BIOFLOC TECHNOLOGY:

PRACTICAL ASPECTS OF CARBOHYDRATE SUPPLEMENTATION

Tran Huu Tinh

PROPOSITIONS

1. Carbohydrate application reduces the carbon footprint of shrimp aquaculture. (this thesis)
2. In biofloc systems, applying less feed does not always result in less production. (this thesis)
3. Seafood consumption is more beneficial to human health than meat consumption.
4. A moratorium on industrial capture fishery is needed for sea life to recover.
5. The adoption of new technologies is a multi-stakeholder effort.
6. Farmers who belong to a cluster perform on average better than independent farmers.
7. Organic farming is the way to go.

Propositions belonging to the thesis, entitled
Biofloc technology: Practical aspects of carbohydrate supplementation
Tran Huu Tinh
Wageningen, 18 November 2022

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Tran Huu Tinh

Thesis

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ABSTRACT

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Biofloc technology emerged recently as a sustainable farming system, allowing improvements of water quality and nutrient use efficiency through carbohydrate addition. This thesis aimed at understanding this technology better and started investigating the combined effects of carbohydrate application level, stocking density and feeding levels on system performance. Subsequently, the effects of carbohydrate type, application frequency and application method to biofloc rearing tanks were explored in separate experiments. Our results showed that the carbohydrate application level and feeding level exhibited a direct positive relationship with shrimp growth and biofloc biomass, while stocking density exhibited an inverse relationship with shrimp growth. The biofloc proximate composition was affected by stocking density and carbohydrate application level, while the biofloc microbial composition was affected by carbohydrate application level and feeding level. We also demonstrated that the choice of organic carbon source plays an important role in the success of the biofloc system. Among the tested carbohydrate sources, corn starch was superior to molasses for enhancing shrimp growth. Nitrogen waste can be efficiently controlled once the biofloc is well established and mature, resulting in relatively small diurnal fluctuation of nitrogen and carbon concentrations in culture water, regardless of the type of carbohydrate applied. In addition, a carbohydrate addition frequency of once per day was optimal and more frequent addition of CHO was not recommended if carbohydrate was to be applied in the culture of Pacific white shrimp. Furthermore, separate carbohydrate addition was better than its inclusion in pelleted feed for biofloc culture of Pacific white shrimp because a high dietary carbohydrate inclusion level results in poorer feed ingestion by the shrimp. The additional carbohydrate enhanced the water quality during culture. Adding carbohydrate did not significantly increase nitrogen retention and resulted in more than 50% carbon loss. Besides, the carbohydrate addition did not affect the microbial composition and prokaryotic microbial community diversity but increased the eukaryotic microbial diversity. To conclude, adding carbohydrate benefited shrimp performance and water quality, but standardizing the technology is difficult, as numerous factors affect system performance. In addition, these factors interact, be it not always, and show temporal variation.

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CHAPTER 1

GENERAL INTRODUCTION

1. Aquaculture and its role

Aquaculture is the fastest growing food production sector, which is partly due to its diversity, including a diversity of farmed species, farming systems and environments. During the last decade, aquaculture production increased by 4.9% annually (FAO/FishStatJ, 2021), and is expected to increase in the coming years, reaching 108 million tons of finfish, crustacean and mollusks in 2030 (FAO, 2020). As the world population is expected to keep increasing, aquaculture will continue growing in the future to meet human food demand.

Among major cultured species, *Litopenaeus vannamei* (commonly known as Pacific white shrimp or whiteleg shrimp) is the third aquaculture species by volume globally, accounting for almost 53% of the aquaculture crustacean production in 2018 (FAO, 2020). Pacific white shrimp is cultured in 37 countries mainly in tropical areas, including Southeast Asia (China, Thailand, India, Indonesia, Malaysia, Vietnam), and South American countries (Brazil, Ecuador, Mexico, the United States of America, Venezuela) (Junning et al., 2019). The species accounted for almost 4% of the world's aquaculture production by volume, and represents 11% of the world aquaculture production by value (Junning et al., 2019).

For the culture of Pacific white shrimp, there is a wide range of culture intensities ranging from extensive (200 – 300 kg ha^{-1} $crop^{-1}$) to intensive (10,000 – 15,000 kg ha^{-1} $crop^{-1}$) (Nguyen et al., 2019). In Vietnam, shrimp culture can be performed in very extensive farms in the south of the Mekong delta, where shrimp are stocked in low numbers per unit surface area, and fed very limited to almost zero formulated feed, relying mainly on natural food (Nguyen et al., 2019). These extensive systems are practiced in (coastal) ponds, under natural conditions without means to influence water quality beyond water exchange. Nevertheless, an important feature of the growth in production is intensification, which reduces resource use per metric ton of shrimp (Boyd et al., 2017; Naylor et al., 2021). In contrast, very intensive operations in indoor biofloc systems, featuring high stocking densities (> 250 post larvae/m^3), high quality pelleted feed, aeration systems, and additives (chemicals, minerals, vitamins, etc.), can be also found in the Mekong delta region, for instance at Viet-Uc Company (Nguyen et al., 2019).

Natural oxygen availability sets the limits to production in extensive ponds to 3 – 5 MT per crop, depending on the capacity of the pond to take up oxygen from primary production and surface water re-aeration (Verdegem et al., 2022). Semi-intensive and

intensive systems have higher oxygen requirements (for respiration) than the natural dissolved oxygen (DO) availability, hence oxygen needs to be added. In biofloc ponds, this is done through aeration, resulting in biofloc production which contributes to water purification (sanitation) and provides additional natural food. Important advantages of biofloc technology are that it can be adjusted to the culture intensity level by controlling the amount of DO added, and it improves the environmental sustainability of intensive shrimp farming through re-use of otherwise unused nutrients and through water purification, leading to a higher culture intensity with less pollution.

In all aquaculture systems, stoichiometry is important. Life organisms need nutrients and energy in the right proportions to build their own tissues. For heterotrophs in a biofloc system, the energy is provided by organic matter, which enters the system either through *in-situ* primary production, or through external nutrient inputs (e.g. manures, feeds) (Ebeling et al., 2006). Besides energy, the nutrients nitrogen and phosphorus are provided nearly exclusively through the feed. For a biofloc system to function well, the balance between energy and nutrient availability must be correct (Emerenciano et al., 2017). However, how to create and maintain such a correct balance is still not fully understood.

2. Biofloc technology

In intensive aquaculture systems, the accumulation of nitrogenous wastes (i.e. total ammonia nitrogen and nitrite) needs to be avoided for the welfare of cultured fish and shrimp. Solutions like water exchange and chemical treatments help to mitigate total ammonia nitrogen buildup, but might cause other concerns such as transmission of diseases or deterioration of the water quality of the surface water surrounding the farm (Ahmad et al., 2017; Cao et al., 2007; Páez-Osuna, 2001; Tovar et al., 2000). Therefore, more environmentally friendly solutions are needed for a sustainable aquaculture growth.

To control nitrogenous wastes, Avnimelech (1999) suggested the manipulation of carbon:nitrogen (C:N) ratios in aquaculture systems. Ammonia-nitrogen removal occurs via three pathways including photoautotrophic removal, autotrophic bacterial removal and heterotrophic bacterial removal (Ebeling et al., 2006). Increasing the C:N ratio through the addition of carbohydrate sources provides energy for heterotrophic bacteria, and stimulates the heterotrophic pathway, which directly assimilates ammonia-nitrogen into microbial biomass. This practice reduces total ammonia and nitrite nitrogen concentrations and improves the water quality in shrimp and fish culture systems

(Avnimelech, 1999; de Lorenzo et al., 2016; Ebeling et al., 2006), allowing intensive aquaculture with limited water exchange during the culture period.

Manipulating the C:N ratio in aquaculture systems by adding carbohydrate sources has attracted much interest among researchers. The diverse varieties of carbohydrate source offer different choices, ranging from liquid to solid, organic to inorganic, or from waste products to high-grade and expensive carbohydrate sources. A summary of studies on different carbohydrates and some of the main observations is shown in Table 1.1. Moreover, carbohydrate addition in general has been reported to improve the immune response of shrimp (Ekasari et al., 2014b; Hostins et al., 2019). However, depending on the type of carbohydrate used, these beneficial effects on immunity to diseases and shrimp survival vary (Ekasari et al., 2014b). Therefore, the diversity of carbohydrate types makes it difficult to standardize the technology, reducing its adoptability.

Biofloc technology started from culturing shrimp and tilapia in low intensity, active sludge water purification systems. When doing so, some basic research questions were never tackled, although the biofloc technology has been around for more than 30 years. Therefore, this thesis examined four hypotheses:

- The biofloc technology benefits culture systems (i.e. improved water quality and shrimp performance) regardless of stocking densities and feeding levels, as the technology allows to maintaining a suitable C:N ratio, irrespective of culture intensity.
- Different carbohydrate sources affect the performance of biofloc systems differently, even though applied at the same C:N ratios.
- Adding once daily the carbohydrate dosage or in different smaller portions each 24-hour period affects biofloc system differently, as water quality (e.g. dissolved oxygen) is affected by the application frequency during the day.
- In biofloc systems, mixing carbohydrate sources in the feed will simplify the CHO application practice, while maintaining the benefits of adding additional CHO.

3. Scope and aims of this thesis

The Pacific white shrimp is an important species with high production and value worldwide. Its culture often employs high-protein complete feed, which is often not well digested and thus wasted, in part because shrimp has a short digestive tract. Biofloc technology seems promising for reducing environmental impacts of shrimp culture by

increasing nitrogen use efficiency through assimilation of nutrients into microbial biomass serving as natural food for shrimp and improving water use efficiency.

Table 1.1 Studies on carbohydrate sources for biofloc systems.

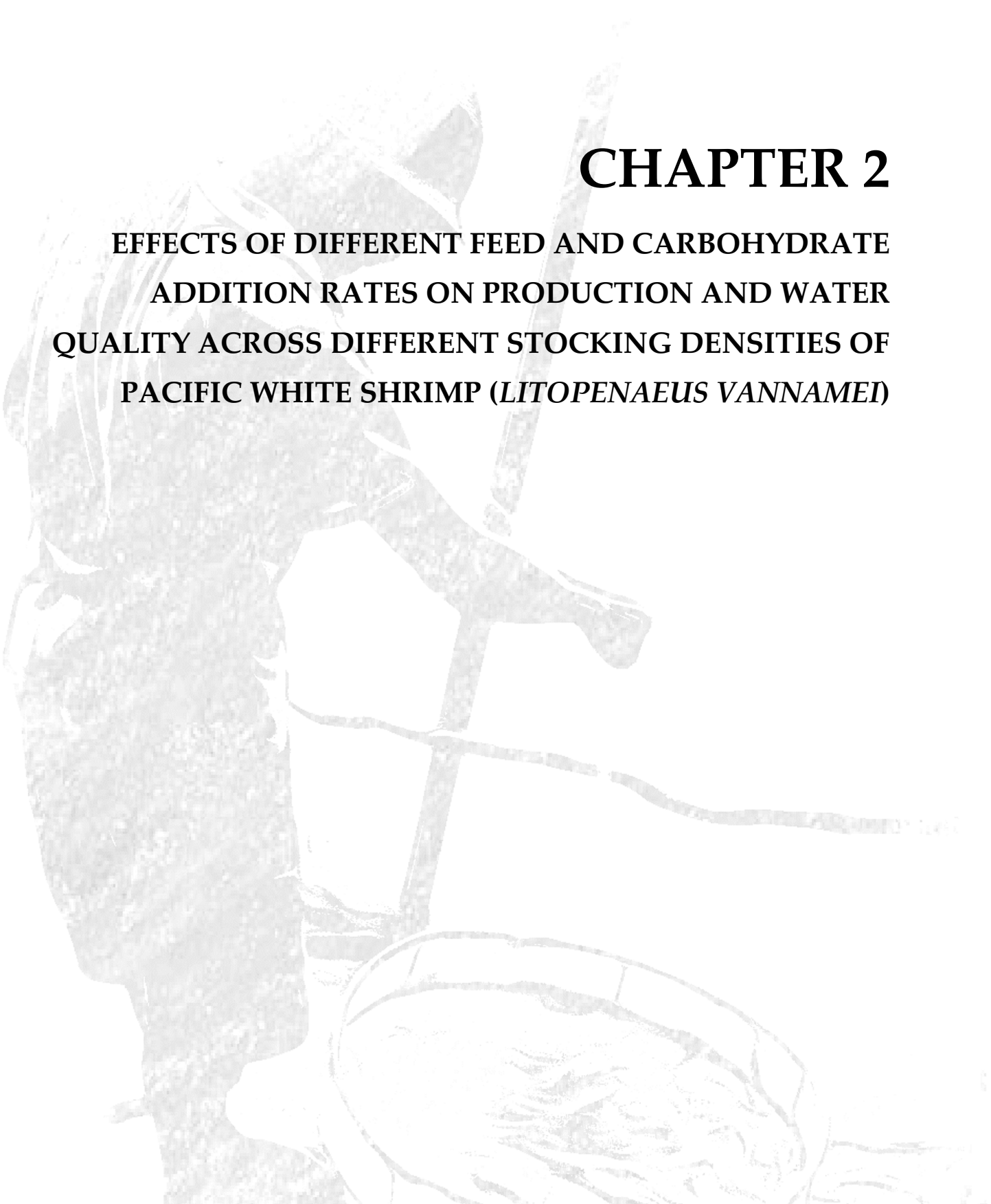
References	Species	Carbohydrate sources	C:N Ratios	Observations
(Li et al., 2018)	*Oreochromis niloticus*	- Longan powder (LP) - Polyhydroxybutyrate-hydroxyvalerate/LP (PHBVL) - Poly(butylene succinate)/LP (PBSL)	23-25	- CHOs affect water quality, microbial community in biofloc and fish gut differently. - Effects of CHO types on fish growth and health require further study.
(Wei et al., 2016)	-	- Glucose - Starch - Glycerol	15	- CHO types affect biofloc nutritional quality, and microbial community and structure.
(Deng et al., 2018)	*Pelteobagrus vachelli*	- Tapioca starch - Plant cellulose - Tapioca starch + Cellulose	13-14	- CHO types affect total ammonia nitrogen levels, and fish growth and survival differently. - CHO types affect microbial diversity and richness unevenly.
(Crab et al., 2010)	*Macrobrachium rosenbergii*	- Acetate - Glycerol - Glycerol + Bacillus - Glucose	10	- Shrimp realized growth based on biofloc only - CHO types affect biofloc nutritional quality.
(Ekasari et al., 2014b)	*Litopenaeus vannamei*	- Molasses - Tapioca - Tapioca-by-product - Rice bran	15	- Survival, yield, protein assimilation differed among CHO types. - Immunity increased with all CHO types
(Serra et al., 2015)	*Litopenaeus vannamei*	- Molasses - Rice bran - Dextrose	6-15	- Different C:N ratio was applied during the culture period. - Degradation rate differs among CHO types - CHO types affect water quality, and shrimp performance differently (due to different degradation rate)

Considering the knowledge gaps about biofloc technology mentioned above, **Chapter 2** investigated the main effects of, and interactions between stocking density, feeding level, and C:N ratio, using a 3 x 3 factorial design. These treatments differed mainly in the amount of input nutrients. A stocking density as low as 27 individual per square meter was used to see how shrimp would perform in the presence or absence of an additional carbohydrate source. It was hypothesized that the carbohydrate addition benefits all treatments, but to different degrees (e.g. CHO addition would improve shrimp growth in low stocking density more than in high stocking density, or vice versa). **Chapter 3**

compared the effect of two types of carbohydrate, representing pure sources (i.e. corn starch) and by-products (i.e. molasses), on biofloc systems. Both CHOs were applied to the system at a similar C:N ratio, however, their effects were hypothesized to differ significantly due to differences in their composition. In **Chapter 4**, the effects of distributing the carbohydrate addition throughout the day were compared to that of the control (without carbohydrate addition). By administrating the same amount of CHO in all treatments per day, but increasing the number of times CHO was applied, we aimed at creating more stable environmental conditions, which ultimately should increase shrimp production. Lastly, **Chapter 5** explored the potential of mixing the carbohydrate otherwise applied separately from the feed directly in the feed pellet. Reference treatments included the normal practice of separate carbohydrate addition, and a control without additional carbohydrate. This method when proven useful would simplify the practice of adding carbohydrate and facilitate more widespread use of biofloc technology throughout the shrimp industry.

CHAPTER 2

EFFECTS OF DIFFERENT FEED AND CARBOHYDRATE ADDITION RATES ON PRODUCTION AND WATER QUALITY ACROSS DIFFERENT STOCKING DENSITIES OF PACIFIC WHITE SHRIMP (*LITOPENAEUS VANNAMEI*)



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Tinh, T.H., Kokou, F., Hai, T.N., Verreth, J.A.J., Verdegem, M.C.J.,. Effects of different feed and carbohydrate addition rates on production and water quality across different stocking densities of Pacific white shrimp (Litopenaeus vannamei)

Abstract

Shrimp culture systems are dynamic with potential interactions among technical parameters. This research investigated the main and interaction effects of stocking density, feeding level, and C:N ratio on the shrimp production, water quality, bioflocs and its associated microbial community, using a 3 x 3 factorial design. Pacific white shrimp were stocked at 27, 120, or 300 ind/m^3, and fed with 100%, 80%, or 60% of the recommended daily feed ration. For each stocking density by feeding level, carbohydrate source was either not added (C:N ratio = 7.4) or added to increase the input C:N ratio to 12, or 16. The results revealed that the shrimp biomass was positively correlated with all three factors. Meanwhile, the shrimp growth was positively correlated with feed and carbohydrate addition rates, and negatively correlated with stocking density. The factors showed independent effects on most shrimp production parameters but demonstrated two-way and three-way interactions on biofloc mass and water quality parameters. The microbial diversity in bioflocs was positively correlated with feeding levels and C:N ratios. To conclude, shrimp growth and biofloc biomass (TSS, VSS) are in a similar way affected by stocking density, feeding level, and C:N ratio, while the biofloc proximate composition is affected by stocking density, and C:N ratio. The microbiota community composition is affected by C:N ratio and feeding level.

Keywords: C:N ratio, factorial design, feeding rate, *Litopenaeus vannamei*, stocking density, microbiota

1. Introduction

In Pacific white shrimp (*Litopenaeus vannamei*) culture, the effects of stocking density, feeding level, and C:N ratio have been described. Shrimp growth and survival often exhibit inverse relationship with stocking density (Araneda et al., 2008; Sandifer et al., 1987; Sookying et al., 2011; Williams et al., 1996). The reduced growth and survival at high density are partly due to crowding leading to competition for space and feed, and due to poor water quality with all these parameters contributing to increased stress (Araneda et al., 2008; Liu et al., 2017; Williams et al., 1996). Liu et al. (2017) reported depressed antioxidant and immune status of shrimp cultured at high density. In addition, increasing stocking density decreased digestive enzyme activity and increased the feed conversion ratio (Liu et al., 2017).

Increasing feeding rate and C:N ratio in the culture system through carbohydrate addition improves shrimp growth. Specifically, a higher C:N ratio improves water quality and biofloc growth (Avnimelech, 1999; Crab et al., 2012; Lara et al., 2017), which may contribute up to 29% of the daily feed intake of Pacific white shrimp (Burford et al., 2004). In systems with high primary production or supplemented with bioflocs, feeding 25% less than the recommended feed ration was reported not to affect shrimp growth (Lara et al., 2017; Roy et al., 2012).

Shrimp culture systems are dynamic with interactions among variables defining the culture system. For instance, Schveitzer et al. (2013a) reported positive effects of substrate addition on shrimp growth, and significant substrate × stocking density interactions on several shrimp production and water quality parameters. The shrimp survival can be also significantly affected by stocking density × water exchange rate interactions (Esparza-Leal et al., 2020). Stocking density × culture condition (e.g. biofloc, artificial substrate) interactions on shrimp growth were reported in other cases (Guemez-Sorhouet et al. 2019). However, the nature of interactions between stocking density, feeding level and C:N ratio remains unclear. Therefore, the current research examined the main effects stocking density, feeding level, and C:N ratio, and their interactions on shrimp performance, water quality, bioflocs, and microbial community composition in Pacific white shrimp biofloc culture systems.

2. Materials and methods

2.1. Experimental design

This experiment was done in the aquatic experimental facilities at College of Aquaculture and Fisheries, Can Tho University, Vietnam, using a three-way factorial design. The feeding rate variable had three levels, including 100% (FL100), 80% (FL80), and 60% (FL60) of the recommended feeding rate by the feed provider (3% - 22% body weight per day depending on shrimp size). The carbon:nitrogen (C:N) ratio variable had three levels, including 7.4 (C:N7), 12 (C:N12), and 16 (C:N16) on weight basis. Each combination of the feeding rate and C:N ratio was tested in three stocking densities, including 27 ind/m^3 (SD27), 120 ind/m^3 (SD120), and 300 ind/m^3 (SD300). The three stocking densities were tested in separate 12-week grow-out trials. In total, this experiment comprised twenty-seven treatments executed in triplicate. During each grow-out trial, the treatment SD120-FL100-C:N7 was executed as inter-phase control, with 3 replicates per trial. The acronyms FL100, FL80, and FL60 refer to tanks where shrimp were fed 100, 80, and 60% of the feed level recommended by the feed company, respectively. The same holds for the acronyms C:N7, C:N12, and C:N16 or SD27, SD120, and SD300.

2.2. Experimental site preparation

The experiment was conducted outdoor to mimic farming conditions. Nevertheless, a transparent plastic film was used to cover the experimental site to prevent the effects of rainwater intrusion, while still allowing ambient light to the experimental tanks. Thirty circular plastic tanks (0.5 m^3 total volume) were cleaned prior to the addition of saline water and employed during each 12-week trial period.

Sea water (80 ppt salinity) was diluted to 15 ppt salinity, disinfected with chlorine and adjusted to 140 mg/L alkalinity using sodium bicarbonate. Three hundred liters treated water were pumped into each experimental tank. All tanks were continuously aerated with one air-stone ca. 10 cm above the bottom in the middle of the tank to maintain the dissolved oxygen level above 6 mg/L. Air was provided to all tanks from one air blower (RB-022, APP Co. Ltd, Taiwan) serving all experimental tanks.

2.3. Experimental animals

For every experimental trial, Pacific white shrimp (*Litopenaeus vannamei*) postlarvae (PL15) were obtained from the same nearby hatchery and nursed in a 2-m^3 tank at 15 ppt salinity for one month. During this period, shrimp were fed three times daily at 08:00, 12:00, and 16:00 hours. Shrimp of 2.5 ± 0.1 cm total length were stocked in the experiment.

Per tank, 8, 36, or 90 shrimp were stocked into each tank to obtain the stocking densities of SD27, SD120, and SD300, respectively.

2.4. Feeding and carbohydrate addition

Shrimps were fed with complete feed (42% protein, C:N ratio 7.4) (Skretting, Vietnam) three times per day at 08:00, 12:00, and 16:00 hours, applied evenly over the whole tank surface area. The shrimp in FL100 tanks were fed 3-20% BW/day according to shrimp size, following the recommendations of the feed company. The shrimp in treatment tanks FL80 and FL60 were fed each day 20% and 40% less feed compared to shrimp in treatment tanks FL100, respectively. The feeding level was adjusted every two weeks, based on the average shrimp individual weight in the inter-trial control treatment SD120-FL100-C:N7.

A mixture of rice bran and cassava (3:7 ratio) was used as carbohydrate (CHO) source to adjust the C:N ratio. Treatment tanks with C:N7 (which received feed with a C:N ratio 7.4) did not receive CHO besides the pelleted feed. To increase the C:N ratio to 12 and 16, 681 and 1278 g of CHO, respectively, was added per kilogram of feed applied. The CHO was weighed and incubated in 60^{o}C water for 24h prior to addition to the treatment tanks. The proximate composition of the feed and carbohydrate applied is shown in Table 2.1.

Table 2.1 The proximate composition of shrimp feed and carbohydrate (CHO) in this research.

Samples	Moisture (%)	Ash (% DM)	Crude Fat (% DM)	Crude Protein (%DM)	NFE (%DM)
Feed	10.9	11.4	8.3	47.3	33.0
CHO	11.3	3.6	3.1	7.0	86.3

2.5. Water quality monitoring

The total ammonia nitrogen (TAN), nitrite, and nitrate concentrations were checked using commercial test kits (sera GmbH, Germany), while the water pH and temperature were monitored on a weekly basis two hours after the mid-day feeding period using electric probe. Every two weeks filtered and disinfected tap water was added to each tank to compensate water loss due to evaporation.

2.6. Sampling and sample analysis

The number of shrimp stocked and the average shrimp body weight and length were determined at the start (Day 0), and from then onwards at bi-weekly intervals (Days 14, 28, 42, 56, and 70), until harvest (Day 84). In the SD27 grow-out trial, 4 shrimp were randomly sampled from each tank for the determination of average weight and length

every two weeks. In the SD120 and SD300 grow-out trials, 10 shrimp per tank were collected for the average weight and length determination. At harvest, the number of shrimp harvested from each tank were counted to determine the survival rate.

Water samples were collected at the end of culture weeks 6 and 12 in each grow-out trial. These samples were analyzed for total suspended solids (TSS), volatile suspended solids (VSS), chlorophyll a (Chla), total ammonia nitrogen (TAN), nitrite nitrogen (NO_2-N) concentration following Standard Methods for the Examination of Water and Wastewater (APHA, 1995).

Biofloc volume was measured every two weeks using Imhoff cones. One liter of water was taken from each tank and let to settle in an Imhoff cone for 20 minutes. The biofloc volume was then recorded as the amount of settled material in mL/L. At the end of the experiment, biofloc samples were collected and analyzed for proximate composition, following the Official Methods of Analysis (AOAC, 2000). During the SD300 grow-out trial, biofloc samples were collected from every tank by filtering 1 L of culture water through 1.5 μm-pore size filters. Biofloc samples were then mixed with DNA-later at the 1:1 ratio and stored at -40^{o}C for the analysis of microbial community composition.

2.7 Microbiota analysis

For microbiota analysis, DNA was extracted from biofloc samples. The biofloc samples, were collected at the end of the experiment by filtering through a 0.22 μm (seston part) pore size sterile filter. The samples were subjected to lysis by lysozyme buffer and proteinase K before DNA extraction using DNeasy PowerSoil kit (Qiagen, Valencia, CA). The harvested DNA was quantified using the nano drop spectrophotometer. Sequencing of the PCR-amplified V4 region of the 16S rRNA (prokaryotic microbial communities), using primers 515 F (5′-CTAGTGCCAGCMGCCGCGGTAA -3′) and 806 R (5′-CTAGGACTACHVGGGTWTCTAAT-3′) was performed using a MiSeq PE300 Next Generation system (Illumina) by Genome Quebec, following the company's protocol.

