was adjusted or not to 15:1 ( $n = 5$ ). A. In each time, differences between means are significant by Student's  $t$ -test ( $p < 0.05$ ), except at days 1, 4, 17, and 19; B. In each time, the differences between means are significant by the Student's  $t$ -test ( $p < 0.05$ ), except at days 1, 4, 6, 10, 12, 25, and 30.

In the last 10 days, however, nitrite declined in T+, but increased in NT+ tanks. In the first tanks, biofloc development might have negatively affected *Nitrobacter* growth, which could explain their higher levels of nitrite at the beginning. Yet after almost 3 weeks, nitrifying bacteria has probably established in T+ tanks because the concentrations of nitrite reduced significantly. It is desirable to have both types of bacteria in aquaculture BFT tanks, that is, heterotrophic and autotrophic bacteria (Azim & Little, 2008; Zhang, Luo, Tan, Liu, & Hou, 2016; Xu et al., 2016). The adjustment of the C:N ratio of water should be moderate to allow the simultaneous growth of nitrifying and heterotrophic bacteria. It is beneficial to maintain some TAN in water to stimulate the development of *Nitrosomonas* and *Nitrobacter* in tanks. While the control of TAN would be carried out by bioflocs and *Nitrosomonas*, nitrite would be kept low by *Nitrobacter*. This microbial management, however, is not so straightforward because the heterotrophic and autotrophic bacteria might compete with each other for space and nutrients (Xu et al., 2016).

In tanks stocked with fish, concentrations of nitrite increased fast in all units up to the 12<sup>th</sup> day (Figure 3). Thereafter, nitrite decreased in all fish-stocked tanks regardless the adjustment of the C:N ratio of water. Final concentrations of nitrite ( $0.73 \pm 0.12$ ;  $0.78 \pm 0.19 \text{ mg L}^{-1}$  for T- and T+, respectively), have not significantly differed between T- and T+ ( $p > 0.05$ ). Therefore, it seems that the adjustment of the C:N ratio of water by daily applications of molasses has no effect on the concentrations of nitrite in water. Initially, nitrite rises high in stagnant tanks because nitrite-oxidizing bacteria, *Nitrobacter*, grows at a slower rate than ammonia-oxidizing bacteria, *Nitrosomonas* (Hargreaves, 2006). These results suggest the adjustment of the C:N ratio of water is an efficient management to reduce TAN, but not nitrite. Ferreira, Lara, Wasielesky Jr, and Abreu (2016) observed a TAN increase in *L. vannamei* BFT tanks up to the 3<sup>rd</sup> rearing week with a subsequent drop. In that study, nitrite concentrations in water increased uninterruptedly until the end. Again, it would be interesting to stimulate both groups of bacteria in BFT tanks, that is, heterotrophic (bioflocs) and autotrophic (nitrifying bacteria) bacteria to control TAN and nitrite, respectively (Krummenauer et al., 2014; Ferreira et al., 2016).

Concentrations of settleable solids (SS) in water increased continuously in all tanks with fish, regardless the C:N ratio of water. Contrarily, SS remained low over the entire experimental period in NT+ tanks (Figure 2). Therefore, it seems clear that the strategy of stocking tilapia some time before the introduction of the main

cultured species fosters the development of bioflocs in BFT aquaculture tanks. After day 12, SS was significantly higher in T+ tanks. Two weeks are probably the minimum period needed for the establishment of bioflocs in BFT tanks, even in those units receiving early tilapia stocking. Xu et al. (2016) reported significant concentrations of SS and TSS in *L. vannamei* BFT tanks of 30 mg L<sup>-1</sup> and 600 mg L<sup>-1</sup>, respectively, after 2 weeks of culture. Ren et al. (2019) observed 438 mg L<sup>-1</sup> TSS in *L. vannamei* BFT tanks after 14 days. In the present study, the highest TSS was found in T+ tanks followed by T- units (Figure 2). Moreover, the lowest TSS was found in NT+. At the end, the final concentrations of TSS in the T+, T-, and NT+ tanks were 236 ± 29 mg L<sup>-1</sup>, 100 ± 32 mg L<sup>-1</sup>, and 40 ± 22 mg L<sup>-1</sup>, respectively (p < 0.05).

## Conclusion

Stable nitrifying and heterotrophic bacterial communities could be attained in BFT aquaculture tanks, before the end of the 4<sup>th</sup> week of culture, if the bacterial growth is promoted by early Nile tilapia stocking.

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