An open-source software package, DADA2 (Callahan et al., 2016), was applied to model and correct Illumina-sequenced amplicon errors. Data were demultiplexed into forward and reverse reads according the barcode sequence into sample identity, and trimming was performed, according to Kokou et al. (2020). For the forward reads and based on the quality profiles, the first 250 nucleotides were kept, and the rest were trimmed, while for the reverse reads, the last 220 nucleotides were kept. DADA2 resolves differences at the single-nucleotide level and the end product is an amplicon sequence variant table,

recording the number of times each exact sequence variant (ESV) was observed in each sample (100% sequence identity). Taxonomy was assigned using the Ribosomal Database Project Classifier (Wang et al., 2007) against the 16S gene reference Silva database (138 version) (McLaren, 2020). Owing to the variation in sequence depths between samples, all samples were normalized to the lowest depth by subsampling.

2.8. Data analysis

Statistical analysis was performed with IBM SPSS Statistics 25 software (IBM Corporation, NY, USA). One-way ANOVA analysis was used to compare the shrimp production among treatments in each grow-out trial. Three-way ANOVA analysis was performed on shrimp production parameters, combining the 3 grow-out trials. Repeated measure three-way ANOVA was performed with bi-weekly shrimp growth data, and water quality parameters. A probability value (P value) of less than 0.05 indicated a significant effect.

For the microbiota alpha-diversity analysis, Shannon H' diversity, richness (observed taxa), and rare taxa abundance were calculated. Non-parametric tests (Wilcoxon test) and linear mixed-effect models (nlme R package (Pinheiro and Bates, 2007) were used to assess alpha-diversity. Adonis implementation of Permanova (Anderson, 2001) (non-parametric permutational multivariate analysis of variance) was used for comparison between groups. Exact sequent variants that had a differential abundance between feeding level and C:N ratio treatments were detected using Deseq2 tool (Love et al., 2014).

3. Results

3.1. Inter-phase controls

Table 2.2 Shrimp production parameters in three repeats of the inter-phase control (treatment SD120-FL100-C:N7). Values are mean ± standard deviation of 3 replicate tanks per repeat.

	Final BW (g/ind)	Final BL (cm/ind)	Total biomass (g)	Survival rate (%)	FCR
Repeat 1	4.7 ± 0.5	8.2 ± 0.2	161 ± 15	95 ± 2	1.06 ± 0.10
Repeat 2	4.3 ± 0.2	8.3 ± 0.1	149 ± 10	96 ± 4	1.10 ± 0.08
Repeat 3	4.6 ± 0.1	8.4 ± 0.1	145 ± 8	92 ± 5	1.19 ± 0.07
P values	0.292	0.501	0.281	0.355	0.212

The shrimp production parameters were not different among different grow-out trial repeats of the inter-phase control ($P > 0.05$) (Table 2.2). In addition, the shrimp growth in terms of weight and length was similar among the trials (Figure 2.1). This showed that the experimental procedure and culture conditions were similar among the grow-out

trials. Therefore, data among inter-phases were combined and analyzed with three-way ANOVA.

3.2. Shrimp production

The stocking density, feeding level, and C:N ratio showed independent effects on most shrimp production parameters (Table 2.3). The stocking density was negatively correlated with shrimp body length and weight, absolute and specific growth rate, and feed conversion ratio, while the feeding level and C:N ratio were positively correlated with those parameters. The shrimp individual body weight and total biomass at harvest are illustrated in Figures 2.2 and 2.3, respectively. The effects of feeding level and C:N ratio on shrimp harvested biomass varied with stocking densities (P_{SD*FL}, $P_{SD*C:N} < 0.05$), and were more pronounced at high stocking density (Figure 2.4). Increasing the feeding level increased the final body weight. In contrast, the feeding level profoundly affected the FCR at low stocking density (Figure 2.5). The shrimp survival rate was above 75% in all treatments (data not shown) and was only affected by the feeding level in this research ($P > 0.05$).

Table 2.3 Probability (P) values for the main and interaction (*) effects of the three independent factors on shrimp production parameters. P values in bold indicate significant effects ($P < 0.05$). The abbreviations are SD = stocking density, FL = feeding level, C:N = carbon:nitrogen ratio, FCR = feed conversion ratio, AGR = absolute growth rate, SGR = specific growth rate.

Parameters	P values						
	SD	FL	C:N	SD*FL	SD*C:N	FL*C:N	SD*FL*C:N
Body length	**0.000**	**0.000**	**0.000**	0.783	0.335	0.490	0.880
Body weight	**0.000**	**0.000**	**0.000**	0.271	0.496	0.329	0.966
Total biomass	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.705	0.498
Survival rate	0.304	**0.004**	0.998	0.310	**0.033**	0.392	0.418
FCR	**0.000**	**0.007**	**0.000**	**0.047**	0.147	0.758	0.484
AGR	**0.000**	**0.000**	**0.000**	0.271	0.496	0.329	0.966
SGR	**0.000**	**0.000**	**0.000**	0.721	0.104	0.239	0.938

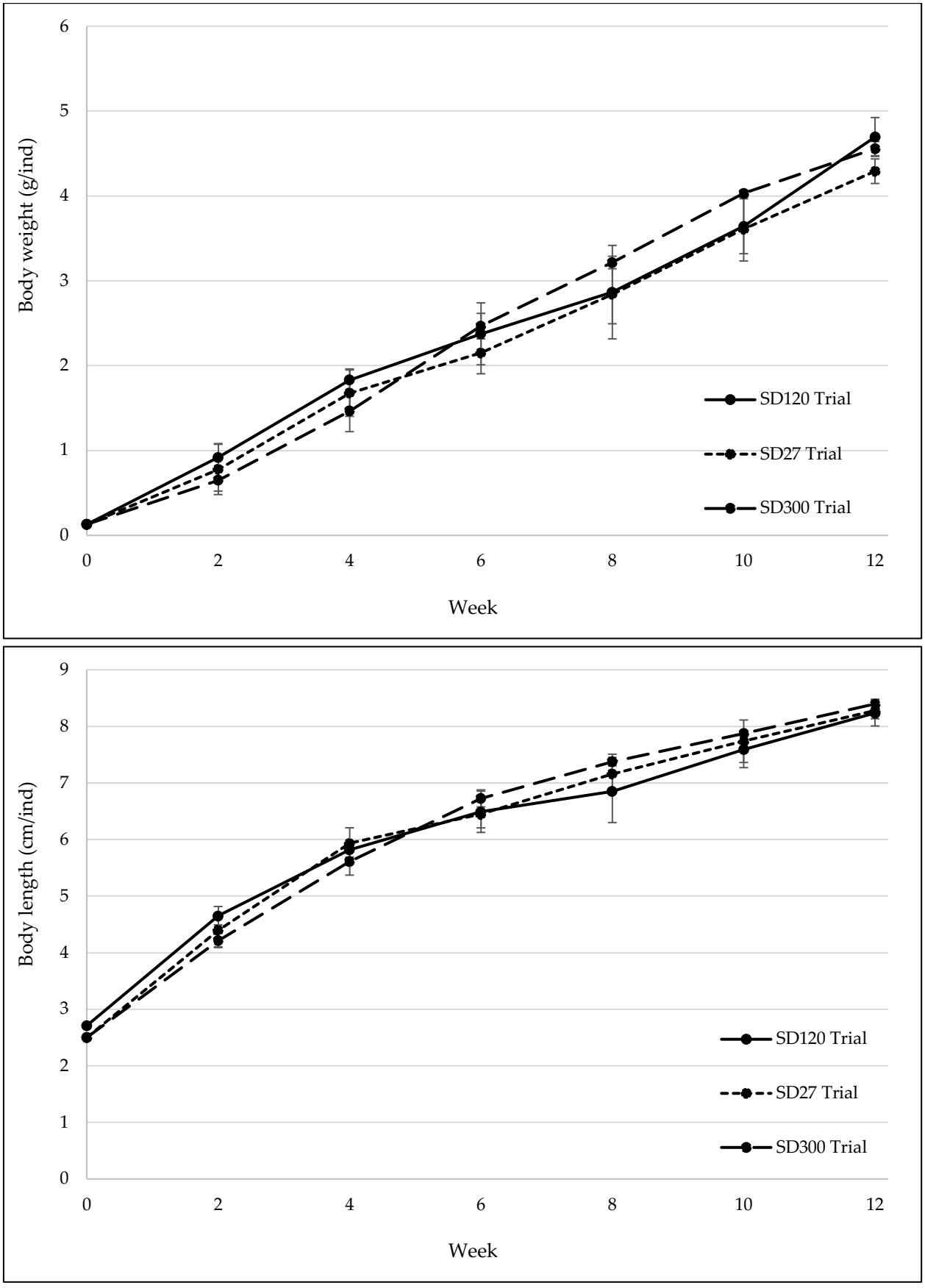


Figure 2.1 Average shrimp weight (top) and length (bottom) among repeats of the inter-phase control (treatment SD120-FL100-C:N7). Data points are mean ± standard deviation of 3 replicate tanks per trial.

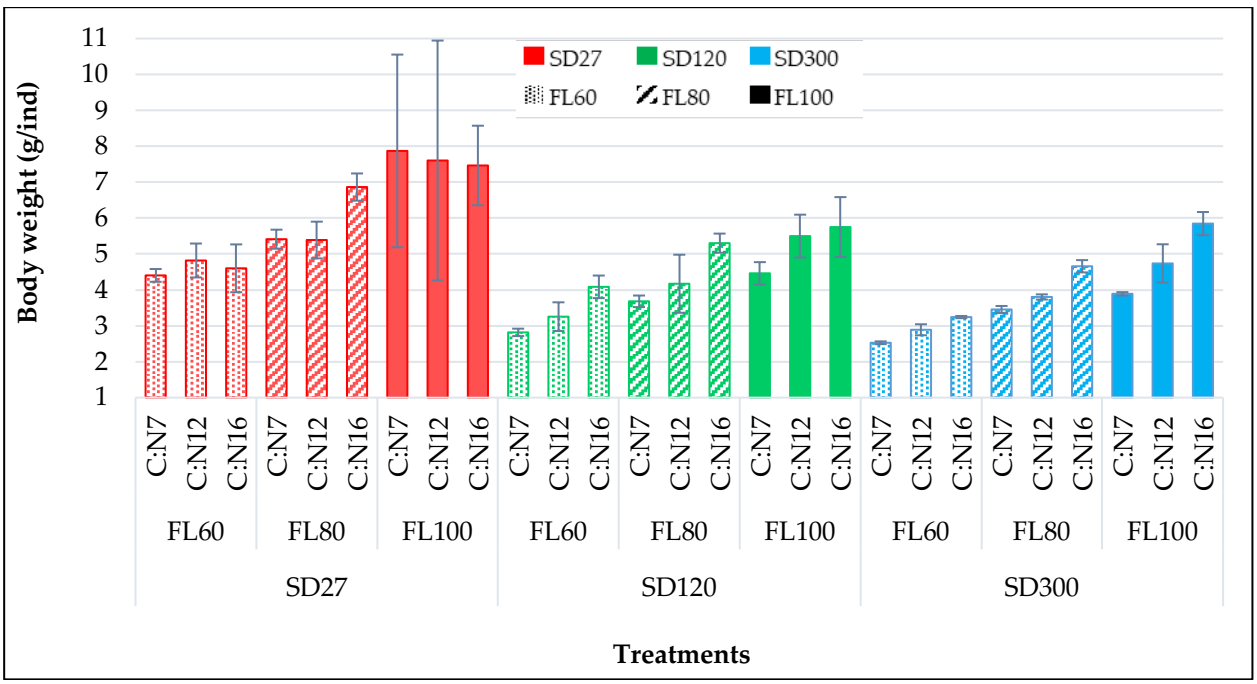


Figure 2.2 Individual shrimp body weight at harvest. Column heights are mean ± standard deviation of 3 replicate tanks per treatment (Stocking density by Feeding level by C:N ratio). Except treatment SD120-FL100-C:N7, the value is mean ± standard deviation of 9 replicate tanks. The abbreviations are SD = stocking density, FL = feeding level, C:N = carbon:nitrogen ratio.

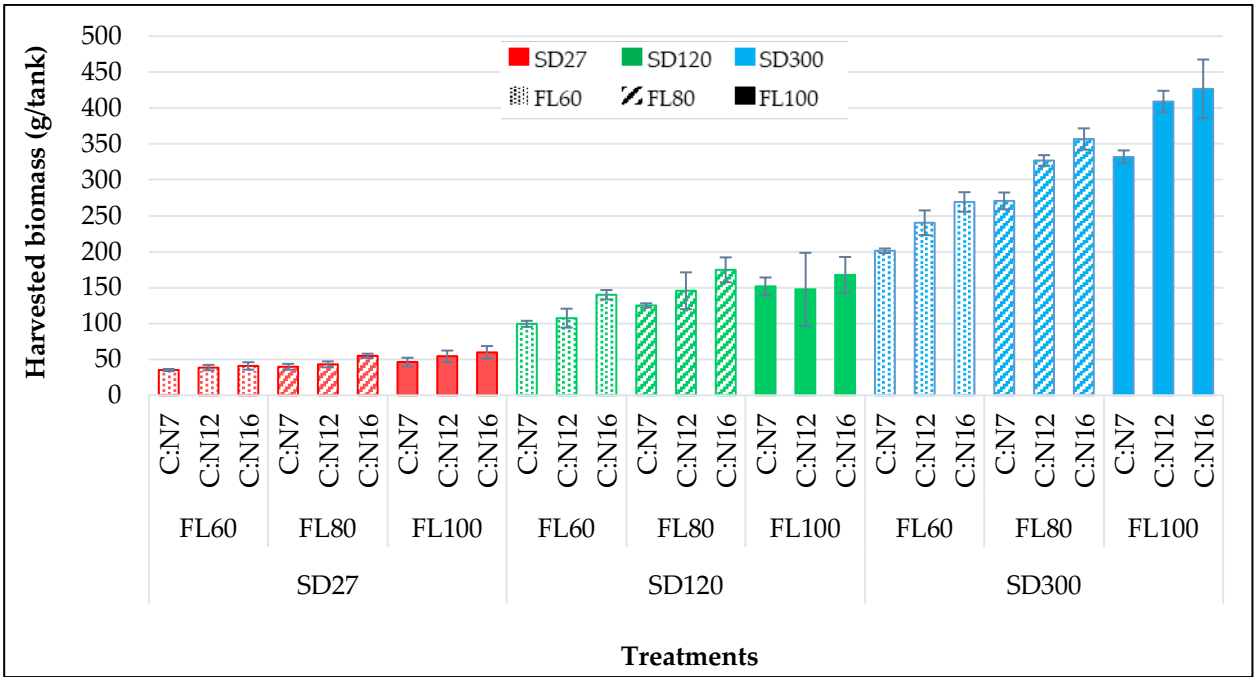


Figure 2.3 Total shrimp biomass per tank at harvest. Column heights are mean ± standard deviation of 3 replicate tanks per treatment (Stocking density by Feeding level by C:N ratio). Except treatment SD120-FL100-C:N7, the value is mean ± standard deviation of 9 replicate tanks. The abbreviations are SD = stocking density, FL = feeding level, C:N = carbon:nitrogen ratio.

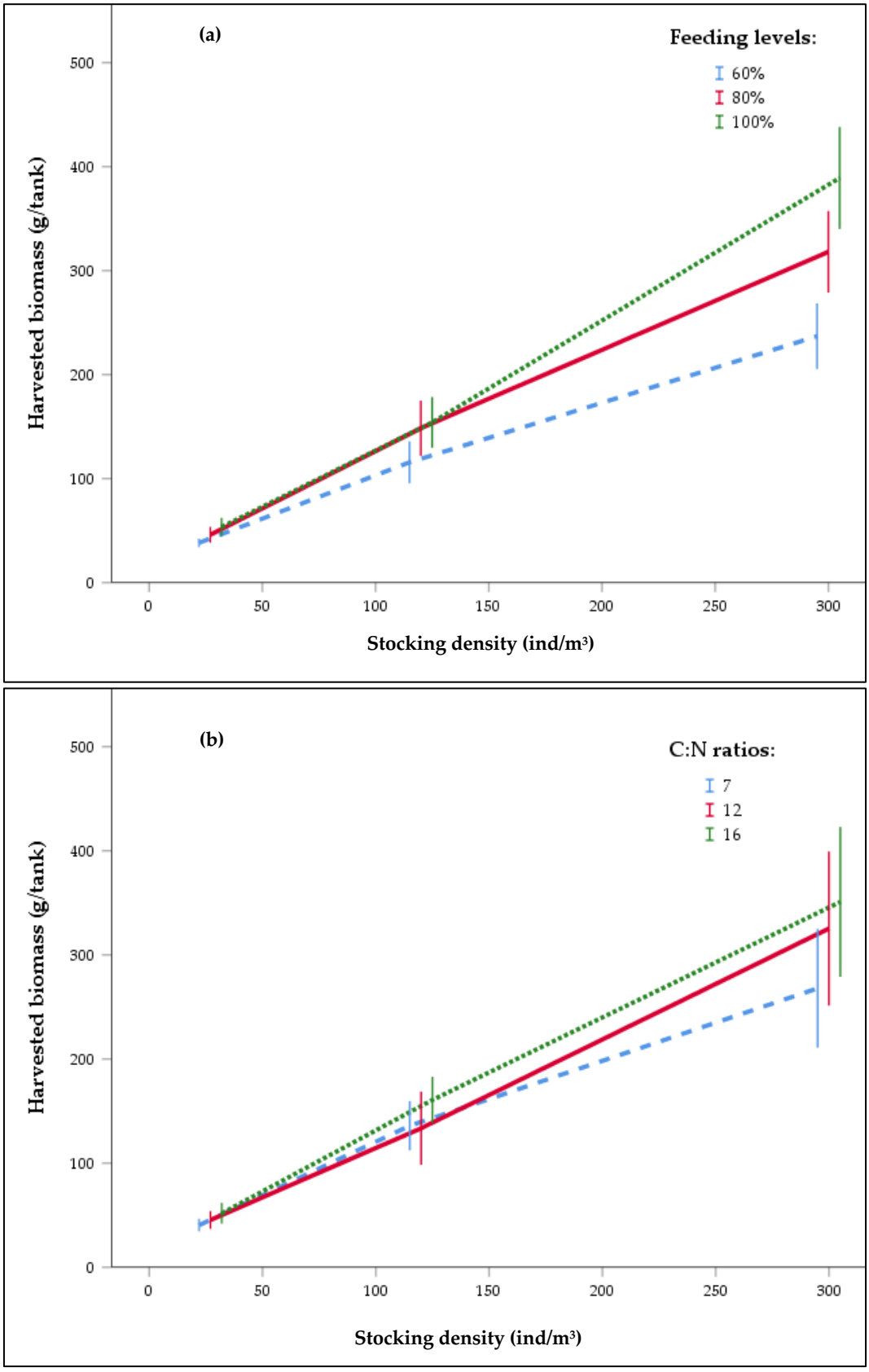


Figure 2.4 Harvested shrimp biomass at three stocking densities (SD27, SD120, and SD300 ind/m³) with varied feeding levels (a) and C:N ratios (b). Values are mean ± standard deviation of 9 tanks per treatment. Except for treatments SD120-FL100 and SD120-C:N7, values are mean ± standard deviation (SD) of 15 tanks. The lines were shifted backward and forward on the X axis to increase the visibility of SD.

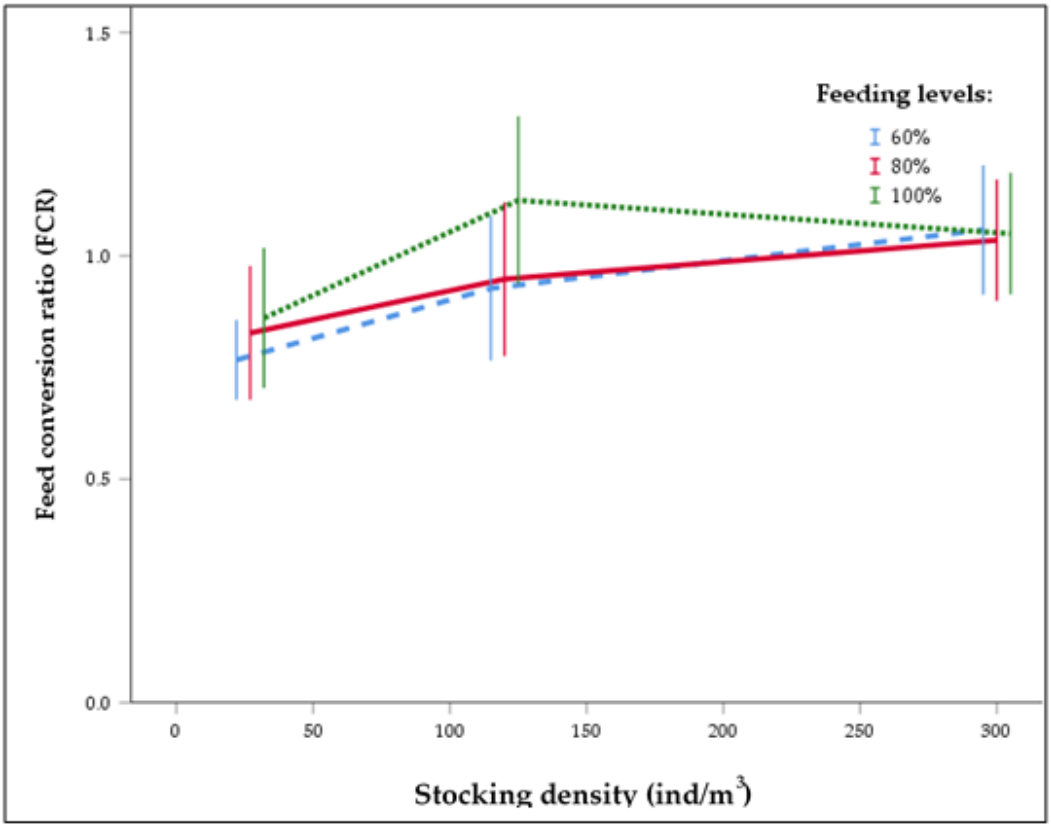


Figure 2.5 Shrimp feed conversion ratio (FCR) at different stocking densities (SD27, SD120, and SD300 ind/m^3) with varied feeding levels. Values are mean ± standard deviation of 9 tanks per treatment. Except for treatment SD120-FL100, the value is mean ± standard deviation of 15 treatment tanks. The lines were shifted backward and forward on the X axis to increase the visibility of SD.

3.3. Biofloc growth and proximate composition

Biofloc mass in terms of total suspended solids (TSS) and volatile suspended solids (VSS) increased significantly during the experiment, here shown for days 42 and 84 of the grow-out trials (Figures 2.6 and 2.7, respectively) (repeated measure three-way ANOVA, $P_{Time} < 0.05$). There was no significant three-way interaction among factors on biofloc TSS and VSS, however, significant interactions between stocking density and feeding level, and stocking density and C:N ratio on both TSS and VSS, and feeding level and C:N ratio on VSS were observed (Table 2.4). Overall, the TSS and VSS increased with increasing stocking density, feeding level, and C:N ratio.

Table 2.4 Probability (P) values for the main and interaction (*) effects of the three independent factors on biofloc growth. P vaues in bold indicate significant effects (P < 0.05). The abbreviations are SD = stocking density, FL = feeding level, C:N = carbon:nitrogen ratio, TSS = total suspended solids, VSS = volatile suspended solids.

Parameters	P values						
	SD	FL	C:N	SD*FL	SD*C:N	FL*C:N	SD*FL*C:N
TSS (mg/L)	**0.000**	**0.000**	**0.000**	**0.000**	**0.011**	0.135	0.277
VSS (mg/L)	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.032**	0.250

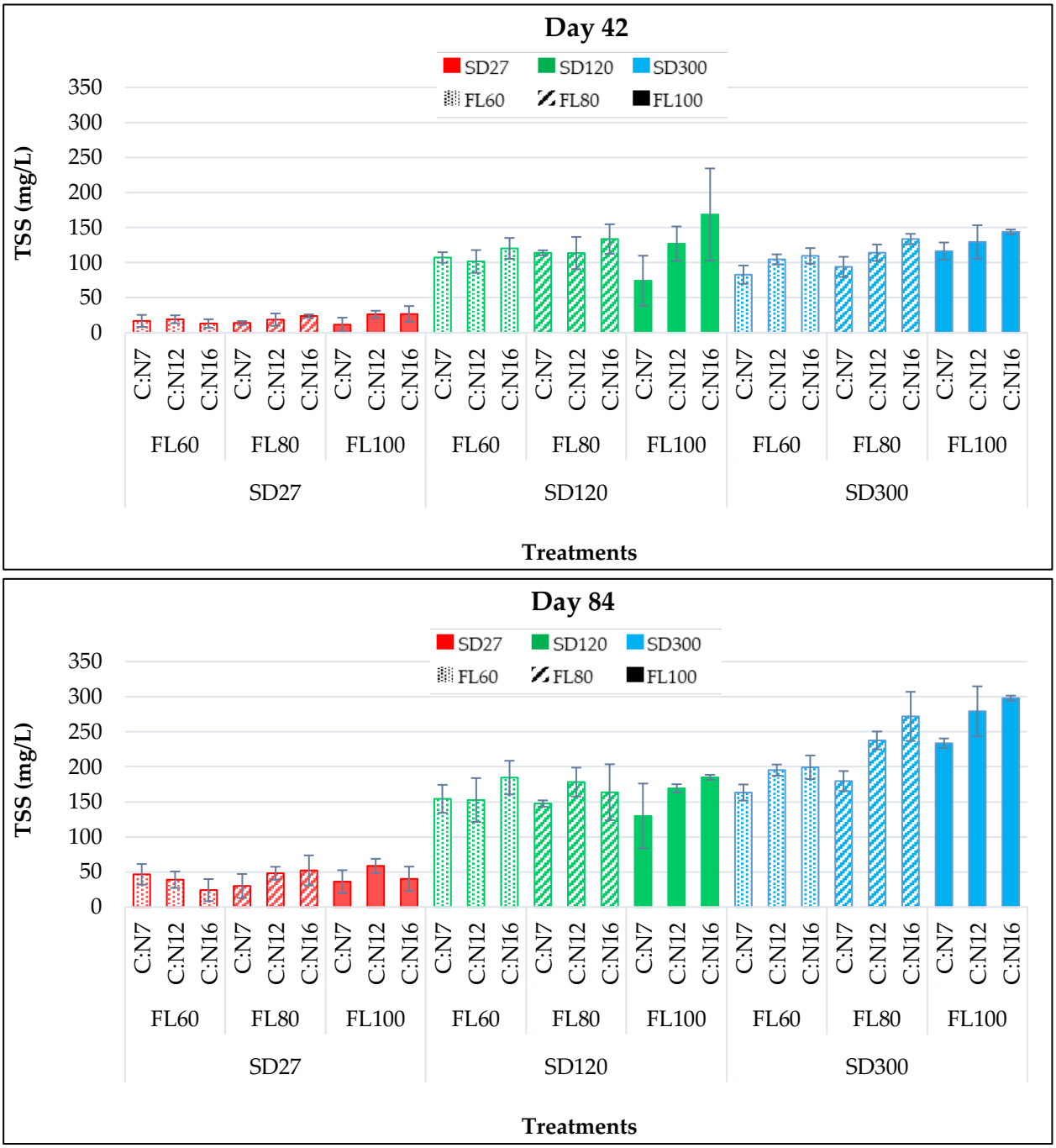


Figure 2.6 The total suspended solids (TSS) on days 42 and 84 of the experiment. Column heights are mean ± standard deviation of 3, except the treatment SD120-FL100-C:N7 of 9, replicate tanks per treatment (stocking density by feeding level by C:N ratio). The abbreviations are SD = stocking density, FL = feeding level, C:N = carbon:nitrogen ratio

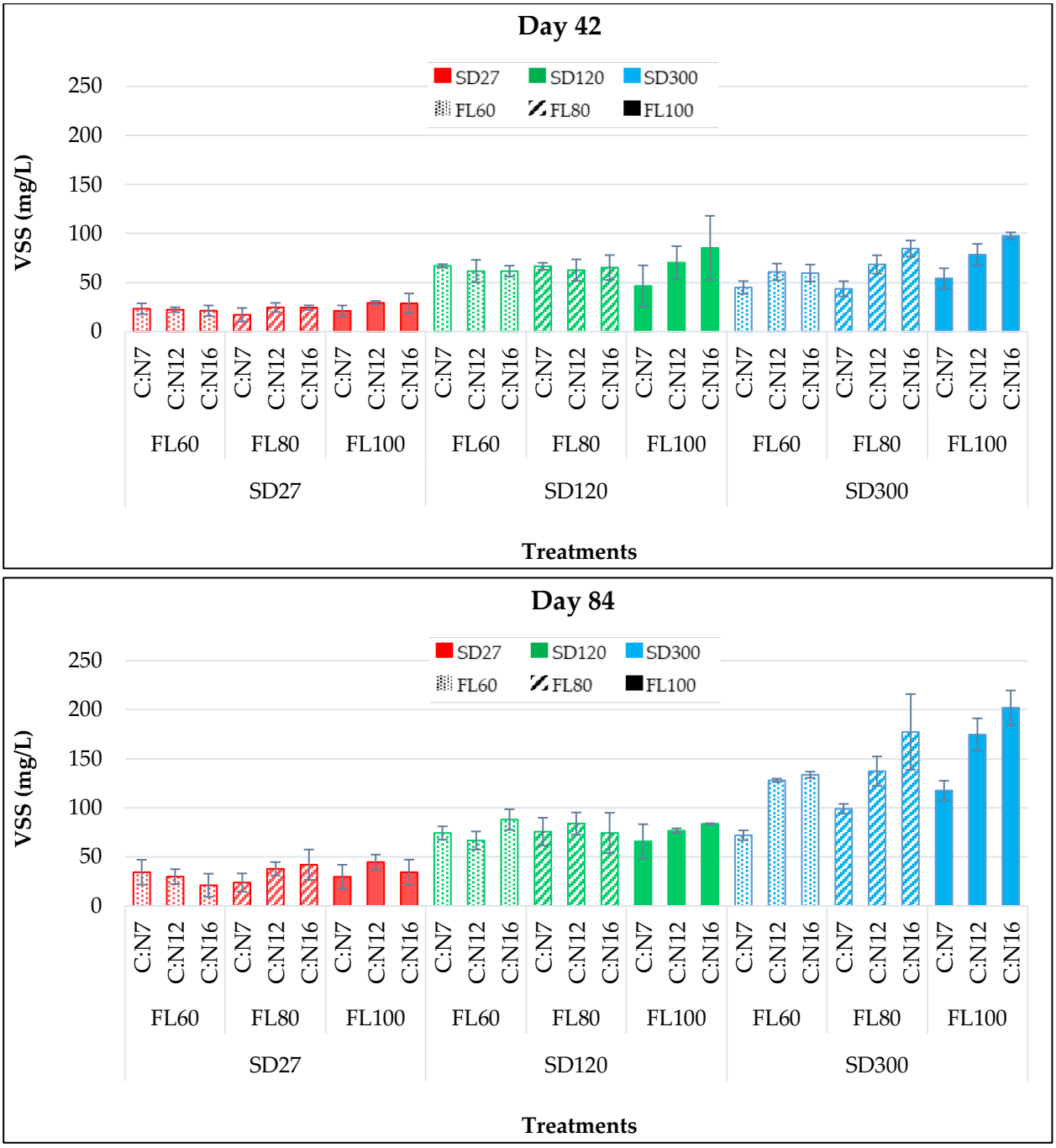


Figure 12.7 The volatile suspended solids (VSS) on days 42 and 84 of the experiment. Column heights are mean ± standard deviation of 3, except the treatment SD120-FL100-C:N7 of 9, replicate tanks per treatment (stocking density by feeding level by C:N ratio). The abbreviations are SD = stocking density, FL = feeding level, C:N = carbon:nitrogen ratio.

The three factors had independent effects on biofloc proximate composition, except on biofloc protein content ($P_{SD*FL*C:N} < 0.05$) (Table 2.5). Specifically, increasing the stocking density reduced the biofloc ash content, and increased the biofloc fat and NFE contents ($P < 0.05$) (Table 2.6). The feeding level showed no effect on biofloc proximate

composition. Meanwhile, increasing the C:N ratio significantly reduced the biofloc ash content and increased the biofloc NFE content (Table 2.6).

Table 2.5 Probability (P) values for the main and interaction (*) effects of the three independent factors on biofloc proximate composition. P values in bold indicate significant effects (P < 0.05). The abbreviations are SD = stocking density, FL = feeding level, C:N = carbon:nitrogen ratio, NFE = nitrogen-free extract.

Parameters	P values						
	SD	FL	C:N	SD*FL	SD*C:N	FL*C:N	SD*FL*C:N
Ash	**0.000**	0.655	**0.003**	0.161	0.209	0.261	0.080
Fat	**0.012**	0.686	0.892	0.220	0.348	0.980	0.861
Protein	**0.000**	0.112	0.351	0.460	0.145	0.169	**0.002**
NFE	**0.000**	0.528	**0.010**	0.298	0.070	0.330	0.371

Table 2.6 Biofloc proximate composition at different stocking densities of *L. vannamei*, and C:N ratios of nutrient inputs. Values are mean ± standard deviation of 27 tanks for the stocking densities 27 and 300 ind/m^3, and of 33 tanks for the stocking density 120 ind/m^3. Within rows, different superscripts indicate significant differences among stocking densities, or C:N ratios (P < 0.05). NFE = nitrogen free extract.

Parameters	Stocking densities (ind/m^3)			C:N ratios		
	27	120	300	7	12	16
Ash (%)	53 ± 5^a	43 ± 11^b	26 ± 13^c	47 ± 15^a	39 ± 15^b	36 ± 15^b
Fat (%)	0.6 ± 0.6^a	0.8 ± 0.8^{ab}	1.2 ± 0.6^b	0.9 ± 0.8	0.9 ± 0.5	0.8 ± 0.8
Protein (%)	17 ± 2^a	21 ± 4^b	12 ± 5^c	17 ± 6	17 ± 6	18 ± 5
NFE (%)	30 ± 3^a	35 ± 11^a	61 ± 12^b	35 ± 15^a	43 ± 17^b	45 ± 14^b

3.4. Water quality

The repeated measure three-way ANOVA showed that the chlorophyll a and TAN levels significantly increased, while the nitrite nitrogen levels decreased during the experiment (P_{Time} < 0.05). The stocking density, feeding level, and C:N ratio showed independent effects on the chlorophyll a concentrations in the water column (Table 2.7). Specifically, the chlorophyll a concentration was not affected by the feeding level, but increased with increasing stocking densities and C:N ratios. There were significant interactions among the three factors on the TAN levels ($P_{SD*FL*C:N}$ < 0.05). Meanwhile, nitrite nitrogen was significantly affected by stocking density and C:N ratio interactions, but not the feeding level.

Table 2.7 Probability (P) values for the main and interaction (*) effects of the three independent factors on water quality. P values in bold indicate significant effects (P < 0.05). The abbreviations are SD = stocking density, FL = feeding level, C:N = carbon:nitrogen ratio, TAN = total ammonia nitrogen, NO_2-N = nitrite nitrogen.

Parameters	P values						
	SD	FL	C:N	SD*FL	SD*C:N	FL*C:N	SD*FL*C:N
Chlorophyll a (μg/l)	**0.000**	0.910	**0.037**	0.680	0.766	0.205	0.296
TAN (mg/L)	**0.000**	**0.029**	0.143	**0.002**	0.220	0.951	**0.044**
NO_2-N (mg/L)	**0.000**	0.199	0.305	0.064	**0.014**	0.284	0.075

3.5. Microbiota

Linear mixed effects model analysis for the biofloc microbial community alpha-diversity (Shannon H' and rare taxa abundance), revealed significant effects coming from both C:N ratio and feeding levels. Although no treatment effect was observed on the microbial richness and Shannon H' (Figure 2.8A, 2.8B; P > 0.05), a significant effect of the C:N ratio in the abundance of rare taxa with increasing feeding levels was observed (Figure 2.8C; P = 0.0206).

Looking at the beta-diversity (microbial composition), the microbial communities were affected by both the C:N ratio and the feeding level (Two-way Permanova analysis; P = 0.001; Table 2.8). Significant differences were mainly found between C:N ratio 7 and 16 (P = 0.003) and feeding level 60 and 100 (P = 0.006), as indicated by Permanova (Bray-Curtis metric) and Principal component Analysis (Figure 2.9).

Table 2.8. Permanova results for biofloc microbiota based on Bray–Curtis distances.

	d.f.	**SS**	**MS**	**PseudoF**	**R^2**	***P*-value**
Feeding level	1	0.3920	0.39195	2.7103	0.09534	0.001**
C:N	1	0.4625	0.46254	3.1984	0.11251	0.001*
Feeding level x C:N	1	0.2198	0.21975	1.5195	0.05345	0.056
Residuals	21	3.0369	0.14462		0.73870	
Total	24	4.1112			1.00000	

* and ** Statistical significance at $P < 0.05$ and 0.01, respectively. Permutations n = 999. d.f., degrees of freedom; SS, sum of squares; MS, mean sum of squares.

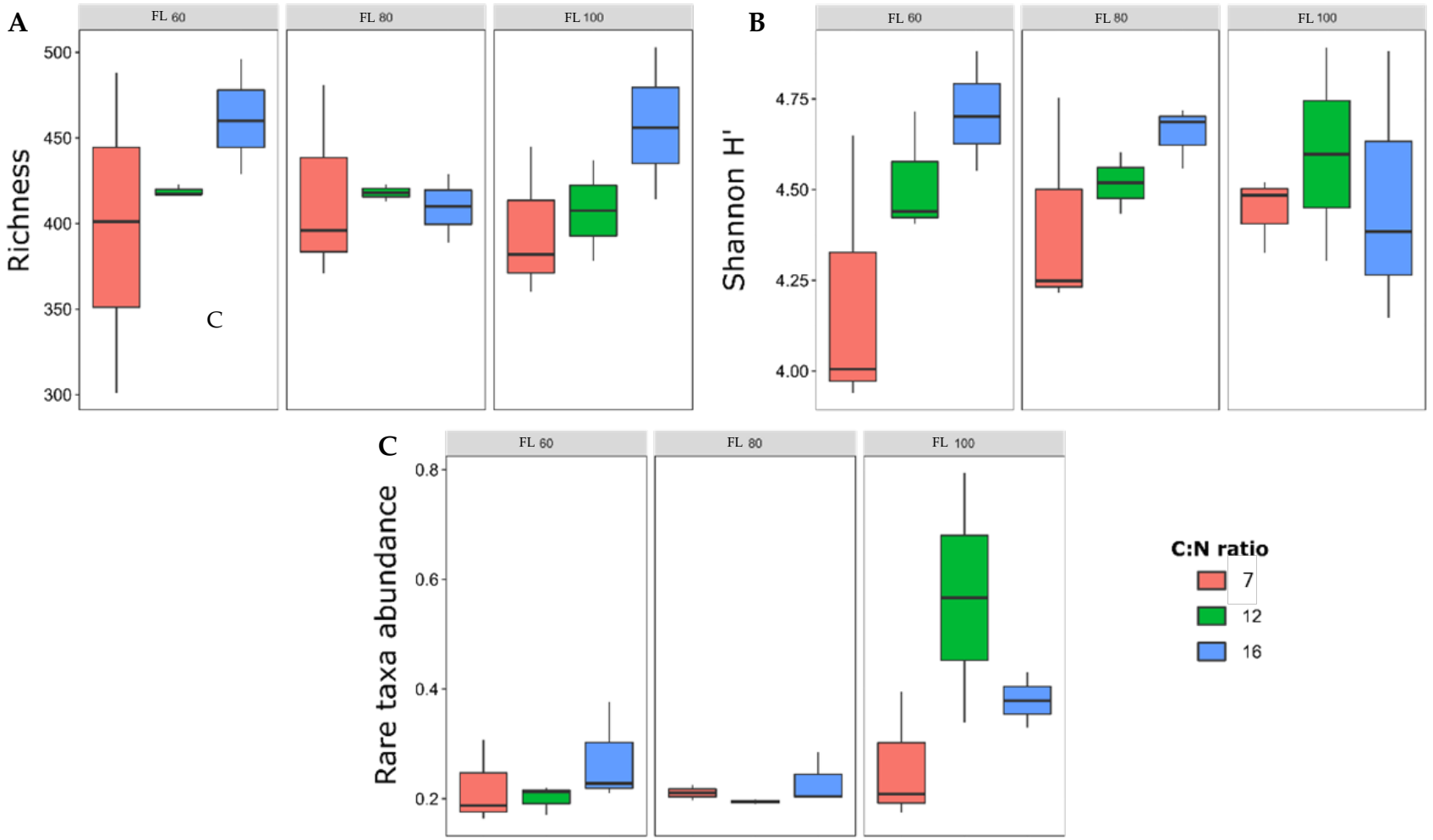


Figure 2.8 Alpha diversity of the biofloc microbial communities in the different treatments, referring to feed levels FL60, FL80 and FL100 and carbon:nitrogen ratios C:N7, C:N12 and C:N16 . Data are shown as box plots; the horizontal line indicates the median and the whiskers indicate the lowest and highest points within 1.5× the interquartile ranges of the lower or upper quartile, respectively.

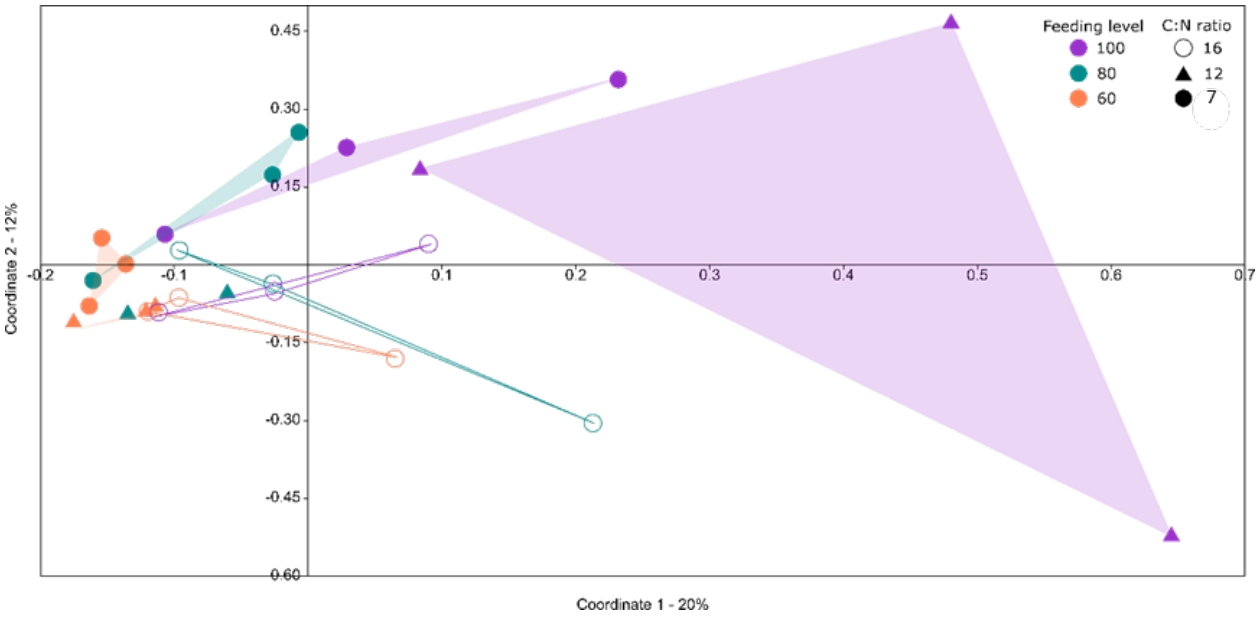


Figure 2.9 Principal coordinate analysis (PCoA) using Bray-Curtis metric. There was a significant clustering (Two-way Permanova, P = 0.001) affected by both the C:N ratio and the feeding level.

The relative abundance of the most abundant prokaryotic phyla and genera are presented in Figure 2.10. Among the phyla, Proteobacteria was the most abundant, followed by Bacteroidota, Actinobacteria, Planctomycetote and Chloroflexi. Regarding genera, a large portion of the microbial communities was not assigned to a genus (60% of the relative abundance), while the most abundant genus was *Maricauda*, *Pirelulla*, *Pseudoalteromonas* and *Ruegeria*. Looking at specific taxa that were affected by feeding level and C:N ratio, we detected several high abundant taxa using the DESeq2 analysis and SIMPER, respectively ($P < 0.05$; Figure 2.11). *Pirellula* and *Maricauda* genus increased with feeding level and C:N ratio (Figures 2.11B, 2.11C), while on the other hand *Pseudoalteromonas* decreased with feeding level but increased with C:N ratio (Figure 2.11A).

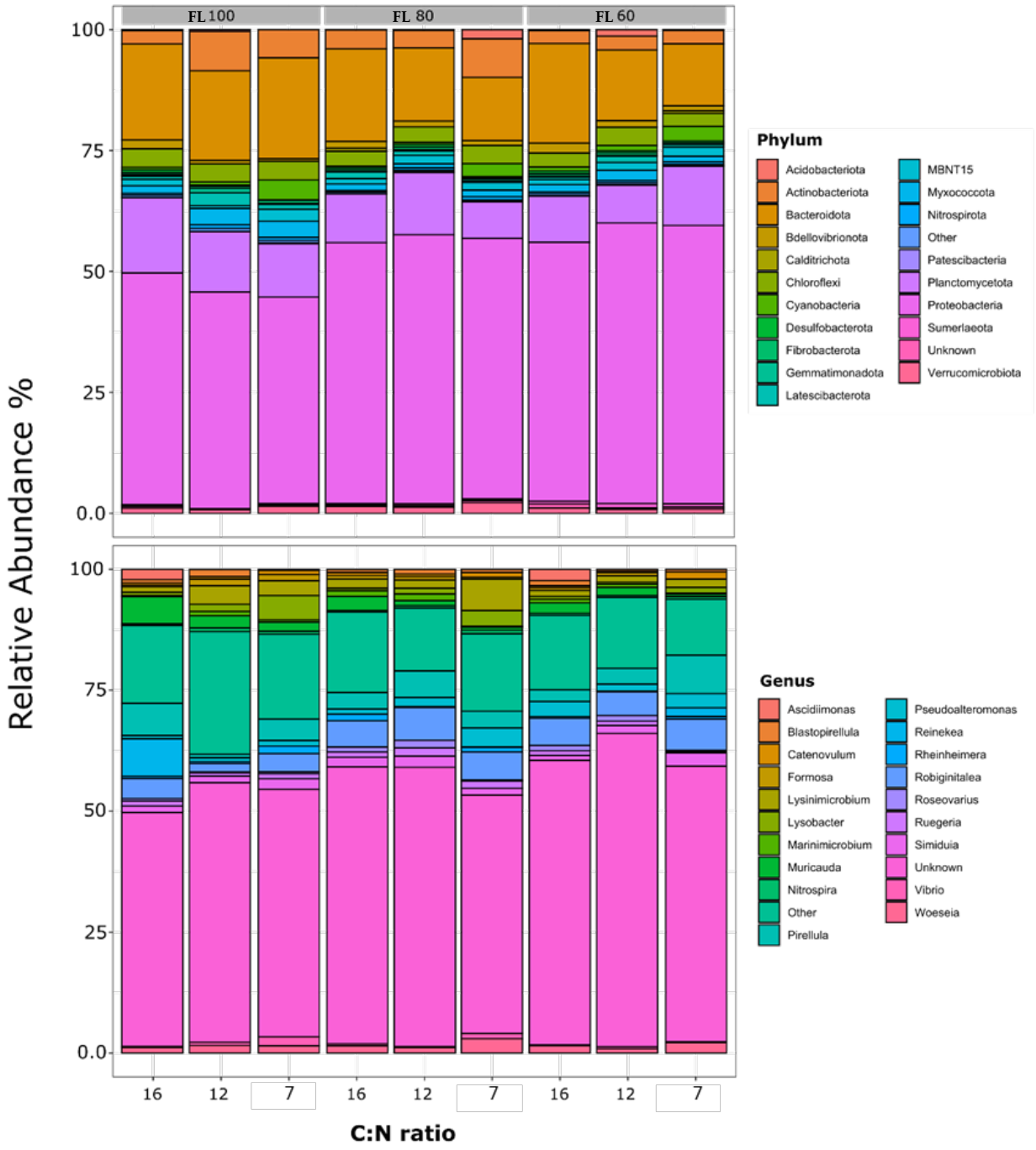


Figure 2.10 Prokaryotic microbial composition of the biofloc samples at the phylum (top) and genus level (bottom) among different treatments, referring to feed levels FL60, FL80 and FL100 and carbon:nitrogen ratios C:N7, C:N12 and C:N16 .

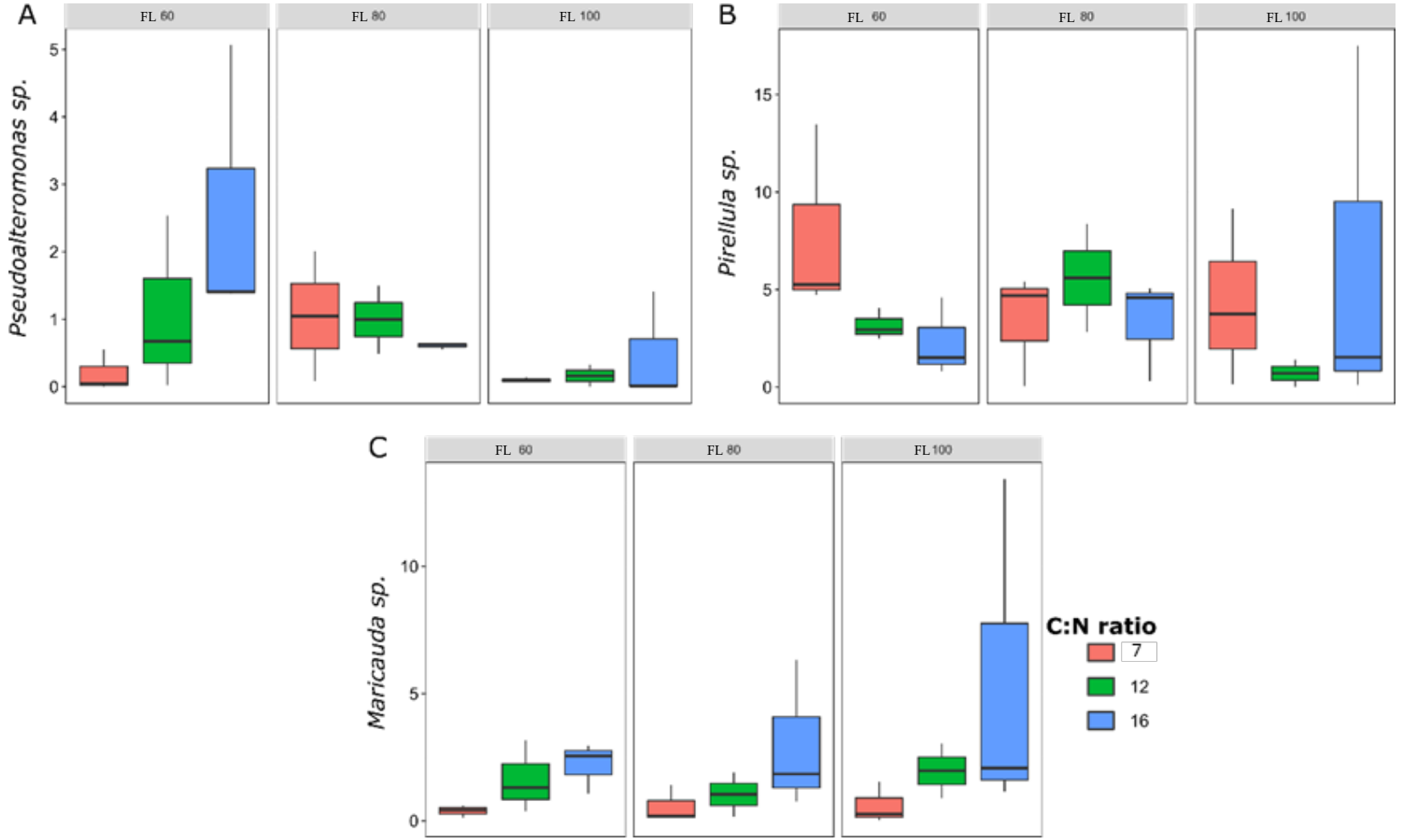


Figure 22.11 Relative abundance of significantly affected microbial taxa within the biofloc communities, as indicated by DESeq analysis ($P < 0.05$), referring to feed levels FL60, FL80 and FL100 and carbon:nitrogen ratios C:N7, C:N12 and C:N16 **.** Data are shown as box plots; the horizontal line indicates the median and the whiskers indicate the lowest and highest points within 1.5× the interquartile ranges of the lower or upper quartile, respectively. In the Y-axis, relative abundance is expressed in %.

4. Discussion

4.1 Shrimp performance

Shrimp exhibited a better growth with low stocking density, and high feeding level and C:N ratio in this research. The inverse relationship between stocking density and shrimp growth and survival was previously reported in conventional ponds (Sandifer et al., 1987; Sookying et al., 2011), recirculating systems (Williams et al., 1996), and biofloc systems in seawater (Krummenauer et al., 2011) or freshwater (Araneda et al., 2008). Reductions in growth and survival of cultured animals are due to increased competition for space and feed, and reduced water quality at high stocking density (Araneda et al., 2008; Liu et al., 2017; Williams et al., 1996). At high stocking density, cultured animals show depressed digestive enzyme activities, antioxidant status and immunology parameters (Liu et al., 2017), which could potentially explain our results.

In systems with high primary production, the feeding rate was reported to not significantly affect growth and survival, while the FCR significantly increased with increasing feeding rate (Roy et al., 2012). Similar results were found in biofloc systems with a TSS concentration of 246 ± 67 mg/L (Lara et al., 2017). The biofloc might contribute up to 29% of daily food intake (Burford et al., 2004). Guemez-Sorhouet et al. (2019) demonstrated that in biofloc systems, shrimp store a surplus of glycogen and carbohydrates in their hepatopancreas, which may increase their resistance to stress. In this research, promoting the biofloc development by increasing the C:N ratio also showed positive effects on shrimp growth. However, lowering the feeding level significantly reduced shrimp growth and survival. This could be due to low biofloc concentrations in most treatment tanks during the experiment, only at the end reaching 300 mg/L in some tanks, which was not able to compensate the effects of reduced feeding level. Schveitzer et al. (2013b) suggested a suitable TSS concentration of 400-600 mg/L for Pacific white shrimp culture. Higher TSS concentration may negatively affect shrimp survival and final biomass (Schveitzer et al., 2013b). Overall, in systems with abundant natural food, the daily feed ration can be lowered to as low as 60% without affecting the shrimp growth, while enhancing profitability (Roy et al., 2012).

Shrimp culture systems are dynamic, and interactions among variables in the systems have been of interest to many researchers. Moss and Moss (2004) reported significant effects of both stocking density and culture condition (i.e. substrate addition) on shrimp growth, but no significant density, substrate effect, or density and substrate interaction

on survival or water quality. Meanwhile, Guemez-Sorhouet et al. (2019) demonstrated stocking density and culture system interactions on shrimp growth. In biofloc and biofloc with substrate systems, shrimp growth was negatively correlated with stocking density, while such correlation was not found in clear water systems where nitrite and nitrate concentrations were higher than in other systems (Guemez-Sorhouet et al., 2019). Interactions among stocking density, feeding level, and C:N ratio on shrimp growth were not observed in this research. Nevertheless, we found interactions between the stocking density, feeding level, and C:N ratio on total biomass, survival, and FCR of the shrimp.

4.2 Water

In Moss and Moss (2004), the stocking density, substrate, or their interaction showed no effect on DO concentration and pH, while treatment effects on TAN and nitrite were not reported. Interestingly, Krummenauer et al. (2011) found similar nitrite concentrations among stocking densities, while TAN levels were lower at high stocking density. Contrasting results were reported by Tong et al. (2020), in which the TAN concentration was not affected by the feeding level, and the nitrite concentration increased with feeding level.

The positive effects of increasing the C:N ratio on TAN, nitrite, and nitrate concentration reduction were demonstrated in numerous research (Asaduzzaman et al., 2008; Dauda et al., 2018; Hari et al., 2006). However, results were also reported, in which the addition of carbohydrate showed no significant effects on TAN, nitrite, and nitrate (Pérez-Fuentes et al., 2016; Xu and Pan, 2012; Xu et al., 2016). This could be due to the different carbon sources used (Table 2.9). Tinh et al. (2021a) demonstrated that at the same C:N ratio, corn starch and molasses affected the shrimp culture system differently. In this research, we found significant three-way interactions between stocking density, feeding level, and C:N ratio on TAN concentrations. In addition, the nitrite concentrations were not affected by the feeding level, but significantly influenced by stocking density x C:N ratio interaction. Therefore, differences in the experimenteal design, for example tested species, stocking densities, experimental durations, or biofloc concentration and composition (Table 2.9), could also be the reason for contradictory conclusions on the effect of increasing C:N ration. This again confirmed the dynamics of shimp culture systems, and that biofloc systems are interfered by numerous additional factors, for example dietary ingredient sources (Shao et al., 2017), carbohydrate types (Tinh et al., 2021b), and biofloc levels (Schveitzer et al., 2013b).

Table 2.9 Some parameters of the experimental design among research on C:N ratios

Carbon addition	References	Tested species	Density (ind/m^3)	Duration (weeks)	Tested C:N ratios	Carbon sources
Supported	Asaduzzaman et al., 2008	*M. rosenbergii*	2	16	10, 15, 20	tapioca
	Dauda et al., 2018	*C. gariepinus*	250	6	10, 15, 20	glycerol
	Hari et al., 2006	*P. monodon*	7	16		tapioca
Not supported	Pérez-Fuentes et al., 2016	*O. niloticus*	75	24	10, 12, 15, 17, 21	molasses
	Xu and Pan, 2012	*L. vannamei*	224	4	7, 12, 15	sucrose
	Xu et al., 2016	*L. vannamei*	300	6	9, 10, 12, 15, 18	molasses

4.3 Biofloc

In numerous research, biofloc mass in terms of TSS and VSS are expected to increase with feeding level and C:N ratio (Asaduzzaman et al., 2010; Panigrahi et al., 2018; Pérez-Fuentes et al., 2016; Tong et al., 2020; Xu and Pan, 2012). It was also reported that the feeding level, C:N ratio, or their interaction showed no significant effects on both TSS and VSS (Xu and Pan, 2014). Meanwhile, Xu et al. (2016) found that while the TSS demonstrated no specific correlation with C:N ratio, the VSS and C:N ratio were positively correlated. Interestingly, Liu et al. (2018) demonstrated that the biofloc mass increased more rapidly in carbohydrate-added system, however reached a similar plateau in all treatments at the end of the experiment. In this research, biofloc mass was affected by various two-way interactions between stocking density, feeding level, and C:N ratio. At higher stocking densities, positive correlations between biofloc mass and feeding level or C:N ratio are more likely to be seen.

The biofloc protein content, 17 ± 5% on average of all treatments, was low compared to most previous research. This could be due to the type of carbohydrate used (rice bran – tapioca mixture). Tinh et al. (2021a) reported a biofloc protein content of 39-43%, depending on whether corn starch or molasses was used as the carbon source. The biofloc proximate composition was mainly affected by the stocking density, and to a lesser extent by C:N ratio in this research, while the biofloc protein content was affected by the three-way interaction among factors.

4.4 Microbiota

The microbial diversity within the biofloc, composed of heterotrophic bacteria, microalgae, zooplankton and rotifers (De Schryver et al., 2008), has been reported to be affected by the C:N ratio (Deng et al., 2018; Guo et al., 2020; Panigrahi et al., 2018). This

was also observed in the present study, with diversity increasing with C:N ratio increase. How the feeding level affects the microbial diversity in biofloc, to our knowledge, has not been reported before. In this research, the microbial diversity and abundance of rare taxa increased with feeding level, which could be potentially related to a higher presence of nutrients available to the biofloc due to a higher waste production by the shrimp. High microbial diversity in water and shrimp gut has been reported to increase system stability and shrimp tolerance to diseases (De Schryver and Vadstein, 2014; Rungrassamee et al., 2016). However, in this study we did not explore such an effect on shrimp health.

The effect of stocking density on the biofloc microbiota diversity was not significant in this research, but was previously reported in Deng et al. (2019). The microbial community was more diverse at high stocking density (800 ind/m^3), followed by low and medium stocking densities (400, and 600 ind/m^3, respectively) (Deng et al., 2019). Besides, the types of carbohydrate used play an important role in shaping the microbial community in biofloc systems (Deng et al., 2018; Li et al., 2018). With regard to the microbiota composition, our results are in accordance with previous studies, with Proteobacteria being the most abundant phylum, followed by Bacteroidetes, Planctomycetes and Actinobacteria, and genera such as *Maricauda* and *Ruegeria* (Deng et al., 2019; Gutiérrez et al., 2016; Huang et al., 2020; Tinh et al., 2021c). We observed a significant increase on the abundance of *Maricauda sp*. and *Pseudoalteromonas sp*. taxa with increasing C:N ratio. The marine bacterium *Pseudoalteromonas* and *Muricauda* sp., which has been previously identified as a core biofloc species (Deng et al., 2019; Huang et al., 2020; Tinh et al., 2021c) have antimicrobial properties against pathogenic microbes (i.e *Staphylococcus aureus*) and quorum quenching properties, respectively, while at high C:N ratios both have been reported to increase their biomass (López-Alcántara et al., 2022; Zhang et al., 2022). These altogether show that the microbiota in biofloc systems is sensitive to variations in the system management, and more research is needed to fully understand its mechanism and direction of changes, especially if these are connected to health-promoting communities.

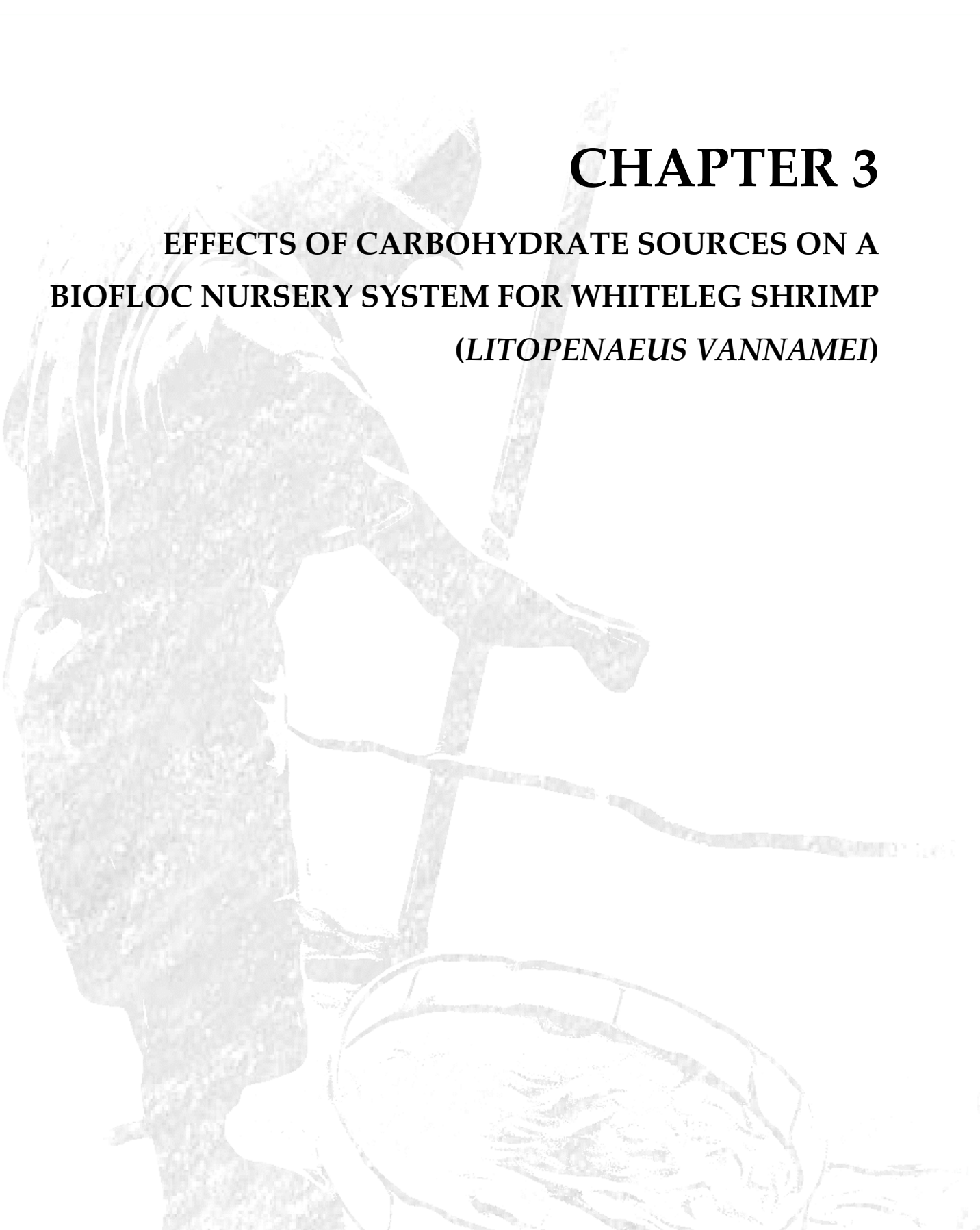
5. Conclusions

Shrimp growth and biofloc biomass increase (TSS, VSS) are in a similar way affected by stocking density, feeding level, and C:N ratio. Meanwhile, the biofloc proximate composition is mainly affected by stocking density, and to a lesser extent by C:N ratio. The microbiota community composition is mainly affected by C:N ratio, with feeding level having a smaller impact. Future research should focus on how the type of

carbohydrates and methods of administration affect shrimp growth and biofloc formation.

CHAPTER 3

EFFECTS OF CARBOHYDRATE SOURCES ON A BIOFLOC NURSERY SYSTEM FOR WHITELEG SHRIMP (*LITOPENAEUS VANNAMEI*)



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Tinh, T.H., Koppenol, T., Hai, T.N., Verreth, J.A.J., Verdegem, M.C.J., 2021. Effects of carbohydrate sources on a biofloc nursery system for whiteleg shrimp (Litopenaeus vannamei). Aquaculture 531, 735795. https://doi.org/10.1016/j.aquaculture.2020.735795

Abstract

In biofloc technology, carbohydrate is added to stimulate the biofloc growth, the latter helps to improve water quality, reduce the need for water exchange and may serve as natural shrimp feed. The large diversity among possible carbohydrate sources makes the selection of a suitable carbohydrate difficult. This study investigated how corn starch addition compared to molasses addition affected water quality, biofloc and periphyton proximate composition, shrimp production parameters, diurnal fluctuations and distribution of carbon and nitrogen in whiteleg shrimp (*Litopenaeus vannamei*) culture system. The results showed that both corn starch and molasses addition treatments resulted in low total ammonia nitrogen levels in the water. The total suspended solids and volatile suspended solids in both treatments increased over time and were not significantly different among treatments. The protein content in the dry matter of the biofloc varied from 34% to 48%, being higher in the molasses treatment. The same was observed for the protein content in the dry matter of the periphyton which ranged between 16% and 26%. The corn starch treatment resulted in significantly higher shrimp growth rate, production, average body weight, and lower FCR compared to molasses addition. Water quality was stable on a daily basis but changed over the weeks. Carbon and nitrogen accumulation in the system was not significantly different among treatments.

Keywords: Biofloc, corn starch, diurnal fluctuation, molasses, nutrient accumulation

1. Introduction

The role of aquaculture in fish supply for human consumption has been increasing since the 1980s (FAO, 2018). To meet the food fish demand of a growing global population, global aquaculture production is expected to increase by 62% from 2010 to 2030, especially in countries like China, India, and in Southeast Asia and Latin America (World Bank, 2013). The future expansion of the aquaculture industry should preferably occur in a resource efficient way (Crab et al., 2012; World Bank, 2013). This includes better use of basic natural resources such as water and land, and fish feed which are the major pre-requisites for aquaculture activities.

The biofloc technology may provide the necessary solutions. The addition of organic carbohydrate (CHO) in the biofloc system provides an energy source for microbial organisms to immobilize ammonia or nitrate into microbial biomass (Avnimelech, 1999). This process helps lowering total ammonia nitrogen and nitrite levels, thereby reducing the need for water exchange (Gao et al., 2012; Hu et al., 2014). At the same time, the generated microbial biomass forms aggregates, called biofloc, which serves as natural food for culture species and increases feed use efficiency (Burford et al., 2004; Emerenciano et al., 2012). The application of biofloc systems is also beneficial for immunological responses of whiteleg shrimp against infectious agents (Ekasari et al., 2014b; Kim et al., 2014; Verma et al., 2016).

The diversity of possible CHO sources causes difficulties in the adoption of the biofloc system. Potential CHO sources may include simple ones such as molasses, glycerol, and glucose, and complex ones such as flour and starch (Crab et al., 2010; Guo et al., 2006; Nikodinovic-Runic et al., 2013). Different CHO sources yield different nutritional values of the biofloc (Crab et al., 2010; Rajkumar et al., 2016; Wei et al., 2016). In addition, they have varying effects on the composition of the microbial community in the biofloc (Deng et al., 2018; Wei et al., 2016), the production (Khanjani et al., 2016; Rajkumar et al., 2016) and the immunity of the cultured fish and shrimp (Ekasari et al., 2014b; Verma et al., 2016). The underlying mechanism for some of these differences may lie in the complexity of CHO structure (Khanjani et al., 2016; Wei et al., 2016). These results altogether indicate the importance of selecting a suitable CHO for a successful biofloc technology.

In biofloc technology, feed and CHO represent major sources of organic material entering the system. Following feeding, the oxygen consumption and ammonia excretion by fish significantly increase, creating high fluctuations in total ammonia nitrogen concentration

on a daily basis (Zakęś et al., 2006). However, following the addition of CHO in tilapia culture the total ammonia nitrogen, nitrite, and nitrate concentrations did not show significant diurnal fluctuations (Hu et al., 2014). Equivalent information regarding diurnal changes of water quality parameters in the biofloc system for whiteleg shrimp remains scarce. Besides, it is currently unknown how the carbon and nitrogen inputs are accumulated in different compartments (e.g. shrimp, biofloc, water) of the biofloc culture of whiteleg shrimp. Further research on nutrient recycling in biofloc system is still needed.

This research aimed at comparing the effects of different CHO on water quality, biofloc and periphyton proximate composition, shrimp production parameters, the diurnal fluctuation of water quality parameters following the addition of feed and CHO, and quantifying the accumulation of nutrients (input carbon, and nitrogen) in different compartments of the biofloc shrimp culture. Corn starch and molasses were used as representatives of complex dietary CHO and simple by-product CHO, respectively.

2. Materials and methods

The experiment was performed at the animal research facility of Wageningen University (Carus), the Netherlands from January to February of 2018.

2.1. Pre-treatment

Six weeks prior to the experiment, biofloc was produced in 6 indoor plastic tanks (900-L total volume; 600-L working volume) stocked with 1.5 kg/m^3 of 93 g individual weight tilapia. The fish were fed twice per day with a 33% protein feed at 2.5% body weight per day, assuming a feed conversion ratio (FCR) of 1.2. Per kilogram of feed fed, 1.52 kg corn starch was added to the tank following each feeding to maintain a C:N ratio of 20 for optimal biofloc growth (Pérez-Fuentes et al., 2016).

The tanks were provided with continuous aeration, and 12h/12h dark/light regime. The salinity of culture water was increased gradually, starting at 0 ppt and reaching 20 ppt by the end of the pre-treatment period. Before pumping water to treatment tanks, tilapia from pre-treatment tank were removed, and pre-treated water was mixed together and adjusted to the salinity of 25 ppt using fine salt (Suprasel® Classic, Akzo Nobel Functional Chemicals B.V., the Netherlands).

2.2. Experimental design

The experiment was conducted with corn starch and molasses as treatments of CHO source, each in triplicate. The same 6 tanks as in the pre-treatment period were used. The tanks were placed indoor with temperature control, 12h/12h dark/light regime providing the average light intensity of 9800 lux. Prior to stocking shrimp, treatment tanks were filled with 4 cm of living sand bottom, 600 L of flocculated water. Culture water was continuously aerated by installation of an air ring (made from PVC tube with holes) at the bottom of the tank.

2.3. Experimental animal and feeding

Shrimp (0.075 ± 0.006 gram per individual) were obtained from CreveTec, Ternat, Belgium, and stocked at the density of 250 shrimp/m^2 into their respective treatment tanks. Shrimp were fed twice per day at 9 AM and 3 PM with 34% protein feed (CreveTec) at the feeding level of 8% BW per day, and assumed FCR of 0.7, reaching a maximum feeding rate of 43g/m^3/day.

After feeding, corn starch and molasses were added immediately to their respective treatments. For each kilogram of shrimp feed fed, 0.6 kg of corn starch or 1.1 kg of molasses were added to maintain an input C:N ratio of 12. A summary of shrimp feed ingredients and proximate composition of shrimp feed and CHO sources can be found in Tables 3.1 and 3.2.

2.4. Water quality monitoring

During the pre-treatment and experimental periods, water temperature and pH were monitored using electric probes, and total ammonia nitrogen concentration was checked daily and nitrite concentration irregularly using test kits (Merck MQuant®). Dissolved oxygen was monitored and maintained at the level above 6 mg/L during the entire duration of the experiment. There was no water exchange during the experimental period.

2.5. Sample collection and analysis

The culture system was divided into 5 compartments, including shrimp, water (filtrate after filtration at 1.5 μm pore size), biofloc (materials remaining on 1.5 μm pore size filter), periphyton (materials sticking on the tank wall), and sediment. Samples were taken periodically to assess the effects of treatments on and the distribution of nutrients in each compartment, as detailed below.

Shrimp samples were collected as a composite sample at the beginning and separately from each tank at the end of the experiment for determination of average body weight and survival rate. Samples were freeze-dried for one week, and grinded prior to the analysis of dry matter and ash (following ISO 6496, 1999), crude protein (following ISO 5983, 2005), energy (following ISO 9831, 1998), total carbon (TC) and total nitrogen (TN) contents (using Dumas analyzer). Samples of feed, corn starch, and molasses were also preserved for the analysis of similar parameters as with shrimp samples. In addition, mineral contents in corn starch and molasses including phosphorus (P), calcium (Ca), iron (Fe), magnesium (Mg), potassium (K), and manganese (Mn) were analyzed using the segmented flow analyzer (SAN++, Skalar Analytical B.V., the Netherlands). Corn starch and molasses were separately dissolved in sterile de-ionized water to a similar concentration, and used for a 5-day biological oxygen demand (BOD_5) assay following the standard protocol (APHA, 1995).

Water samples were collected at the beginning of the experiment and weekly onwards. Unfiltered water samples were analyzed for total suspended solids (TSS), volatile suspended solids (VSS), chlorophyll a, b, and c following the Standard Methods for the Examination of Water and Wastewater (APHA, 1995). Suspended solids using Imhoff cone were not determined because TSS gives a more accurate estimation of biofloc biomass (Xu et al., 2016). The filtrates, after having gone through a glass microfiber filter of 1.5 μm pore size, were acidified with 3N HCl to pH of 2-3, and analyzed for total carbon (TC), inorganic carbon (IC), total nitrogen (TN), total ammonia nitrogen (TAN), nitrite and nitrate (NO_x), phosphate phosphorus (PO_4-P) using a segmented flow analyzer (SAN++, Skalar Analytical B.V., the Netherlands).

Biofloc samples were collected at the beginning of the experiment and the end of weeks 1, 3, and 5 by filtration through glass microfiber filter of 1.5 μm pore size. Samples were kept at -20^{o}C until further analysis of proximate composition (following APHA, 1995), total energy following ISO 9831 (1998), total carbon and total nitrogen contents (using LECO CN 628 Dumas analyzer, LECO Instrumente GmbH., Germany).

Soil samples were collected at the start of the experiment, and the end of weeks 3 and 5. Samples were collected by pressing a Greiner tube on to the bottom surface, and were kept at -20^{o}C until further analysis of proximate composition, total carbon and total nitrogen contents employing the same protocols as with shrimp samples.

Periphyton samples were collected at the end of weeks 3 and 5, as periphyton was not present at the beginning of the experiment. To allow easy periphyton collection, two white plastic solid sheets of 15 cm in width were attached to the basin wall, going all the way from the tank bottom to above the water surface. Periphyton samples were preserved at -20^{o}C prior to the determination of their proximate composition, energy, carbon and nitrogen contents.

2.6. 24h measurements

To examine the diurnal fluctuation of water quality parameters, on day 32 of the experiment, water and biofloc samples were taken one hour after the first feeding (9 AM) and every 3 hours onwards for a period of 24 hours. Water samples were analyzed for TAN, NO_x, TSS, VSS, total carbon and total nitrogen; biofloc samples were analyzed for total carbon and total nitrogen, using the aforementioned methods.

2.7. Data analysis

Statistical analysis was performed using IBM SPSS Statistics 25 software (IBM Corporation, NY, USA). The effects of treatment on shrimp production and proximate composition, and nutrient accumulation were analyzed using One-way ANOVA. The effects of treatment on weekly and diurnal water quality, biofloc-related and periphyton-related parameters were analyzed using Repeated measure ANOVA. A probability value (P) of less than 0.05 was used to indicate significant differences.

Table 3.1 Shrimp diet composition (CreveTec, Ternat, Belgium).

Ingredients	%
Fishmeal	16
Fish oil	1
Wheat gluten	10
Soybean meal	10
Krill protein hydrolysate	1
Wheat flour	27.6
Wheat	20
Wheat bran	10
Cholesterol	0.2
Soya lecithin	0.5
Monocalcium phosphate	1.6
CaCO3	0.4
Premix	1
Lysine HCL	0.3
DL-methionine	0.2
L-Threonine	0.2

Table 3.2 Nutritional composition of shrimp diet and carbohydrates on dry weight (DW) basis.

Proximate compositions	Values
Feed:	
Dry matter (g/kg wet weight)	911
Crude protein (g/kg)	341
Ash (g/kg)	69
Energy (kJ/g)	20
Corn starch:	
Dry matter (g/kg wet weight)	884
Crude protein (g/kg)	3
Ash (g/kg)	1
Energy (kJ/g)	18
Molasses:	
Dry matter (g/kg wet weight)	630
Crude protein (g/kg)	85
Ash (g/kg)	171
Energy (kJ/g)	18

3. Results

3.1. Effects on shrimp production

A summary of shrimp production parameters is shown in Table 3.3. After the 5-week culture period, shrimp body weight in the corn starch treatment (2.47 ± 0.13 g/ind) was significantly higher than that in the molasses treatment (1.32 ± 0.01 g/ind) ($P < 0.05$). Shrimp survival rate was above 90% in both treatments, and significantly higher in the corn starch treatment ($P < 0.05$). Total shrimp biomass in the corn starch treatment was double of that in the molasses treatment. Specific growth rate was significantly higher in the corn starch treatment ($P < 0.05$). Overall, the corn starch addition resulted in better production parameters compared to the molasses addition.

Table 3.3 Shrimp production parameters at the end of 5-week experiment.

Treatments	Body weight (g/ind)	Biomass (g)	Weight gain (g/ind)	Survival (%)	FCR	FCR (incl. CHO)	Growth rate (% BW/day)
Corn starch	2.5 ± 0.1	355 ± 15	2.4 ± 0.1	96 ± 1	1.3 ± 0.1	2.0 ± 0.1	10.1 ± 0.3
Molasses	1.3 ± 0.0	178 ± 4	1.2 ± 0.1	90 ± 2	2.6 ± 0.1	5.5 ± 0.1	8.1 ± 0.1
P values	***	***	***	*	***	***	**

Presented values are the averages ± SD of each treatment, with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
FCR – feed conversion ratio.
FCR (incl. CHO) – feed conversion ratio considering the amount of carbohydrate added.

Table 3.4 Proximate composition of initial and final shrimp samples.

Parameters	Initial sample	Final samples		
		Corn starch	Molasses	P values
Dry matter (g/kg WW)	130	217 ± 8	199 ± 4	**0.033**
Ash (g/kg DW)	188	148 ± 4	156 ± 3	**0.045**
Crude protein (g/kg DW)	741	728 ± 12	742 ± 10	0.212
Total energy (kJ/g DW)	18.9	20.3 ± 0.1	19.8 ± 0.0	**0.001**
Carbon-nitrogen ratio	3.5	3.8 ± 0.1	3.6 ± 0.1	**0.044**

Values are means (±SD). Initial sample was analyzed on a batch sample of post-larvae (n = 1). Probability (P) values given only relate to final samples. P values ($P < 0.05$) in **bold** indicate significant effects.

Proximate analysis showed that on average, final shrimp samples had higher C:N ratio, dry matter and energy contents, but lower ash content compared to the initial shrimp sample (Table 3.4). Shrimp in the corn starch treatment contained more dry matter and total energy, and a higher C:N ratio, compared to shrimp in the molasses treatment ($P < 0.05$). Meanwhile, shrimp in the corn starch treatment contained significantly less ash

than in the molasses treatment ($P < 0.05$). Crude protein content in shrimp was not different among treatments ($P > 0.05$).

3.2. Effects on water quality

Overall, the total ammonia nitrogen levels remained low throughout the experimental period. The TAN concentration was highest at the beginning of the experiment (Figure 3.1). The TAN concentration decreased from 0.38 mg/l to less than 0.1 mg/L in both treatments during the first week and remained below that level subsequently. Measured TAN concentrations in the molasses treatment in weeks 3, 4, and 5 were significantly lower than those in the corn starch treatment ($P < 0.05$).

In both treatments, the NO_x-N concentration increased during the first week of the experiment (Figure 3.2). The NO_x-N concentration declined from weeks 1 to 4, and increased in the last week of the experiment. The differences in the NO_x-N concentration were not significant between the two treatments ($P > 0.05$). When verified, the NO_2-N concentration was always below 0.5 mg/L.

The fluctuation of dissolved nitrogen showed relatively similar patterns among treatments (Figure 3.3). Organic and inorganic nitrogen increased in the first week of the experiment, and then decreased form week 1 to week 4. In week 5, organic and inorganic nitrogen content in water showed a slight increase in both treatments. The inorganic nitrogen in water was not different among treatments at all sampling points ($P > 0.05$), while the organic nitrogen in the molasses treatment was significantly higher than that in corn starch treatment from week 2 onwards ($P < 0.05$).

The dissolved inorganic carbon remained relatively constant throughout the experimental period (Figure 3.3), but was significantly higher in the molasses treatment than in the corn starch treatment in weeks 4 and 5 ($P < 0.05$). Meanwhile, the organic carbon (OC) in water of the molasses treatment kept increasing and was higher than that of the corn starch treatment in weeks 2, 3, 4, and 5 ($P < 0.05$). Chlorophyll a concentration was not different among treatments, showing an increase between weeks 1 and 3, and a decrease between weeks 3 and 5 (Figure 3.4).

Repeated measures ANOVA showed that all measured parameters changed significantly over weeks ($P < 0.05$) (Table 3.5). The effects of treatments on TC, IC, OC, ON, TAN in water were significantly different from each other ($P < 0.05$). However, their effects on TN, IN, NO_x-N, PO_4-P, and chlorophyll parameters were not different ($P > 0.05$).

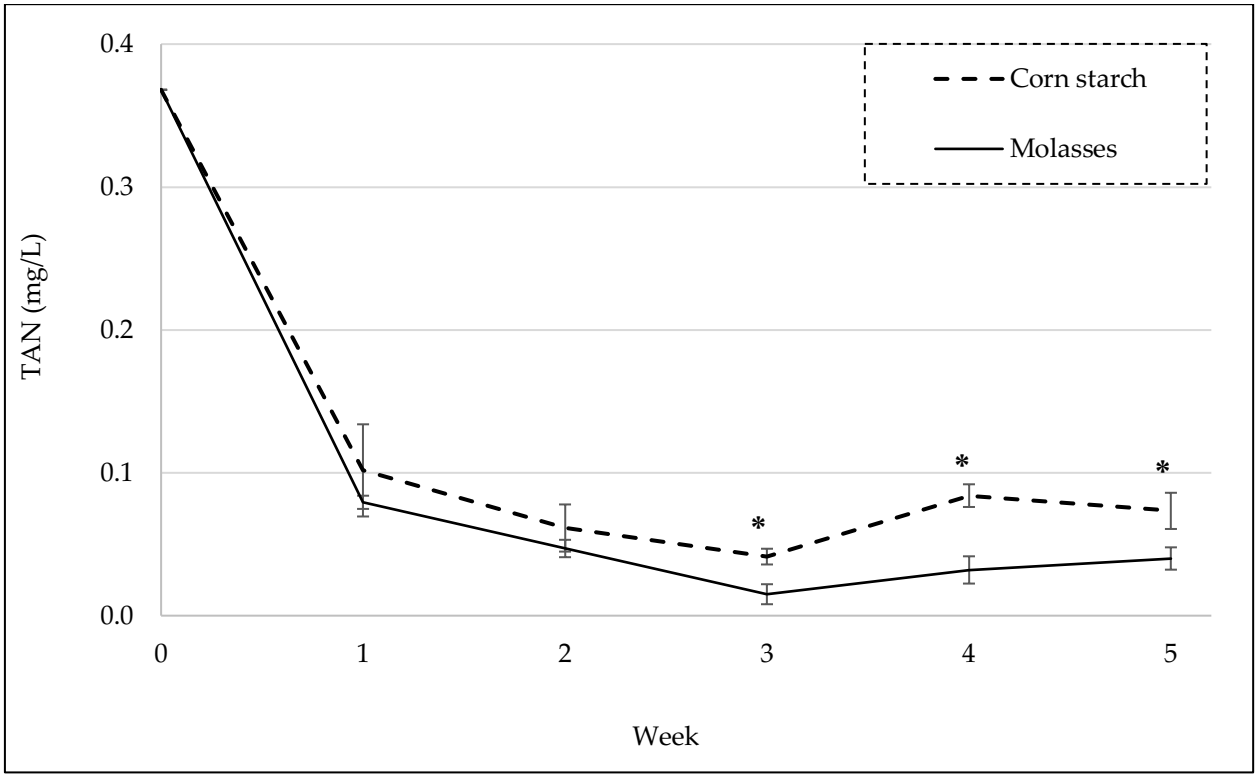


Figure 3.1 Weekly changes of total ammonia nitrogen (TAN) concentration in water by treatment (carbohydrate source). Values are means (±SD) of three replicate tanks per sampling time in each treatment. The asterisks (*) indicate weeks with significant difference among treatments ($P < 0.05$).

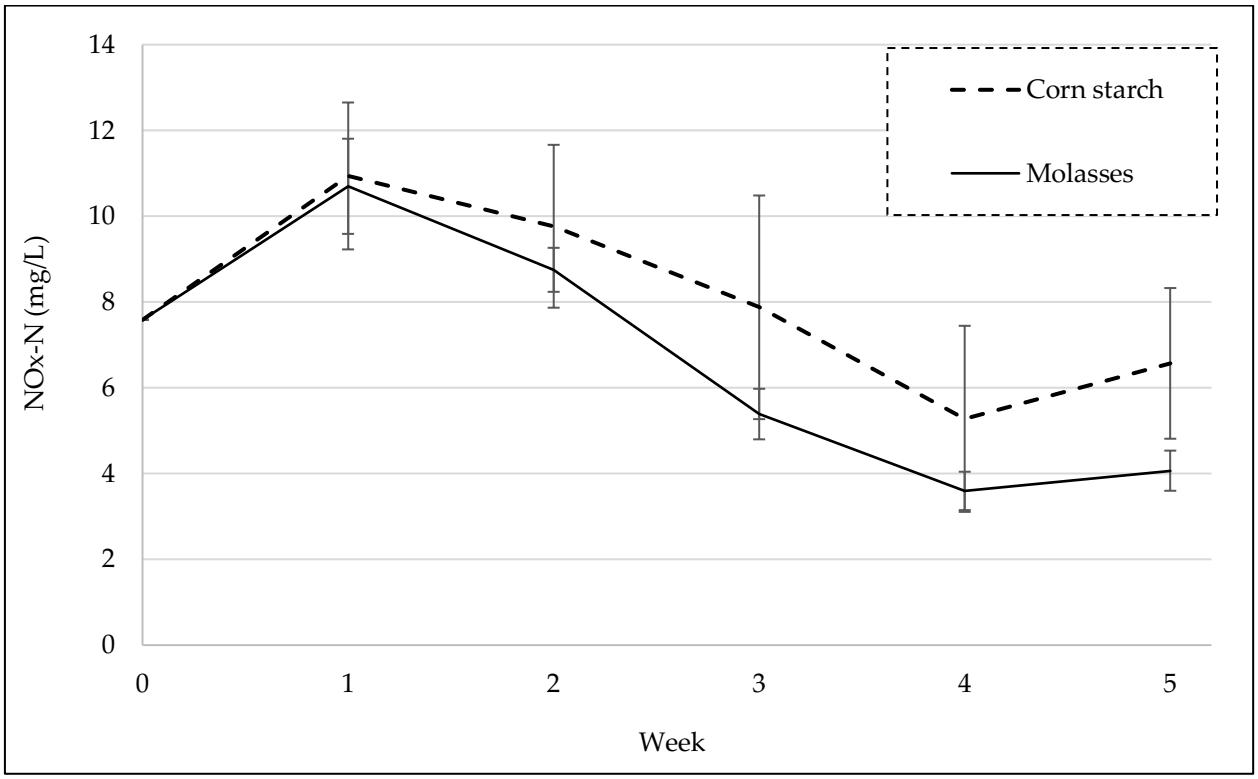


Figure 3.2 Weekly changes of nitrite and nitrate nitrogen (NO_x-N) concentration in water by treatment (carbohydrate source). Values are means (±SD) of three replicate tanks per sampling time in each treatment.

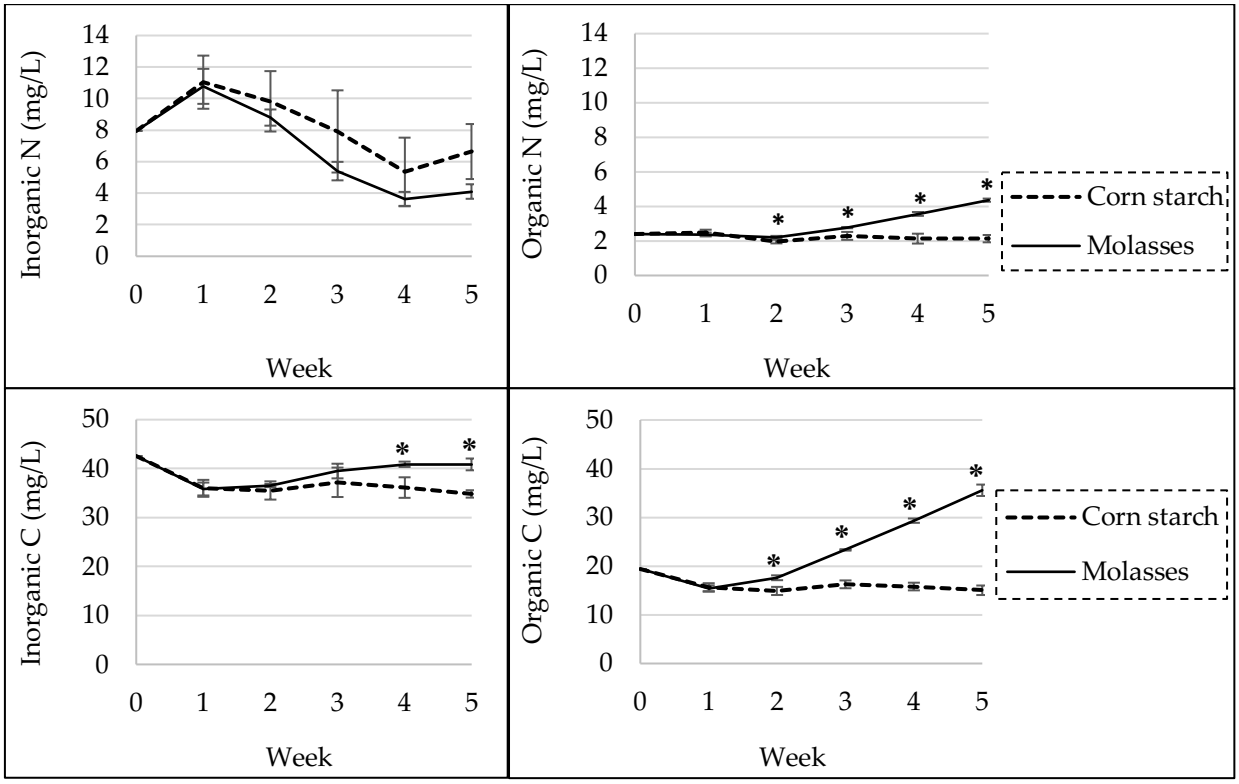


Figure 3.3 Weekly changes of dissolved nitrogen (N) and carbon (C) in water by treatment (carbohydrate source). Values are means (±SD) of three replicate tanks per sampling time in each treatment. The asterisks (*) indicate weeks with significant difference among treatments ($P < 0.05$).

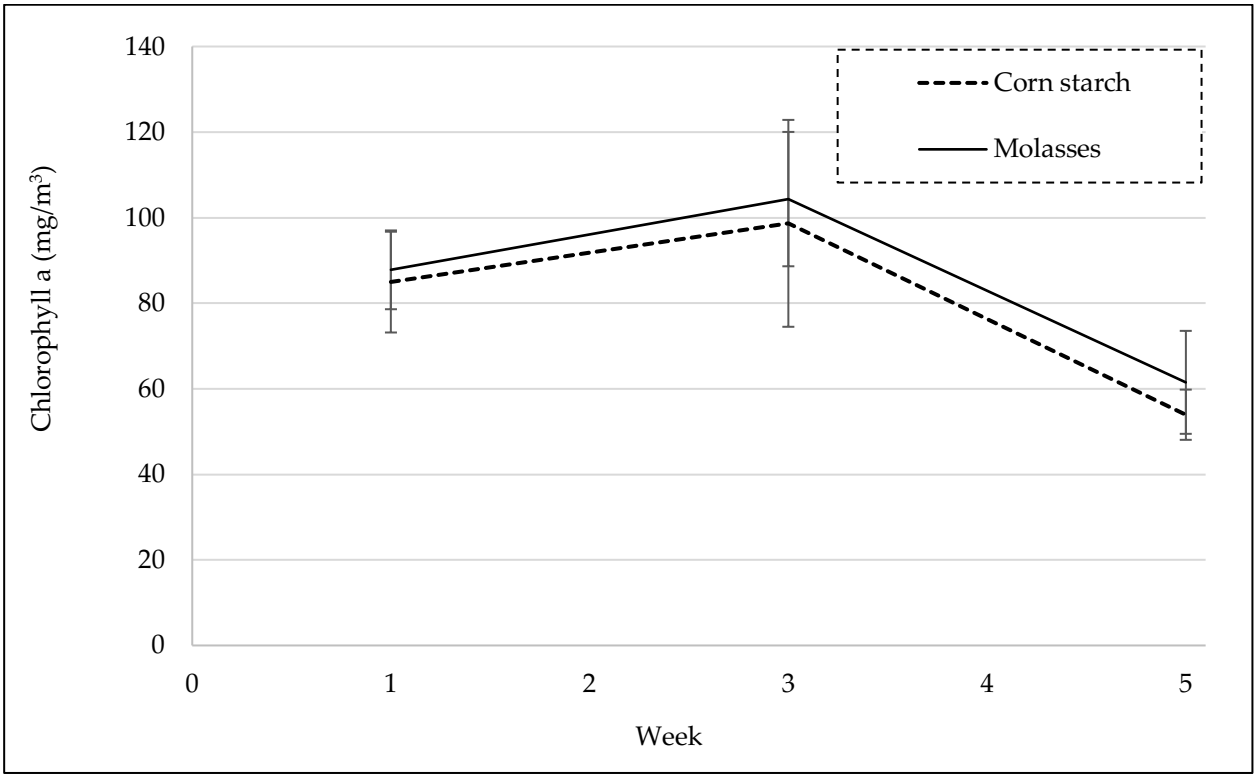


Figure 3.4 Changes of chlorophyll a concentration in water by treatment (carbohydrate source). Values are means (±SD) of three replicate tanks per sampling time in each treatment.

Table 3.5 Summary of selected parameters in water compartment by treatment (carbohydrate source). Values are means (±SD) of six sampling times for each treatment. Probability (P) values in bold indicate significant effects ($P < 0.05$).

Parameters	Treatments		P values		
	Corn starch	Molasses	Treatment	Time	Interaction
TC (mg/L)	52 ± 2	63 ± 10	**0.000**	**0.000**	**0.000**
IC (mg/L)	36 ± 2	39 ± 2	**0.036**	**0.006**	**0.005**
OC (mg/L)	16 ± 2	24 ± 7	**0.000**	**0.000**	**0.000**
TN (mg/L)	10 ± 3	10 ± 2	0.510	**0.000**	0.457
IN (mg/L)	8 ± 3	7 ± 3	0.205	**0.000**	0.168
ON (mg/L)	2.2 ± 0.3	3.1 ± 0.8	**0.000**	**0.000**	**0.000**
TAN (mg/L)	0.07 ± 0.03	0.04 ± 0.02	**0.005**	**0.000**	0.196
NO_x-N (mg/L)	8 ± 3	7 ± 3	0.211	**0.000**	0.177
PO_4-P (mg/L)	1.1 ± 0.3	1.0 ± 0.2	0.192	**0.000**	0.817
Chl a (mg/m^3)	79 ± 24	85 ± 22	0.608	**0.000**	0.920
Chl b (mg/m^3)	61 ± 21	70 ± 24	0.094	**0.002**	0.940
Chl c (mg/m^3)	95 ± 30	111 ± 39	0.115	**0.002**	0.984

3.3. Effects on biofloc production

The biofloc growth was evaluated based on changes in TSS and VSS concentrations in the water. At all sampling points, TSS concentration was not different between the two treatments ($P > 0.05$) (Table 3.6). TSS concentration decreased from the beginning to the first week of the experiment and increased from the end of week 1 onwards (Figure 3.5). Changes in VSS concentration showed similar pattern to that of TSS (Figure 3.5) and were not significantly different among the two treatments ($P > 0.05$).

The average protein content in biofloc in both treatments was higher than 40% of the dry matter (Table 3.6). Biofloc protein content significantly changed during the culture period ($P_{Time} < 0.05$). Molasses yielded a biofloc with significantly higher protein content ($P < 0.05$) than in the corn treatment. Ash and total energy contents, and the C:N ratio of biofloc did not change significantly in time and were not significantly different among treatments ($P > 0.05$).

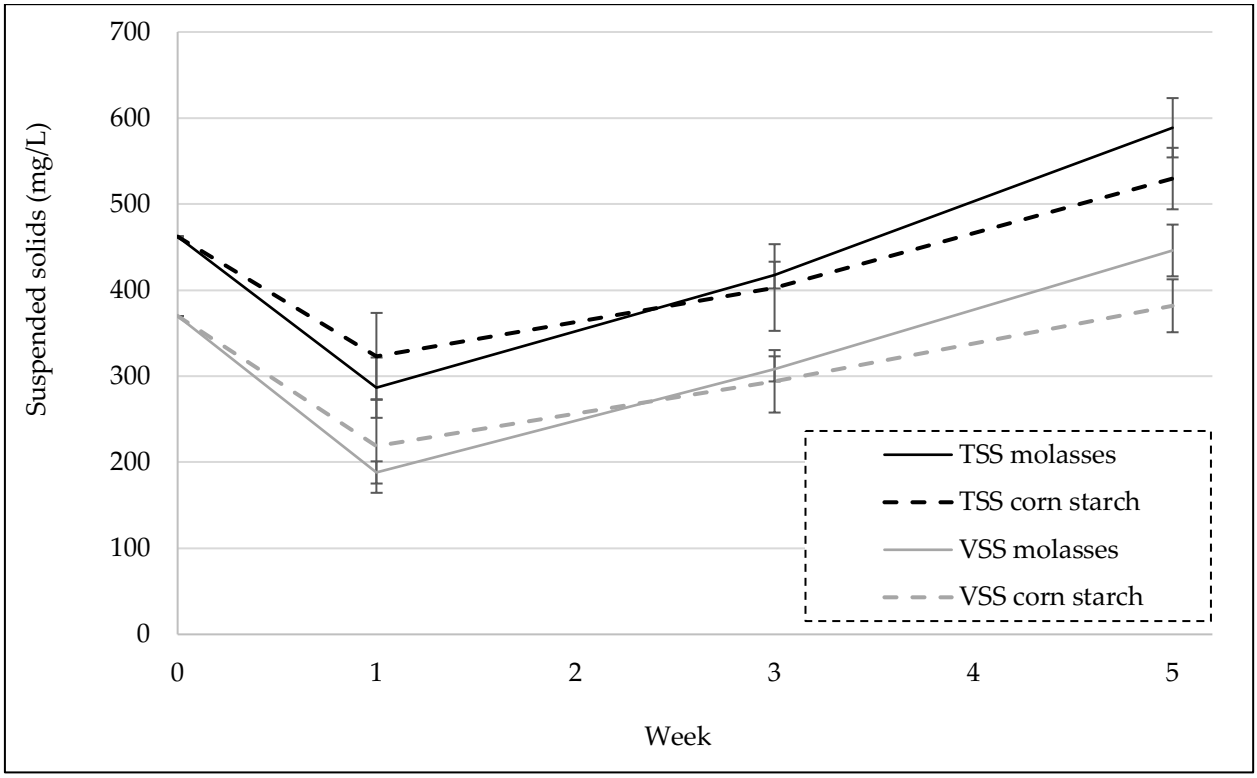


Figure 3.5 Changes of total suspended solids (TSS) and volatile suspended solids (VSS) in water of corn starch and molasses treatments. Values are means (±SD) of three replicate tanks per sampling time in each treatment.

Table 3.6 Biofloc biomass in terms of total suspended solids (TSS) and volatile suspended solids (VSS), and biofloc proximate composition by treatment (carbohydrate source). Values are means (±SD) of six sampling times for each treatment. Probability (P) values in bold indicate significant effects (P < 0.05).

Parameters	Treatments		P values		
	Corn starch	Molasses	Treatment	Time	Interaction
TSS (mg/L)	419 ± 99	431 ± 134	0.709	**0.000**	**0.000**
VSS (mg/L)	298 ± 79	314 ± 113	0.542	**0.000**	**0.006**
Ash (g/kg DW)	292 ± 56	281 ± 60	0.678	0.083	0.715
Crude protein (g/kg DW)	394 ± 28	426 ± 53	**0.001**	**0.000**	0.097
Total energy (kJ/g DW)	10 ± 3	13 ± 7	0.077	**0.000**	**0.013**
Carbon:nitrogen ratio	6.5 ± 1	6.8 ± 1.1	0.579	0.397	0.473

3.4. Effects on periphyton production

Periphyton was removed from all tank walls at the beginning of the experiment to ensure similar starting conditions. At the end of week 3, the periphyton biomass was not different among treatments (P > 0.05), but was significantly higher in the molasses treatment at the end of week 5 (P < 0.05) (Figure 3.6). Repeated measures ANOVA showed that total periphyton biomass increased over time and was significantly higher in the molasses treatment (P < 0.05) (Table 3.7). Periphyton in the molasses treatment had

significantly higher protein content, and significantly lower C:N ratio compared to those of the corn starch treatment ($P < 0.05$). The ash and organic matter contents in periphyton were comparable among treatments ($P > 0.05$).

Table 3.7 Proximate composition of periphyton by treatment (carbohydrate source). Values are means (±SD) of two sampling times for each treatment. Probability (P) values in bold indicate significant effects ($P < 0.05$).

Parameters	Treatments		P values		
	Corn starch	Molasses	Treatment	Time	Interaction
Total biomass (g DW/tank)	19 ± 8	33 ± 15	**0.032**	**0.013**	0.057
Ash (g/kg DW)	550 ± 39	487 ± 43	0.121	0.302	0.668
Crude protein (g/kg DW)	192 ± 23	237 ± 18	**0.042**	0.813	0.495
Organic matter (g/kg DW)	450 ± 39	513 ± 43	0.121	0.302	0.668
Carbon:nitrogen ratio	6.4 ± 0.1	6.2 ± 0.1	**0.043**	0.112	0.764

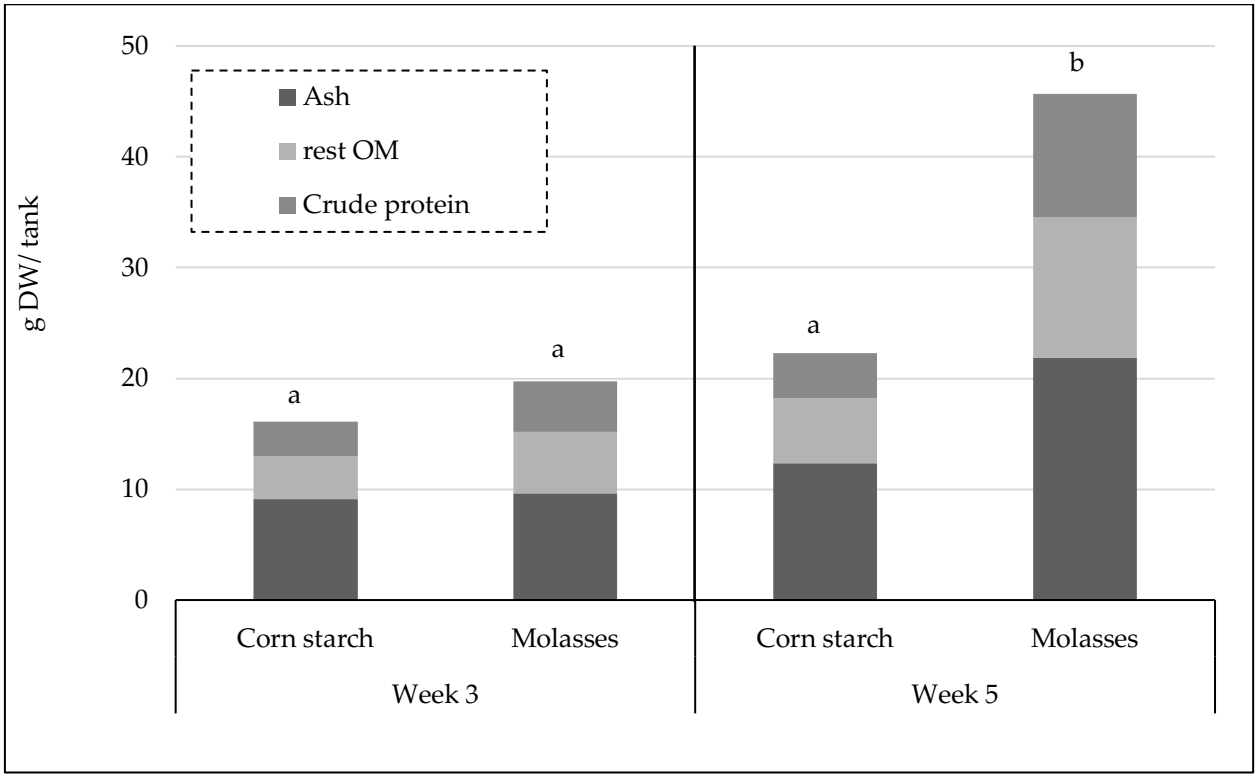


Figure 3.6 Absolute periphyton biomass at the end of weeks 3 and 5 by treatment (carbohydrate source). Values are means of three replicate tanks per sampling time for each treatment. For each week, different letters on the top of columns indicate significant differences ($P < 0.05$).

3.5 Diurnal fluctuations of water quality and biofloc

3.5.1 Water quality parameters

Figure 3.7 showed that within 24h period, carbon and nitrogen contents in water were relatively stable. The total dissolved nitrogen in water was comparable among treatments, while the total dissolved carbon in water in the corn starch treatment was

lower than that in the molasses treatment on the sampling day (Table 3.8) ($P > 0.05$). This was consistent with the results of the weekly measurements shown in Figure 3.3. However, the repeated measure analysis showed that TC, OC, TN, ON, TAN, and PO_4-P changed significantly during the day ($P < 0.05$).

3.5.1 Biofloc

Biofloc volume in terms of suspended solids was measured at the beginning and end of the 24h measurement. In both treatments, there was a relatively small increase in TSS after 24 hours, however, the difference between biofloc volume at the start and end of the 24h period was insignificant ($P > 0.05$). Biofloc carbon and nitrogen contents were not significantly different among different sampling times of the day in both treatments ($P > 0.05$), averaging 339 g carbon and 57 g nitrogen per kilogram of biofloc. The average C:N ratio of biofloc in both treatments was 5.9 ± 0.3., and constant during the day ($P > 0.05$).

3.6 Nutrient accumulation

During the experiment, the absolute amount of carbon and nitrogen in the system increased in both treatments (Figure 3.8). One-way ANOVA showed that the percentages of both retained carbon and nitrogen were not significantly different among treatments ($P > 0.05$). Carbon retention efficiency in corn starch and molasses treatments were 15 and 17% of total carbon input, respectively. The majority of the retained carbon in the corn starch treatment accumulated in the shrimp and in the sediment. In the molasses treatment, retained carbon was more equally distributed in all compartments of the culture system.

Table 3.8 Summary of selected parameters from 24h water measurement by treatment (carbohydrate source). Values are means (±SD) of nine sampling times for each treatment. Measurement was done on day 32, at 3-hour intervals. Probability (P) values in bold indicate significant effects ($P < 0.05$).

Parameters	Treatments		P values		
	Corn starch	Molasses	Treatment	Time	Interaction
TC (mg/L)	52 ± 1	77 ± 2	**0.000**	**0.011**	0.391
IC (mg/L)	37 ± 2	42 ± 1	**0.007**	0.074	0.653
OC (mg/L)	15 ± 1	35 ± 1	**0.000**	**0.001**	**0.013**
TN (mg/L)	8.1 ± 1.7	7.7 ± 0.4	0.737	**0.000**	0.108
IN (mg/L)	5.9 ± 1.7	3.5 ± 0.4	0.132	0.088	0.513
ON (mg/L)	2.2 ± 0.3	4.2 ± 0.3	**0.000**	**0.000**	**0.003**
TAN (mg/L)	0.1 ± 0.03	0.1 ± 0.04	0.150	**0.000**	**0.000**
NO_x-N (mg/L)	5.8 ± 1.8	3.5 ± 0.4	0.132	0.165	0.529
PO_4-P (mg/L)	1.0 ± 0.29	0.9 ± 0.04	0.687	**0.000**	0.920
TSS (mg/L)	505 ± 34	554 ± 51	0.211	0.065	0.391

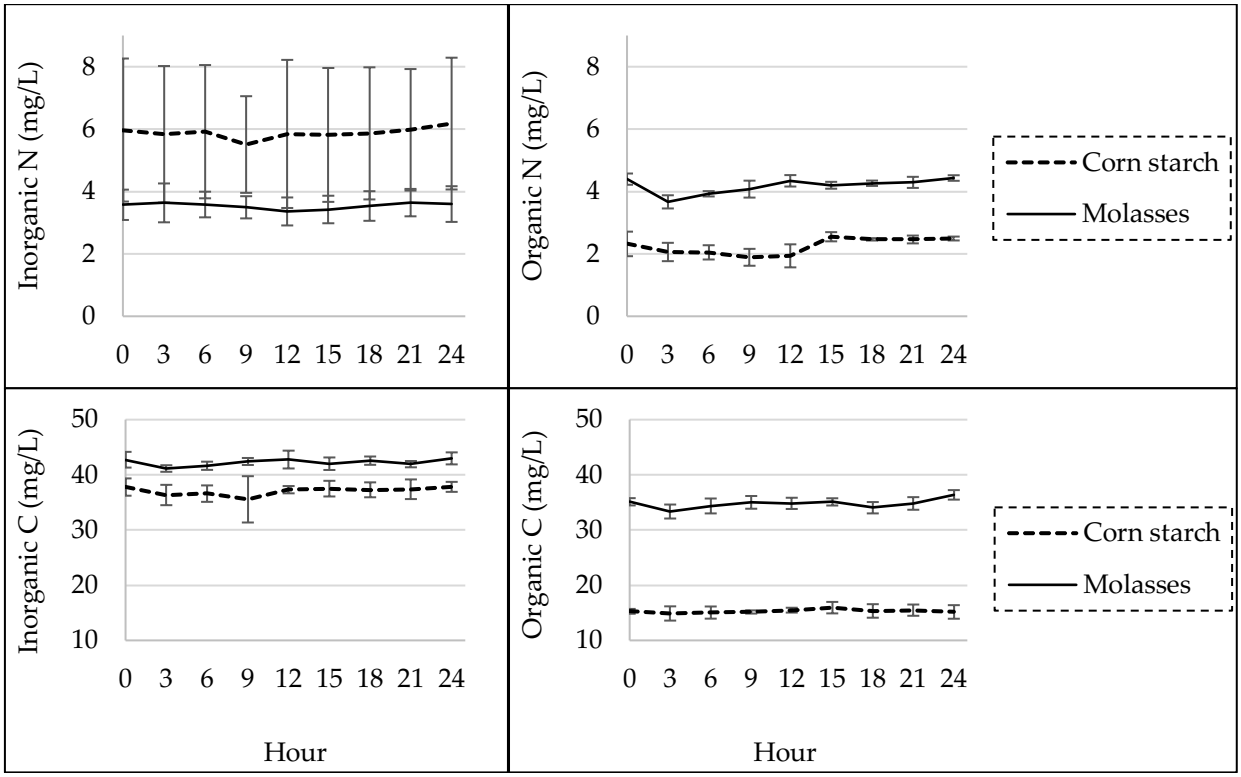


Figure 3.7 Diurnal fluctuation of dissolved nitrogen (N) and carbon (C) in water by treatment (carbohydrate source). Values are mean (±SD) of three replicate tanks per sampling time in each treatment. Measurement was done on day 32, at 3-hour intervals. Shrimp feeding was done one hour prior to and five hours after the first sampling.

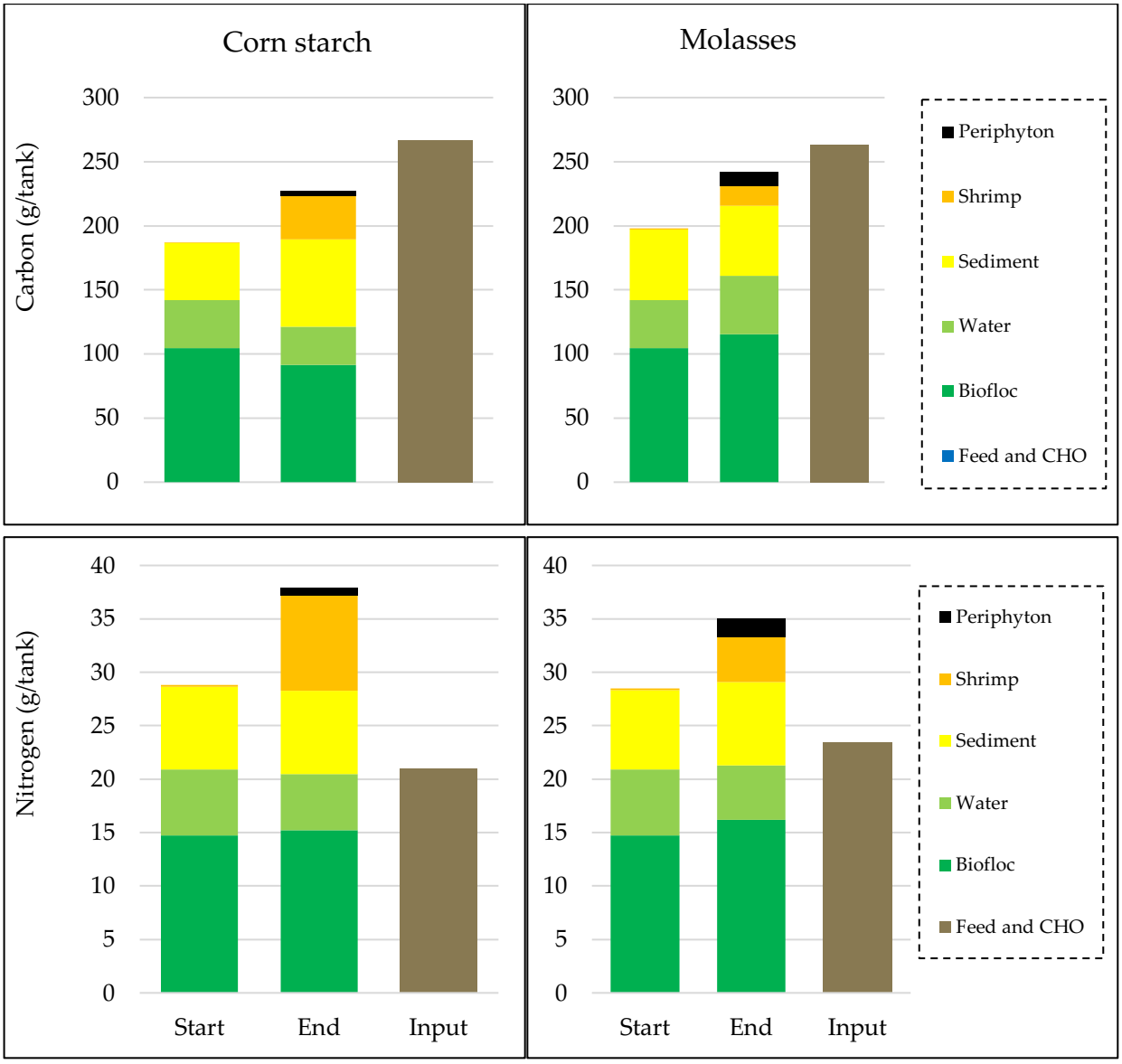


Figure 3.8. Carbon and nitrogen distributions in different compartments of the culture system, and total nutrient input from feed and carbohydrates. Values are means of three replicate tanks by treatment (carbohydrate source).

Regarding nitrogen, 43 and 28% of the input were retained in corn starch and molasses treatments, respectively. In the corn starch treatment, retained nitrogen mainly accumulated in the shrimp. Meanwhile, accumulated nitrogen in the molasses treatment was more equally distributed among shrimp, biofloc and periphyton.

3.7 Mineral content and BOD5 of corn starch and molasses

Molasses contained higher concentrations of all analyzed minerals, especially potassium, compared to corn starch (Table 3.9). In total, corn starch and molasses treatments received 260 and 484 g/tank, respectively, for the whole experimental duration. Consequently, the molasses treatment had greater absolute mineral inputs and concentrations in water compared to corn starch treatment. Regarding the 5-day biological oxygen demand

(BOD_5), each milligram dry matter of molasses consumed 0.1 ± 0.02 mg oxygen, while this value for corn starch was 0.2 ± 0.04 mg oxygen ($P < 0.05$).

Table 3.9 Mineral content, absolute input, and concentration in water of carbohydrate sources

	P	Ca	Fe	Mg	K	Mn
Mineral content of carbohydrates (g/kg):						
Corn starch	0.13	0.08	0.001	0.02	0.06	0
Molasses	0.24	0.56	0.080	0.30	34.2	0.03
Molasses/corn starch	1.9	6.7	80	12	561	-
Absolute input from carbohydrates (g):						
Corn starch	0.03	0.02	0	0.01	0.02	0
Molasses	0.12	0.27	0.04	0.140	16.5	0.02
Molasses/corn starch	4.0	14	-	14	825	-
Concentration of minerals provided from carbohydrates in water (mg/L):						
Corn starch	0.05	0.03	0	0.02	0.03	0
Molasses	0.20	0.45	0.07	0.23	27.5	0.03

4. Discussion

4.1 Shrimp

The survival percentage (90-96%) and specific growth rate (8-10% BW/day) of shrimp in this research were high and comparable to previous studies on white leg shrimp nursery (Arias-Moscoso et al., 2018; Correia et al., 2014; Khanjani et al., 2016; Serra et al., 2015). In research covering a 3-4 month whole culture cycle, lower survival percentages of shrimp (82-93%) were reported with an overall specific growth rate of 7.6% BW/day (Khoa et al., 2020; Krummenauer et al., 2016).

In this research, a C:N ratio of 12 was used for both treatments. This ratio was of intermediate level, and recommended for biofloc culture of whiteleg shrimp (Panigrahi et al., 2019; Xu et al., 2018, 2016). Nevertheless, the addition of corn starch yielded significantly better shrimp production. The hypothesis for this production enhancement was not clear. It was thought to be due to the higher stability of environmental conditions in the corn starch treatment, in particular the dissolved organic carbon and nitrogen, compared to that in the molasses treatment (Figure 3.3). Stable water quality reduces stress and improves growth and survival of cultured shrimp (Janeo et al., 2009). Lower and more stable organic carbon and nitrogen concentrations in the water of corn starch treatment suggests that the microbial community in this system differed in composition and was better in functionality than in the molasses treatment (Jiang et al., 2020; Moss

and Pruder, 1995). However, the conclusion that molasses should be replaced with corn starch in biofloc technology was not yet drawn, as one may argue that corn starch is a dietary ingredient for both human and animal, and of greater value compared to molasses, which is a by-product from sugar industry (Guo et al., 2006; Nikodinovic-Runic et al., 2013).

The two carbohydrate sources used in this research differed significantly in mineral content, especially in potassium. Previous research also reported that iron, potassium and manganese concentrations in molasses were approximately 17, 50, and 70 times, respectively, higher than in starches (Heuzé et al., 2016, 2015; Prasanthi et al., 2017). Besides, the phosphate concentration in the molasses treatment was higher than in the corn starch treatment and previously reported values in conventional shrimp pond (Casillas-Hernández et al., 2007). However, whether these differences contributed to the difference in shrimp growth among treatments is unclear. Overall, beside maintaining a suitable C:N ratio, the choice of CHO source is of prime importance in biofloc technology, as CHOs have different effects on biofloc nutritional content (Crab et al., 2010; Rajkumar et al., 2016; Wei et al., 2016), microbial diversity (Deng et al., 2018; Wei et al., 2016) and production of culture animal (Rajkumar et al., 2016). The latter was also found in the present study.

4.2 Water

Between the two CHOs, molasses contained mostly simple sugars, and was expected to be more easily utilized by microbial organisms, resulting in more rapid improvement in water quality (Heuzé et al., 2015; Wei et al., 2016). This effect was observed in weeks 3, 4, and 5 when significantly lowered TAN levels were obtained in the molasses treatment compared to the corn starch treatment. However, both treatments resulted in low TAN of 0.02 - 0.1 mg/L, which was sufficient for shrimp growth (Lin and Chen, 2001). However, the BOD_5 of corn starch was twice as high as the BOD_5 of molasses (0.2 and 0.1 mg O_2/mg DM, respectively), indicating differences in microbial activities. This may concur with a shift in microbial community composition, as different CHO sources have varied effects on biofloc microbial community (Deng et al., 2018). However, since both treatments in this research reacted similarly in term of water quality (e.g. TSS, VSS, TAN), it would be safe to assume that the switch of carbon source did not create imbalances. In the aerated biofloc systems used, the higher oxygen demand in the corn starch treatment did not cause problems.

In new systems, where there is no or low level of biofloc, biofloc concentration is often seen to increase continuously during the culture period (Xu et al., 2016; Xu and Pan, 2014a). However, the TSS and VSS concentrations in this experiment decreased during the first week. This is due to the high biofloc concentration (> 460 mg/L) relative to the low amount of shrimp feed at the beginning of this experiment. Therefore, the nutrient input was too low to maintain the initial biofloc volume. Over the experimental period, total dissolved nitrogen decreased while TSS and periphyton biomass increased, suggesting that waste nitrogen was immobilized into microbial biomass.

Towards the end of the experiment, both dissolved organic carbon and nitrogen in the molasses treatment increased. However, the mechanisms of this occurrence could not be properly elucidated. One possibility is that while both treatments received the same amount of feed, shrimp growth in the molasses treatment was slower. Therefore, part of the feed in the molasses treatment was not eaten, and degraded releasing also organic carbon and nitrogen.

4.3 Diurnal fluctuation

The repeated measures analysis of 24h water measurements showed that dissolved carbon and nitrogen fluctuated during the day ($P < 0.05$). In systems where feed and CHO are added twice per day, significant fluctuation of carbon and nitrogen contents following the addition of feed and CHO is expected. Nevertheless, Figure 3.7 showed that the total carbon and nitrogen in water remained stable over 24h. This may be due to the fact that these measurements were done toward the end of the experiment (day 32) when carbon and nitrogen input from carbohydrate (8.7 mg/L and 0.14 mg/L, respectively) were small compared to the amount present in the water at sampling time (65.5 mg/L for carbon and 8.13 mg/L for nitrogen). Although daily fluctuations were not significant over time, differences in organic and inorganic C and N concentrations were observed, as well as differences between corn starch and molasses treatments (Figure 3.3).

4.4 Biofloc

Biofloc protein content was higher than 390 g/kg dry weight in both treatments. The average biofloc concentration reached 434 mg/L, presenting an extra source of nutrients for the cultured shrimp. The higher biofloc protein content observed in the molasses treatment likely stemmed from the fact that molasses contains more protein on dry weight basis (8.5%) than corn starch (0.3%), which was directly available to biofloc. This concurs with Kumar et al. (2015) also reporting that CHO with a high protein content

resulted in biofloc with a high protein content. A regression of CHO protein content against protein content of biofloc from this study and Kumar et al. (2015) yielded a correlation coefficient of 0.91 ($R^2 = 0.83$). However, Kumar et al. (2015) used a feeding level of 1.5% body weight and maintained an assumed C:N ratio of 10, making combining the different treatments difficult. Therefore, it remains unclear how protein in CHO may change the protein concentration (on dry matter basis) in the biofloc.

We observed that as the biofloc concentration increased, its protein content increased, while its ash content decreased. This may be due to changes in the biofloc microbial composition. At high biofloc concentration, the bacteria part in biofloc became dominant over the algal content (Xu et al., 2016). In this research, algae in the system was outcompeted from week 3 onwards, showing a decrease in chlorophyll a concentration (Figure 3.4) when biofloc concentration reached 403 mg/L in the corn starch and 417 mg/L in the molasses treatments (Figure 3.5). A balanced biofloc system where neither algae nor bacteria is dominant is more beneficial for shrimp (Xu et al., 2016). Biofloc concentration of 400-600 mg/L is suitable for whiteleg shrimp culture (Schveitzer et al., 2013b).

4.5 Periphyton

Periphyton production in this research reached 22-46 g DW/tank (Figure 3.6) at the end of the experiment, with protein contents ranging from 19% to 24% (Table 3.7). Periphyton protein content, similarly to biofloc protein content, was significantly higher in molasses treatment. Studies on effects of carbohydrate type on periphyton were scarce. However, periphyton nutritional values were shown to be dependent on substrate type (Azim et al., 2005). Periphyton protein level in this research was comparable to the 25% found in whiteleg shrimp pond, and represented an extra source of nutrient for the culture animal (Kumar et al., 2017). Direct contribution of periphyton to intensive whiteleg shrimp culture has not been studied. However, it was shown that promoting periphyton growth by substrate addition increased whiteleg shrimp production in less intensive system (Kumar et al., 2017). In intensive system the aeration rate has a significant effect on evaporation, constantly reducing water level (Li et al., 2008). We observed that shrimp grazed on periphyton which mostly grew on tank wall at water-air interface, therefore, maintaining a constant culture water level may increase the availability of periphyton to the culture animal.

4.6 Nutrient accumulation

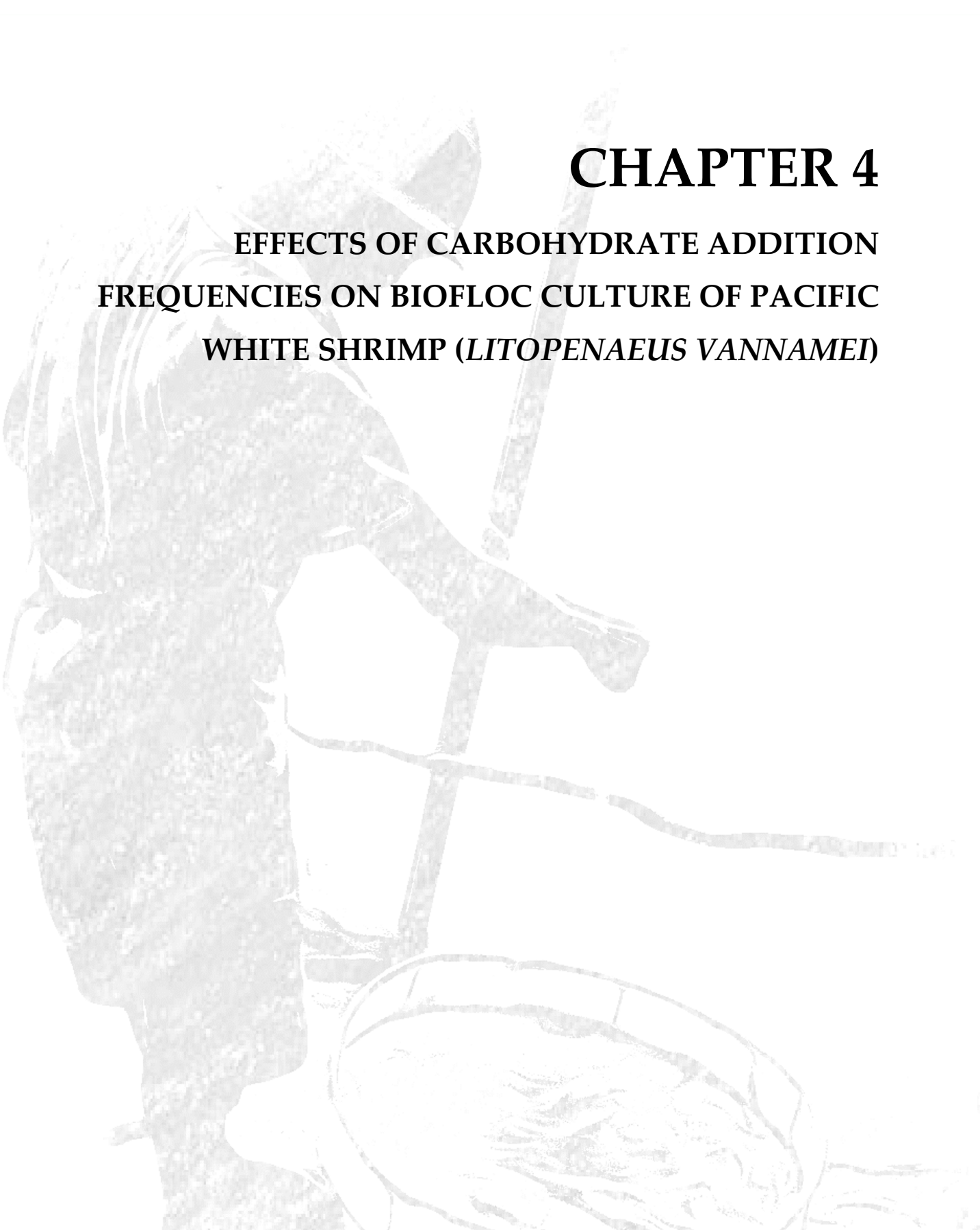
This research demonstrated that only 15-17% of carbon, and 28-43% of nitrogen input remained in the system at the end of the experiment. The nitrogen accumulated in shrimp in the corn starch treatment of this research is comparable to that in da Silva et al. (2013). However, the total 43% of the nitrogen input accumulated in the tank was slightly more than half of the 80% accumulation of the nitrogen input reported by (da Silva et al., 2013). The other 20% was assumed to be lost through denitrification and volatilization. In conventional culture ponds without CHO addition, 23% of carbon and 35% of nitrogen inputs (i.e. from feed and fertilizer) were assimilated in shrimp (Dien et al., 2018). This indicates that our system was less efficient in carbon use compared to the conventional system. Hu et al. (2014), utilizing a tilapia culture model, showed that the addition of CHO increased daily CO_2 emissions by 91%, however, it reduced the daily N_2O emissions by 83%, both of which are among major greenhouse gasses from aquaculture activities. Although this phenomenon has not been investigated in biofloc culture of shrimp, a similar trend can be expected to occur. Therefore, it can be controversial whether the adoption of the biofloc system should be encouraged, since this system was proven to be more efficient in water and nitrogen use, however, less efficient in organic carbon retention which may possibly have adverse effects on global warming (Gao et al., 2012; Hu et al., 2014). By employing mesocosms, the distribution of input nutrients among compartments in the system could be accounted. The unaccounted amount is considered lost through valorization. More insight in which factors contribute to a higher retention of nitrogen in a biofloc system merits further investigation.

5. Conclusions

The choice of organic carbon source plays an important role in the success of the biofloc system. Corn starch was superior to molasses for enhancing the growth of whiteleg shrimp. Once the biofloc is established, nitrogen waste can be efficiently controlled, resulting in relatively little diurnal fluctuation of nitrogen and carbon in culture water. However, the nutrient loss in biofloc systems, especially carbon loss is high, and ways to reduce C-loss from culture systems should be explored. Further research on improving nutrient use efficiency, either directly by culture animals or indirectly by trapping nutrients and making them available for other uses, is necessary.

CHAPTER 4

EFFECTS OF CARBOHYDRATE ADDITION FREQUENCIES ON BIOFLOC CULTURE OF PACIFIC WHITE SHRIMP (*LITOPENAEUS VANNAMEI*)



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Abstract

In Pacific white shrimp culture, adding organic carbohydrate to the pond besides feed improves water quality and shrimp performance. While the feeding frequency has significant effects on shrimp production, how often carbohydrate should be added remains unknown. This research investigated the effects of carbohydrate addition frequency of 1, 3, and 6 times per day on Pacific white shrimp production. Tapioca powder was used as carbohydrate source to maintain an input C:N ratio of 12. The same daily ration of tapioca was applied but divided into equal portions according to the application frequency. The shrimp performance was better in carbohydrate-added systems than in a conventional system without additional carbohydrate, but the carbon retention efficiency was lower. Splitting daily dosage of carbohydrate into more frequent additions had no significant effect on Pacific white shrimp performance, and carbon and nitrogen retentions. A carbohydrate addition frequency of once per day is recommended for good performance of Pacific white shrimp in biofloc rearing systems.

Keywords: biofloc technology, carbohydrate addition frequency, *Litopenaeus vannamei*, nutrient retention.

1. Introduction

During the last decade, biofloc technology (BFT) emerged as a potential solution for improving the sustainability of aquaculture (Bossier and Ekasari, 2017). In this technology, carbon is added through organic carbohydrate additions, to provide energy for the assimilation of nitrogenous compounds into microbial biomass (Avnimelech, 2009). This reduces the levels of total ammonia nitrogen, the need for water exchange, and increases the concentration of flocculated materials (biofloc) which can serve as natural food for the cultured animals (Crab et al., 2012).

The application of biofloc technology has been tested in an extensive range of culture species, including Pacific white shrimp, *Litopenaeus vannamei*. This species stood at number one in value and number six in production, accounting for nearly 4% (or 4.45 million tons) of world aquaculture production in 2017 (Junning et al., 2019), and continued growing reaching 4.97 million tons in 2018 (FAO, 2020). Cultured Pacific white shrimp are often fed multiple times per day or continuously in intensive systems. The majority of research recommended a feeding frequency of 3-4 times daily, mostly during day time, to ensure good growth (de Lima et al., 2009; Peixoto et al., 2018; Pontes et al., 2008; Robertson et al., 1993; Wasielesky Jr. et al., 2020). This spaced feeding stimulates foraging and ingestion activities (de Lima et al., 2009; Pontes et al., 2008), and enzyme production (Peixoto et al., 2018) in Pacific white shrimp. Higher feeding frequencies may not be advantageous for growth and survival while being less cost-effective (Carvalho and Nunes, 2006; Velasco et al., 1999). Recent research, however, recommends on-demand feeding system for improved shrimp growth (Reis et al., 2020).

In biofloc technology research, the organic carbon source is often added to the system once daily, regardless of feeding frequencies (Panigrahi et al., 2019; Xu and Pan, 2012; Xu et al., 2016). By splitting daily dosage of CHO into more frequent applications per day, less organic carbon enters the biofloc system each time, which may help reduce the fluctuation of oxygen. As this organic carbon is primarily consumed by heterotrophic bacteria, frequent CHO addition increases the availability of food over a longer time period. This may boost the growth of heterotrophic bacteria, which have a lower alkalinity requirement compared to autotrophic bacteria (Ebeling et al., 2006), and help to reduce fluctuations in pH and alkalinity. A stable culture environment promotes growth and survival of the cultured animals (Janeo et al., 2009). However, more frequent addition of CHO will also increase labor cost, and therefore may reduce the adoption of the technology. Lower CHO addition frequencies when proven to be more beneficial to

shrimp culture system would allow the simplification and facilitate the dissemination and adoption of the biofloc technology. Therefore, this research aimed at comparing the effects of different CHO addition frequencies on Pacific white shrimp production, water quality, biofloc and periphyton in a mixed photoautotrophic-heterotrophic biofloc system with minimum water exchange.

2. Materials and methods

2.1. Experimental design

This experiment was done at the aquatic experimental facilities in College of Aquaculture and Fisheries, Can Tho University, Vietnam. The experiment consisted of 3 treatments of carbohydrate (CHO) addition frequency in triplicate, including one addition per day after mid-day feeding (CHO 1), three additions per day after every feeding (CHO 3), and six additions per day before and after every feeding (CHO 6). Another treatment where no CHO was added (CHO 0) was included to demonstrate the effect of CHO addition as Xu et al. (2016) showed that system without CHO addition performed equally well. The shrimp were fed three times daily at 08.00, 12.00, and 16.00. The experiment lasted for 6 weeks from August to September, 2018.

2.2. Experimental site preparation

The experiment was conducted outdoor. The experimental site was covered with a transparent plastic film to prevent the effects of rainwater intrusion, while providing the ambient lighting to experimental tanks. Twelve plastic tanks (0.5 m^3 total volume, 0.3 m^3 working volume) were cleaned prior to the addition of saline water and employed for the experiment.

Sea water (80 ppt salinity) was diluted to 15 ppt salinity, and alkalinity was adjusted to 140 mg/L by adding sodium bicarbonate. Three hundred liters of disinfected and filtered water were pumped into each experimental tank. All tanks were continuously aerated to maintain the dissolved oxygen level above 6 mg/L.

2.3. Experimental animals

As base population, two thousand Pacific white shrimp post-larvae of day 15 were obtained from a nearby hatchery and nursed in a 2-m^3 tank at 15 ppt salinity for one month. During this period, shrimp were fed to satiation three times per day with a 48% protein feed. Rice flour was added to the nursing tank after every feeding to maintain an input C:N ratio of 12. The amount of rice flour needed was calculated using the following formula, assuming the 48% protein feed contained 50% carbohydrate (%$Carbon_{feed}$) and

7.7% nitrogen ($\%Nitrogen_{feed}$), and rice flour contained 80% carbohydrate ($\%Cabon_{CHO}$) and 0.5% nitrogen ($\%Nitrogen_{CHO}$):

$$(1) \qquad C:N\ ratio = \frac{(Weight_{feed}\ \text{x}\ \%Carbon_{feed}) + (Weight_{CHO}\ \text{x}\ \%Carbon_{CHO})}{(Weight_{feed}\ \text{x}\ \%Nitrogen_{feed}) + (Weight_{CHO}\ \text{x}\ \%Nitrogen_{CHO})}\ \text{x}\ 100$$

As part of the nitrogen in feed was retained by the shrimp, the actual input C:N ratio entering the system would be higher. After one month of nursing, groups of 36 shrimp of similar size (0.61 ± 0.02 g average individual weight) were randomly taken from the base population, weighed for initial total biomass and stocked into each experimental tank, concurring to a stocking density of 120 ind/m^3. Shrimp were fed three times daily at 08.00, 12.00, and 16.00 with a commercial feed of 43% protein (Skretting, Vietnam) during the experiment. A feeding table was made assuming a feed conversion ratio (FCR) of 1, a feeding rate of 5.5% shrimp body weight (BW), and a mortality rate of 5% over 6-week culture period. Tapioca powder was used as the CHO source during the experimental period. Lab analysis prior to the experiment showed that the 43% protein feed contained 33% carbohydrate and 6.9% nitrogen, and tapioca powder contained 66% carbohydrate and 0.5% nitrogen. To maintain an input C:N ratio of 12, the amount of tapioca powder needed was 846g per kilogram of feed, calculated using formula (1).

2.4. Sampling and sample analysis

During the experiment, total ammonia nitrogen (TAN), nitrite nitrogen (NO_2-N), and nitrate nitrogen (NO_3-N) were monitored daily with test kits (Sera GmbH, Germany). Water samples were collected on a weekly basis from each tank. The sampling took place 30 minutes after the first feeding of the day. All samples were stored at 4^{o}C if immediate analysis was not possible. Water samples were analyzed for total suspended solids (TSS), volatile suspended solids (VSS), chlorophyll a (Chla), total nitrogen (TN), TAN, NO_2-N, NO_3-N (APHA, 1995), and total carbon (TC) (QuickTOCultra, LAR Process Analysers AG, Germany).

The number and weight of shrimp were determined only at stocking and harvest to minimize handling stress. Shrimp samples were collected at the beginning and end of the experiment. Biofloc and periphyton samples were collected at the end of the experiment. Shrimp, biofloc, and periphyton samples were immediately sent to Mekonglab Testing Center (NhoNho Technology Ltd Co., Vietnam) after their collection for the proximate analysis of crude protein (ISO 5983, 2005b), crude fat (ISO 6492, 1999), ash (ISO 5984, 2002), and total carbon (QuickTOCultra, LAR Process Analysers AG, Germany).

2.5. Data analysis

Shrimp specific growth rate (SGR) was calculated as follow:

$$(2) \qquad SGR\ \left(\%\frac{BW}{day}\right) = \frac{\ln(\text{final BW}) - \ln(\text{initial BW})}{\text{Days of culture}} \text{ x } 100$$

The nitrogen content of shrimp, biofloc, and periphyton were approximated to be 16% of crude protein by weight. Carbon and nitrogen retention efficiencies in the shrimp, water, biofloc, and overall system were calculated by dividing the difference between the starting (in water and stocked shrimp) and the harvested (in water, biofloc, and harvested shrimp) amounts by the total amount of input from feed and CHO, as simplified in the equation below:

$$(3) \qquad Retention\ efficiency\ (\%) = \frac{\text{Total at harvest (g)} - \text{Total at start (g)}}{\text{Total input (g)}} \text{ x } 100$$

Statistical analysis was performed with IBM SPSS Statistics 25 software (IBM Corporation, NY, USA). The effects of treatment on Pacific white shrimp production parameters, proximate compositions of shrimp, biofloc and periphyton, and nutrient retention efficiency were compared using One-way ANOVA. The effects of treatment on water quality parameters were analyzed with Repeated measure ANOVA. The probability value (P value) of less than 0.05 was used to indicate significant differences. Least significant difference (LSD) post-hoc test was performed when a significant effect was found.

3. Results

3.1. Effects on Pacific white shrimp production parameters

Shrimp production parameters are summarized in Table 4.1. The harvested biomass, survival rate, and FCR were not different among treatments ($P > 0.05$). The shrimp specific growth rate was not different among treatments but tended to be higher shrimp where additional CHO was added ($P < 0.1$). Besides, the CHO addition resulted in higher FCR-CHO (FCR in which carbohydrate was considered as feed in the calculation) ($P < 0.05$). Shrimp final body weight was significantly improved by the CHO addition but was similar among different addition frequencies ($P < 0.05$).

The proximate composition of harvested shrimp was similar among all treatments ($P > 0.05$), ranging in dry weight (DW) between 77.6-78.4% for crude protein, 5.0-5.2% for crude fat and 13.4-14.3% for ash (Table 4.2). Shrimp contained a similar carbon to nitrogen ratio among treatments ($P > 0.05$).

Table 4.1 Pacific white shrimp production parameters in the six-week experiment.

Treatments (CHO/day)	Final BW (g/ind)	Harvested biomass (g)	Survival rate (%)	FCR	FCR-CHO	SGR (% BW/day)
CHO 0	7.2 ± 0.6[a]	241 ± 18	93 ± 4	0.98 ± 0.08	0.98 ± 0.08[a]	5.9 ± 0.2
CHO 1	8.3 ± 0.3[b]	266 ± 22	89 ± 10	0.87 ± 0.08	1.62 ± 0.15[b]	6.2 ± 0.1
CHO 3	8.1 ± 0.2[b]	223 ± 61	88 ± 2	1.14 ± 0.42	2.11 ± 0.77[b]	6.2 ± 0.1
CHO 6	8.0 ± 0.2[b]	250 ± 30	86 ± 8	0.94 ± 0.12	1.74 ± 0.22[b]	6.2 ± 0.1
P values	**0.039**	0.547	0.712	0.522	**0.001**	0.053

Values are means (± SD) of three replicate tanks per treatment (CHO addition times/day). BW = body weight, FCR = feed conversion ratio, FCR-CHO = feed conversion ratio accounting CHO as feed, SGR = specific growth rate. Probability (P) values in bold indicate significant effects ($P < 0.05$). Different superscripts indicate significant differences among treatment means by column.

Table 4.2 Proximate composition of shrimp at the end of the six-week experiment.

Treatments (CHO/day)	DW (%)	Crude protein (% DW)	Crude fat (% DW)	Ash (% DW)	C:N ratio
CHO 0	21.6 ± 0.5	78.9 ± 1.1	5.0 ± 0.3	14.3 ± 1.2	3.7 ± 0.2
CHO 1	22.0 ± 0.9	77.3 ± 1.6	5.2 ± 1.3	14.1 ± 0.9	4.4 ± 0.8
CHO 3	21.8 ± 0.4	79.7 ± 1.4	5.2 ± 1.4	14.1 ± 0.9	3.4 ± 0.3
CHO 6	22.4 ± 0.9	79.7 ± 1.9	5.2 ± 0.6	13.9 ± 0.5	3.4 ± 0.3
P values	0.947	0.531	0.179	0.985	0.071

Values are means (± SD) of three replicate tanks per each treatment. DW = dry weight.

3.2. Effects on water quality

During the experiment, environmental parameters fluctuated in the ranges of 5.9-6.8 mg/L for oxygen, 7.9-8.5 for pH, and 27-30°C for temperature. Repeated measure ANOVA showed that all measured water quality parameters, except organic nitrogen (ON), changed significantly over the weeks ($P_{Time} < 0.05$) (Table 4.3). The effects of treatment on TSS, TAN, TN and ON were not significant ($P_{Treatment} > 0.05$). Chlorophyll a concentration was significantly higher in the CHO 0 treatment, while VSS and TC concentrations were significantly higher in treatments where CHO was added ($P_{Treatment} < 0.05$). The treatments showed different effects on inorganic nitrogen (IN) and NO_3-N in water at different moments in time during the experiment ($P_{Treatment \times Time} < 0.05$).

The VSS concentrations increased over the weeks in all treatments, reaching the highest level of 198 mg/L at the end of the experiment (Figure 4.1). Increasing the CHO application frequency from 1 to 6 times per day showed no effect on the VSS. The TSS concentrations (Figure not shown) showed a similar pattern, and had a correlation coefficient of 0.93 with VSS. The chlorophyll a concentration showed more fluctuations, ranging between 110-337 μg/L (Figure 4.2). The chlorophyll a concentration increased more rapidly in the CHO 0 treatment, reaching the highest level of 337 μg/L after one

week and remaining relatively constant until the end of the trial. Meanwhile, different CHO addition frequencies showed similar effect on Chla concentration.

Table 34.3 Summary of water quality parameters in the six-week experiment.

Parameters	Treatments (CHO/day)				P values		
	CHO 0	CHO 1	CHO 3	CHO 6	Treatment	Time	Treatment x Time
TSS (mg/L)	142 ± 70	152 ± 78	160 ± 71	174 ± 88	0.145	**0.000**	0.424
VSS (mg/L)	82 ± 35^{a}	103 ± 50^{b}	105 ± 42^{b}	100 ± 45^{b}	**0.003**	**0.000**	0.127
Chla (µg/L)	295 ± 30^{a}	216 ± 71^{ab}	203 ± 53^{b}	187 ± 47^{b}	**0.023**	**0.000**	0.203
TAN (mg/L)	1.4 ± 1	1.9 ± 1.8	1.7 ± 1.5	1.9 ± 1.6	0.566	**0.002**	0.389
NO_2-N (mg/L)	1.6 ± 2^{a}	0.7 ± 1.6^{b}	1.3 ± 2.8^{ab}	1.2 ± 2.8^{ab}	**0.042**	**0.000**	0.122
NO_3-N (mg/L)	2.2 ± 2.8^{a}	0.9 ± 0.4^{b}	1.1 ± 0.7^{b}	1.4 ± 1.1^{b}	**0.001**	**0.000**	**0.000**
TN (mg/L)	7.6 ± 1.3	8.1 ± 1.5	8.6 ± 2.7	8.6 ± 1.3	0.607	**0.013**	0.585
IN (mg/L)	5.2 ± 5.7^{a}	3.6 ± 3.4^{b}	4.1 ± 4^{ab}	4.5 ± 4.5^{ab}	**0.036**	**0.000**	**0.023**
ON (mg/L)	4.9 ± 1.4	5.4 ± 0.8	6.1 ± 1.4	5.6 ± 1.2	0.676	0.381	0.410
TC (mg/L)	38 ± 4^{a}	43 ± 5^{b}	42 ± 4.9^{b}	42 ± 5.1^{ab}	**0.014**	**0.000**	0.100

Values are means (± SD) of measurements from week 1 to 6 by treatment (CHO addition times/day). Probability (P) values in bold indicate significant effects ($P < 0.05$). Different superscripts indicate significant differences among treatment means on one row.

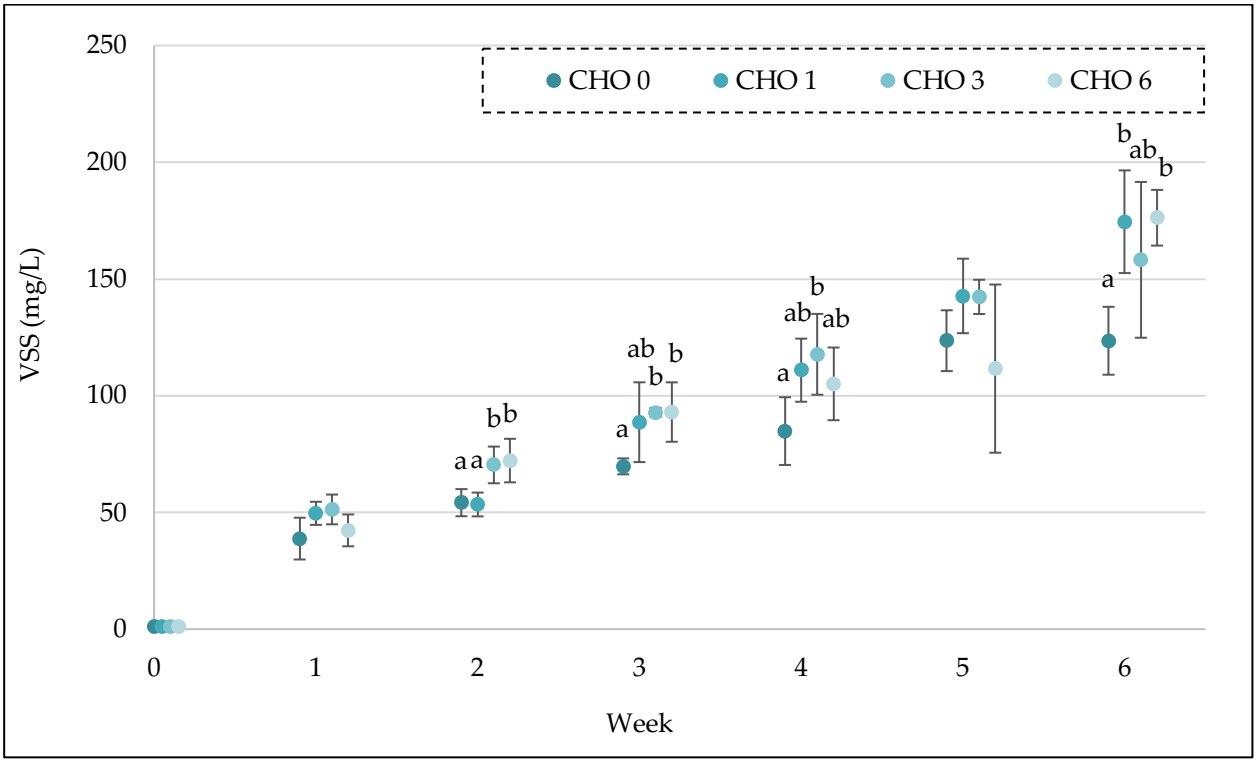


Figure 4.1 Weekly changes of volatile suspended solids (VSS) by treatment (CHO addition times/day). Values are means (± SD) of three replicate tanks per treatment. Samples from different treatment tanks were taken on the same day weekly. Data points in each sampling day were presented one beside the other to

increase visibility to avoid overlapping of error bars in the graph. For each week, different letters above data points indicate significant differences

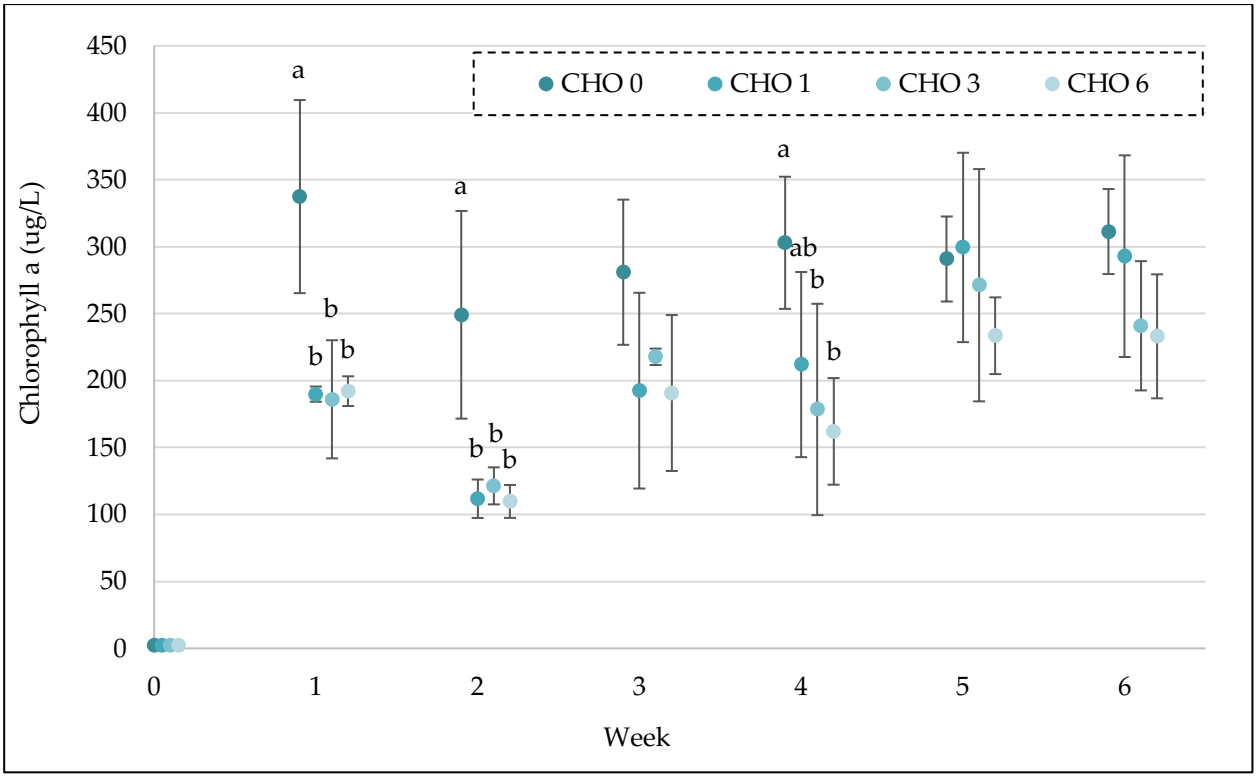


Figure 4.2 Weekly changes of chlorophyll a concentration in water by treatment (CHO addition times/day). Values are means (± SD) of three replicate tanks per treatment. Samples from different treatment tanks were taken on the same day weekly. Data points in each sampling day were presented one beside the other to increase visibility to avoid overlapping of error bars in the graph. For each week, different letters above data points indicate significant differences

3.3. Biofloc and periphyton proximate compositions

Biofloc and periphyton proximate compositions are shown in Table 4.4 and Table 4.5, respectively. One-way ANOVA showed that biofloc protein and fat contents, and C:N ratio were not different among treatments. Biofloc ash content was significantly higher in the CHO 0 treatment than in treatments where CHO was added once or thrice per day ($P < 0.05$), but was similar to that in treatment where CHO was added six times per day ($P > 0.05$). Regarding periphyton proximate composition, no significant difference was identified among treatments ($P > 0.05$).

3.4. Nutrient retention efficiency

Carbon retention in terms of percentage of input and absolute amount is shown in Figure 4.3. One-way ANOVA showed that the CHO 0 treatment had significantly higher carbon retention (% input) in shrimp, biofloc, and water and significantly lower carbon loss (unaccounted carbon in periphyton and through evaporation) compared to other

treatments (P > 0.05). Different CHO addition frequencies showed similar effect on carbon retention efficiency. In term of absolute amount, adding CHO once per day resulted in higher carbon retention in shrimp, and lower carbon loss compared to adding 3 or 6 times daily. Nitrogen retention in terms of percentage of input and absolute amount (Figure 4) was similar among all treatments (P > 0.05).

Table 4.4 Biofloc proximate composition at the end of the six-week experiment.

Treatments (CHO/day)	Moisture (%)	Crude protein (% DW)	Crude fat (% DW)	Ash (% DW)	C:N ratio
CHO 0	92.2 ± 0.9	29.7 ± 0.9	2.8 ± 0.8	45.3 ± 2.0^{a}	3.24 ± 0.42
CHO 1	92.7 ± 1.2	29.0 ± 3.6	3.5 ± 0.9	37.3 ± 3.2^{b}	3.77 ± 1.02
CHO 3	91.7 ± 1.1	27.6 ± 2.2	2.5 ± 0.8	36.3 ± 3.8^{b}	3.56 ± 0.12
CHO 6	93.0 ± 0.3	28.1 ± 2.2	2.7 ± 0.2	39.5 ± 0.9^{ab}	3.75 ± 0.86
P values	0.433	0.705	0.453	**0.014**	0.781

Values are means (± SD) of three replicate tanks per treatment (CHO addition times/day). Probability (P) value in bold indicates significant effect (P < 0.05). Different superscripts indicate significant differences among means in a column.

Table 4.5 Periphyton proximate composition at the end of the six-week experiment.

Treatments (CHO/day)	Moisture (%)	Crude protein (% DW)	Crude fat (% DW)	Ash (% DW)	C:N ratio
CHO 0	88.4 ± 1.9	51.7 ± 3.7	6.7 ± 1.0	40.1 ± 4.5	2.49 ± 0.61
CHO 1	90.5 ± 0.4	52.7 ± 4.9	6.3 ± 2.1	42.6 ± 4.0	2.66 ± 0.62
CHO 3	90.2 ± 0.9	56.2 ± 8.4	5.5 ± 0.8	40.9 ± 1.9	2.42 ± 0.71
CHO 6	90.3 ± 1.1	57.9 ± 7.4	7.1 ± 0.3	41.8 ± 0.9	2.45 ± 0.42
P values	0.194	0.625	0.485	0.784	0.959

Values are means (± SD) of three replicate tanks per treatment (CHO addition times/day).

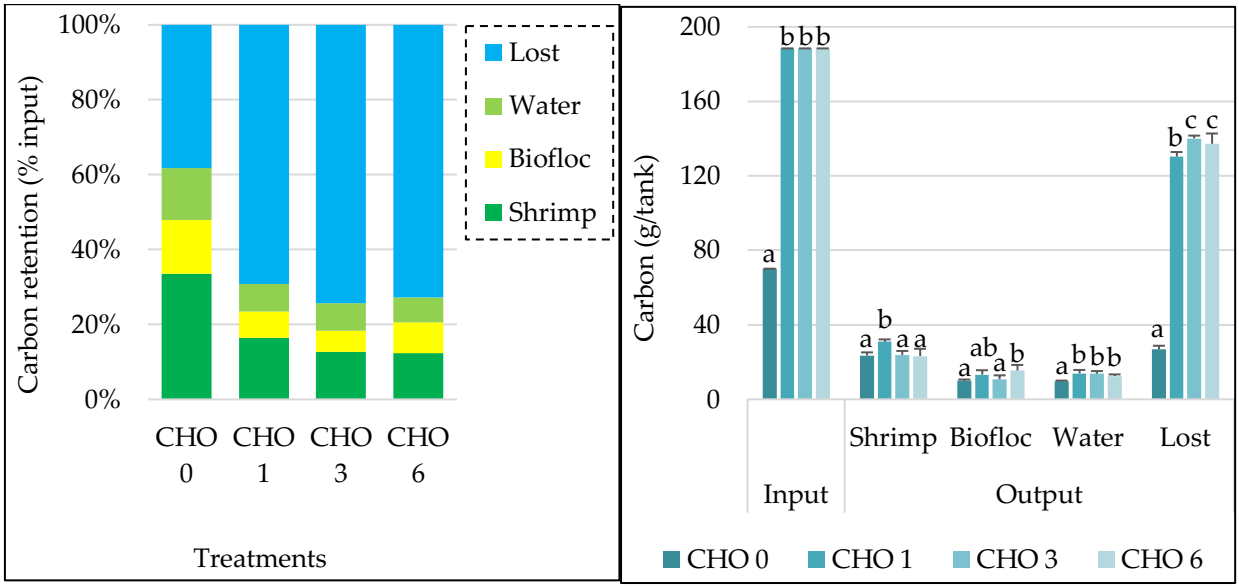


Figure 4.3 Carbon retention in terms percentage of input (left) and absolute amount (right) at the end of the six-week experiment. Values are means of three replicate tanks per treatment (CHO addition times/day). Columns in a group with different superscripts are statistically different.

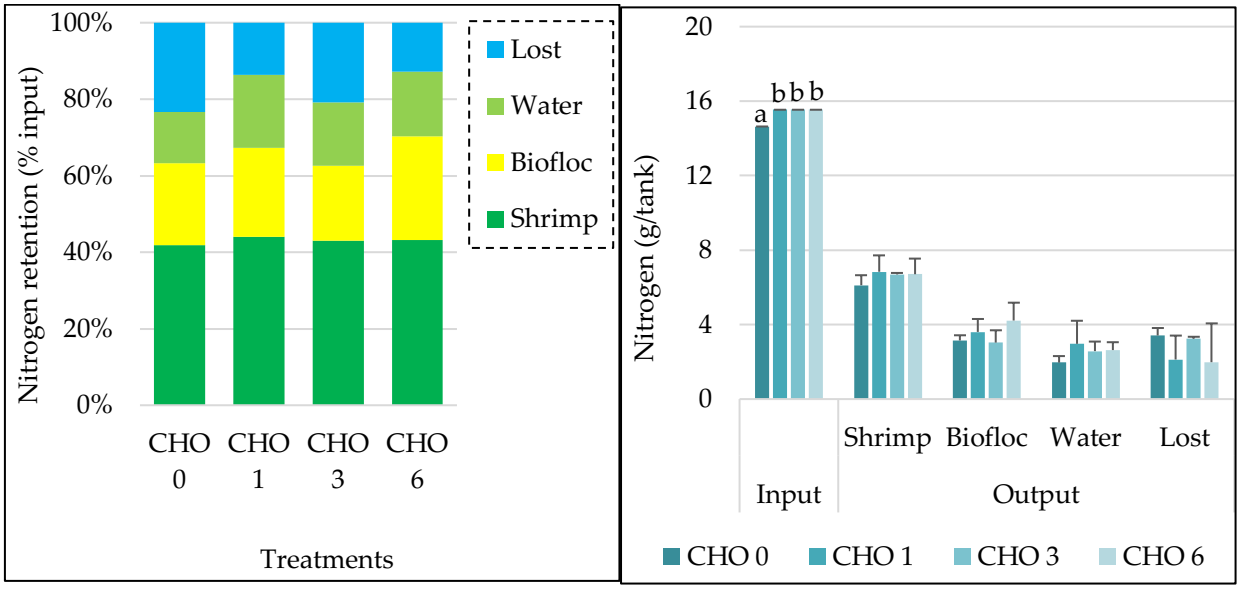


Figure 34.4 Nitrogen retention in terms percentage of input (left) and absolute amount (right) at the end of the six-week experiment. Values are means of three replicate tanks per treatment (CHO addition times/day). Columns in a group with different superscripts are statistically different.

4. Discussion

The average final shrimp biomass by treatment was 14% better than predicted according to the feeding table provided by the feed company, in spite of the 5% higher than anticipated mortality. The slightly higher mortality increased the amount of feed per shrimp explaining partially the 21% increase in average individual body weight by treatment reached at the end of the experiment (Liu et al., 2017; Sookying et al., 2011).

Carbohydrate addition significantly increased the individual final shrimp body weight ($P < 0.05$) and numerically improved the SGR ($P < 0.1$). No differences were observed in survival and FCR with CHO addition, in contrast to Gao et al. (2012) and Panigrahi et al. (2019) who observed improved survival and FCR when administrating CHO. While the total ammonia nitrogen was similar among treatments, significantly lower nitrite and nitrate nitrogen concentrations where CHO was added, especially at the frequency of once per day, indicated a more active bacterial community in these treatments. Besides, significantly higher VSS found in CHO-added treatments suggested a more abundance bacterial content in biofloc compared to biofloc in the CHO 0 treatment.

The addition of carbohydrate prevented the nitrite production, improving the water quality in this research. To test the effects of splitting daily CHO ration, we maintained a C:N ratio of 12 as higher ratios (e.g. 15 or 18) adversely affected shrimp growth and yield (Xu et al., 2016). Panigrahi et al. (2019), however, suggested an optimal C:N ratio of 15 for improved shrimp growth, water quality, and immunity to bacteria, while Xu and Pan (2012) demonstrated that both C:N ratios of 15 and 20 resulted in similar shrimp growth performance. Emerenciano et al. (2017) suggested an optimal C:N ratio should not be fixed, but rather adjusted during the culture period to maintain good water quality and to meet the nutritional needs of fish or shrimp and biofloc. Therefore, much effort is still needed in creating a standard protocol for biofloc technology that can be widely applied.

Most research on biofloc technology used the CHO addition frequency of once per day, but differed in the time of administration, for example at morning feeding at 6 AM (Gao et al., 2012), after morning feeding at 9 AM (Panigrahi et al., 2019), at mid-day at 12 PM (Xu et al., 2016), or at afternoon feeding at 14 PM (Xu and Pan, 2012). The current research, to our knowledge, was the first to describe the effect of CHO addition frequency in a biofloc system. In shrimp ponds where dissolved organic carbon is low, microalgae dominate (Xu et al., 2016). Meanwhile, autotrophic bacteria thrive when there is a lot of total ammonia nitrogen and little organic carbon. In biofloc systems with CHO addition, heterotrophic bacteria become dominant over autotrophic bacteria due to their faster growth rate and more efficient nutrient use (Hargreaves, 2006). However, splitting daily dosage of CHO to 1 to 3 or 6 addition times per day, which may ensure the continuity of energy source for bacteria growth showed no beneficial effect on shrimp performance, and biofloc growth. Therefore, a CHO addition frequency of once per day is advisable for good shrimp performance and economic labor cost.

As expected, VSS increased over the experimental period, as also reported by Xu et al. (2016). The addition of CHO slowed down the algal growth in the first two weeks, but its effects reduced towards the end of the experiment as the chlorophyll a concentration became similarly high among treatments (Figure 4.2). This suggests that the used C:N ratio of 12 was low to maintain the dominance of bacteria over algae. Besides, the sharp increase of chlorophyll a in week 1 (Figure 4.2) indicated that the environmental conditions (i.e light intensity, low organic carbon at the start) in this experiment were favorable for algal growth and may have facilitated algae in the algae-bacteria interaction in CHO-added treatments. By aiming for an intermediate C:N ratio of 12 (Xu et al., 2016), and spreading the application of CHO in time, we aimed to maintain a more stable water quality, while keeping algae production going. The results showed that increasing the number of daily CHO applications has minor effects on system performance. However, it should be noted that these results were for a semi-intensive biofloc system. In intensive and super-intensive biofloc systems, the feed and CHO loads are often near the carrying capacity, and therefore, changing the frequency of adding CHO may have significant effects.

In the present study, the biofloc proximate composition was not different among treatments with and without CHO addition. This finding contrasted most previous research where biofloc nutritional values increased when CHO was added (Khanjani et al., 2016; Rajkumar et al., 2016; Xu and Pan, 2012, 2014a). The contribution of biofloc to the daily nitrogen retention in shrimp may range between 18-29% in high density culture, and tended to increase with the biofloc concentration (Burford et al., 2004). In semi-intensive culture, natural biota may occupy 91% of stomach contents of the shrimp (Gamboa-delgado et al., 2003). Both biofloc and periphyton presented nutritious food sources for the cultured animal (Bossier and Ekasari, 2017; Wasielesky et al., 2006). While the biofloc protein content in our research was comparable to those in previous research (Khanjani et al., 2016; Kumar et al., 2018; Xu and Pan, 2012, 2014a), the periphyton protein content was almost double that of biofloc and the reported figure for periphyton (25% of dry matter) in substrate-added Pacific white shrimp culture (Kumar et al., 2017). It is possible that part of the residual feed was trapped in the rim of periphyton at the water-air interphase, resulting in high periphyton protein content. In tilapia ponds with substrate, the periphyton protein ranges from 14% to 17% dry matter (Azim et al., 2003). The introduction of substrate to promote periphyton growth improved Pacific white shrimp production in both conventional (Audelo-Naranjo et al., 2012; Kumar et al., 2017)

and biofloc systems (Schveitzer et al., 2013a). Nevertheless, the nutritional contribution of periphyton to shrimp growth in biofloc system merits further research.

Nutrient retention efficiency

Feed constituted more than 50% of production cost in shrimp culture (Cruz-Suárez et al., 1994). Therefore, increasing feed use efficiency is one way to make aquaculture activities profitable. This can be achieved by employing biofloc systems, where shrimp use feed more efficiently and retain more nitrogen due to the ingestion of biofloc as natural food and the enhanced enzymatic activities of the shrimp (Kumar et al., 2018; Xu and Pan, 2012). In aquaculture systems, ammonia nitrogen is removed through photoautotrophic uptake by algae, autotrophic bacterial conversion, and heterotrophic bacterial uptake (Ebeling et al., 2006). The addition of CHO facilitates the third process in which nitrogen is converted to bacterial biomass and carbon is released as carbon dioxide. In closed culture systems, the nitrogen is lost from the system as a result of denitrification which might occur following nitrification. The nitrogen retentions in shrimp and biofloc, and the total nitrogen loss in this research (Figure 4.4) were comparable to previous reports for the biofloc system (Audelo-Naranjo et al., 2010; da Silva et al., 2013; Kumar et al., 2018). However, the nitrogen retentions were not different among the CHO 0 and biofloc treatments, and among CHO addition frequencies. This may have stemmed from the relatively similar biofloc growth and nutritional value among treatments, and the short experimental duration. A 120-day experiment at C:N ratio of 15 showed that biofloc system had 5% higher nitrogen retention compared to a conventional system (Kumar et al., 2018). Increased nitrogen retention due to carbohydrate addition was also reported in other shrimp species (Hari et al., 2006). Nevertheless, our research demonstrated that the nitrogen retention in shrimp was high, and the nitrogen and carbon retentions were not affected by CHO addition frequency.

Regardless of CHO addition frequencies, biofloc culture of Pacific white shrimp showed double carbon loss compared to conventional culture. This is in line with what was found for tilapia culture where the carbon fraction lost from the biofloc system doubled that from the conventional system (Hu et al., 2014). We found that Pacific white shrimp assimilated 34% of the total carbon input in conventional culture, while the reported value for *Penaeus monodon* was 16-21% (Sahu et al., 2012). In biofloc culture, 846g of tapioca powder per kilogram of feed fed was added to maintain a C:N ratio of 12, more than 70% of which was not accounted for. While the economic impact of carbon loss can be reduced

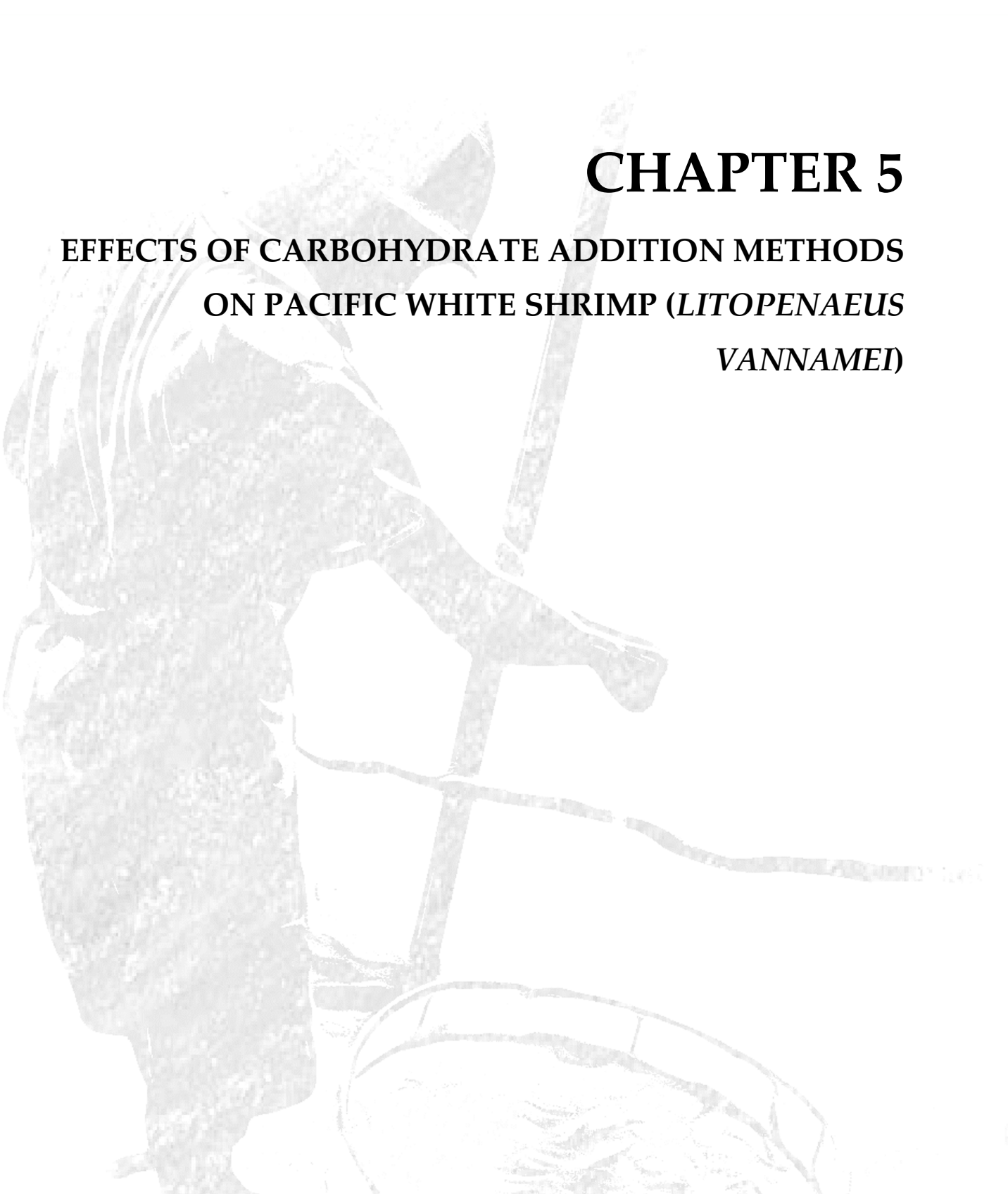
by using cheap by-product carbon sources (e.g. molasses), such high percentages of carbon loss may cause concerns over the environmental impacts of biofloc systems. Therefore, further research is needed to optimize the use of nutrients, specifically carbon, if the biofloc technology is to be widely disseminated.

5. Conclusions

The current research showed that an addition frequency of once per day is optimal and more frequent addition of CHO is not recommended if carbohydrate is to be applied in the culture of Pacific white shrimp.

CHAPTER 5

EFFECTS OF CARBOHYDRATE ADDITION METHODS ON PACIFIC WHITE SHRIMP (*LITOPENAEUS VANNAMEI*)



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Abstract

The addition of external carbohydrate in Pacific white shrimp (*Litopenaeus vannamei*) culture has shown positive effects on water quality and shrimp performance. However, this practice requires additional skills and labor, and therefore may not be widely adopted. This research investigated the potential to combine the carbon source and the feed in one pellet in shrimp culture. The carbon source used in this experiment was corn starch. The experiment was executed in 6 indoors mesocosms tanks, with full control of the water source, temperature, and light intensity and duration. The three treatments including COM-Feed (commercial feed), COM-Feed + CHO (commercial feed with separate corn starch addition), and CHO-Feed (pelleted diet made by incorporating additional corn starch into the commercial feed) were randomly assigned to the mesocosms tanks and run two times consecutively. Adding corn starch separately resulted in higher shrimp biomass gain and protein efficiency ratio ($P < 0.05$), compared to dietary corn starch addition and no corn starch addition. Carbohydrate addition caused a significant drop in inorganic nitrogen and orthophosphate concentrations in the water. The treatments had no effect on the biofloc and periphyton growth, and the proximate composition of shrimp, biofloc, and periphyton, however increased the eukaryotic microbial diversity in the bioflocs. Meanwhile, the carbohydrate addition reduced the dietary energy and carbon utilization efficiencies regardless of the carbohydrate addition methods.

Keywords: Biofloc, Carbohydrate addition method, *Litopenaeus vannamei*, Microbial communities, Nutrient budget, Periphyton

1. Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is an important farmed species, accounting for more than 52% of global crustaceans production in 2018 (FAO, 2020). Intensive farming of this species relies heavily on protein rich feed because the natural productivity of the pond food web is insufficient to sustain the desired high productivity. Xia et al. (2010) shows that Pacific white shrimp performs best with feed of 38-40% dietary protein levels. However, the use of high protein feed in large quantity is often associated with total ammonia nitrogen and nitrite accumulations in water. Exposure to high concentrations of these substances may negatively affect the metabolic responses of Pacific white shrimp (Racotta and Hernández-Herrera, 2000).

Manipulating the C/N ratio in the system through organic carbohydrate addition directly into the water provides energy for bacteria to assimilate nitrogenous compounds into microbial biomass (Avnimelech, 1999). By increasing the C/N ratio in the system, the total ammonia, nitrite, and nitrate nitrogen concentrations are significantly reduced (Panigrahi et al., 2019). This practice improves water quality and Pacific white shrimp production (Panigrahi et al., 2019; Ren et al., 2019). The beneficial effects of carbohydrate additions into the water were demonstrated in several fish culture systems (e.g. Magondu et al., 2013; Pérez-Fuentes et al., 2016) and shrimp culture systems (Asaduzzaman et al., 2008; Hari et al., 2004; Panigrahi et al., 2019; Xu and Pan, 2012). In a minimal water exchange system for Pacific white shrimp, a C/N ratio of 15 was optimal for shrimp survival, growth, and immune activity (Panigrahi et al., 2019).

Adding extra carbohydrate into the water in addition to feeding requires additional skills and labor which may not be favored by farmers. If the additional carbohydrate can be mixed in the feed to produce pellets that provide jointly carbohydrates for the microbes in the pond and nutrients for the shrimp, system management is simplified and more easily adopted. However, according to Xia et al. (2010), the inclusion of carbohydrate in the feed at the expense of protein increases the overall feed digestibility, but reduces the protein digestibility. This may increase nutrient excretion and reduce shrimp growth, at the same time provide more substrate for microbial growth and promote the development of bioflocs. Guo et al. (2006) also demonstrated that the dietary corn starch inclusion levels affects the feed digetibility, shrimp growth rate, survival and feed conversion ratio (FCR), being optimal at 10-15% dietary content (Guo et al., 2006). It is unknown how the inclusion of carbohydrate source in the diet will affect the shrimp

culture system, compared to biofloc system. This research therefore investigated the effect of carbon supplementation as well as the potential of including the carbohydrate in the feed for biofloc culture of Pacific white shrimp.

2. Materials and methods

2.1. Biofloc stimulation

Four weeks prior to the experimental start, biofloc growth was stimulated in three indoor tanks (with a working volume of 0.8 m^3 per tank) stocked with Nile tilapia (*Oreochromis niloticus*). The fish, which were obtained from the Carus Experimental Station of Wageningen University, were stocked at a density of 0.5 kg/m^3, corresponding to 400 gram of fish per tank. Each tank was provided with continuous aeration, and 12h/12h dark/light regime. The fish were fed a diet containing 33% protein, twice daily at 08.00 and 16.00 hours, at 2.5% body weight per day, assuming an FCR of 1.0. Native corn starch was added one hour after each feeding to maintain a C/N ratio of 20 of the combined feed and carbohydrate input to stimulate the biofloc development.

Tilapia tanks were first filled with 160 L of freshwater. The water salinity in these tanks was gradually increased by addition of salt water to reach the target salinity of 25 ppt after 4 weeks to a final volume of 800 L/tank. At the start of the shrimp experiment, tilapia were removed and the biofloc water from these three tanks was pooled (total 2.4 m^3) and well mixed. The mixed biofloc water was equally distributed over the six experimental tanks (400 L per tank). Saline and tap water were added to bring the water volume to 600 L per tank and salinity of 25 ppt.

2.2. Experimental design

The experiment consistent of three different treatments. The first treatment ("COM-Feed") consisted of feeding a pelleted diet that mimicked a commercial diet having a C/N ratio of 7.6 g/g. For the second treatment ("CHO-Feed"), consisted of feeding a pelleted diet with a high C/N ratio (14.6 g/g). The pelleted "CHO-Feed" consisted of 50% "COM-Feed" and 50% gelatinized corn starch. For the third treatment ("COM-Feed + CHO") the same amount of "COM-Feed" and corn starch were added to the tanks, but starch was directly added to the tanks instead of being included into the pelleted diet. At the tank level also for the "COM-Feed + CHO" treatment the C/N ratio was 14.6 g/g.

In order to increase the number of replicates (i.e., tanks) per treatment, the experiment consisted of two trials, and data from both trials were combined for statistical analysis. Each trial lasted 4 weeks and was performed with 6 tanks (i.e., 6 experimental units).

Within each trial, each treatment was randomly assigned to the experimental units in duplicate. The experimental conditions were identical between both trials. All tanks were connected to one central air blower, which continuously aerated through a round plastic pipe positioned at the bottom of each tank. The pipe was 0.6 m in diameter with eight 0.5 cm holes, allowing equal air diffusion in the water column and maintaining an oxygen concentration above 6 mg/L. A lighting regime of 12h light and 12h dark was applied, and the temperature was kept constant with a heater at 27°C.

2.3. Experimental animals and feeding

Pacific white shrimp (*Litopenaeus vannamei*) were obtained from CreveTec bvba, Ternat, Belgium. From a base population, 300 shrimp of similar initial weight (3.2 ± 0.04 and 3.4 ± 0.05 g for trial 1 and 2, respectively) were selected and randomly stocked into the experimental tanks (50 shrimp per tank). Two experimental diets were produced by steam pelleting using a die size of 2 mm resulting in sinking pellets by Research Diet Services (Wijk bij Duurstede, The Netherlands). The ingredient and analyzed nutrient composition of both diets are shown in Table 5.1. During the experiment, shrimp were fed with commercial feed or corn starch-mixed feed according to their assigned treatment twice daily at 08.00 and 16.00 hours. The amount of feed for treatments COM-Feed and COM-Feed + CHO was calculated using a feeding rate of 5% BW/day, an assumed feed conversion ratio (FCR) of 1.3 and a survival rate of 95% for the whole experimental period. Additionally, the treatment COM-Feed + CHO received the same amount of corn starch after every feeding as the amount of COM-feed administered. Treatment CHO-Feed received double the amount of feed to ensure similar protein input among treatments, and similar input C:N ratio to treatment COM-Feed + CHO.

2.4. Water quality monitoring

During the experiment, temperature, salinity and pH in experimental tanks were measured daily using a multi-parameter electronic meter (WTW Multi3630IDS™). Water quality parameters including NH_4^+, NO_2^- and NO_3^- were monitored by sensitivity test using the MColortest™ kit, while the biofloc volume was measured with Imhoff cones.

2.5. Sample collection and analysis

Water samples were collected weekly, starting on the day of stocking. Plastic bottles of 2 L were held in the middle of the water column, and then opened to fill with culture water. A magnet was inserted in each bottle for magnetic stirring at the speed of 350 rpm to keep the biofloc in suspension during the sub-sampling process. Sub-samples of 100 mL were

taken from each bottle and filtered through 1.5 μm pore size filter paper. The filtrates were acidified to pH of 2-3 with HCl and immediately submitted to CBLB laboratory (Chemical Biological Soil Laboratory, Wageningen University, The Netherlands) for the following analyses: total carbon (TC), inorganic carbon (IC), total nitrogen (TN), total ammonia nitrogen (TAN), nitrite and nitrate (NO_x), phosphate phosphorus (PO_4-P) using a segmented flow analyzer (SAN++, Skalar Analytical B.V., The Netherlands). The chlorophyll a contents were determined every two weeks using unfiltered water samples (APHA, 1995).

Table 5.1 Ingredients and nutrient composition of the experimental diets used in this experiment.

	Commercial feed (COM-Feed)	**Corn starch-mixed feed (CHO-Feed)**	**Corn starch (CHO)**
Ingredient composition (%):			
Gelatinized corn starch	---	50	100
Wheat	20.58	10.29	
Wheat flour	15	7.5	
Wheat gluten	10	5	
Wheat bran	15	7.5	
Soybean meal	12	6	
Fishmeal LT (CP > 680)	20	10	
Fishmeal hydrolysate (CPSP)	1	0.5	
Soy lecithin	0.7	0.35	
Fish oil	2	1	
Cholesterol	0.3	0.15	
Calcium carbonate	0.5	0.25	
Mono calcium phosphate	1.2	0.6	
L-lysine	0.2	0.1	
DL-Methionine	0.3	0.15	
L-Threonine	0.2	0.1	
Premix	1	0.5	
Yttrium oxide	0.02	0.01	
Total	**100**	**100**	
Nutrient composition (g/kg DW):			
Dry matter (g/kg WW)	934	928	873
Crude protein	394	200	1
Crude fat	70	39	6
Ash	72	37	1
Energy (kJ/g)	20	19	17
Total carbon (g/kg WW)	447	433	398
Total nitrogen (g/kg WW)	59	30	1
C/N ratio	7.6	14.6	294

Biofloc samples were collected at the start, middle and end of the experiment. The samples were taken by filtering 100 mL of culture water through 1.5 μm pore size filter

paper and rinsing with 100 mL demineralized water to eliminate the salt content. Filter papers containing biofloc were used for determination of biofloc biomass in terms of total suspended solids (TSS) and volatile suspended solids (VSS) (APHA, 1995), proximate composition (AOAC, 2000), and TC and TN contents (using LECO CN 628 Dumas analyzer, LECO Instrumente GmbH., Germany).

A hundred shrimp were collected at the start as a composite sample and all remaining shrimp in each experimental unit were collected at the end of the experiment. Periphyton samples were collected from each treatment tank only at the end by carefully scratching the tank walls. All shrimp and periphyton samples were preserved at -20^{o}C until further analysis. These samples were freeze-dried for one week prior to analysis of their proximate composition (AOAC, 2000), TC and TN contents (using LECO CN 628 Dumas analyzer, LECO Instrumente GmbH., Germany). During the experiment, roughly 10 gram of each feed and CHO were taken daily. These daily samples were pooled per diet and at the end of the experiment analyzed for their proximate composition, and TC and TN contents employing the same methods as for shrimp and periphyton, without prior freeze-drying.

For microbiota analysis, DNA was extracted from biofloc samples. The biofloc samples, were collected at the end of the experiment by filtering through a 0.45 (particulate part) and a 0.22 μm (seston part) pore size sterile filter. The samples were subjected to lysis by lysozyme buffer and proteinase K before DNA extraction using DNeasy Blood & Tissue kit (Qiagen, Valencia, CA). The harvested DNA was quantified using the nano drop spectrophotometer. Sequencing of the PCR-amplified V4 region of the 16S rRNA (prokaryotic microbial communities), using primers 515 F (5′-CTAGTGCCAGCMGCCGCGGTAA -3′) and 806 R (5′-CTAGGACTACHVGGGTWTCTAAT-3′), and of the 18S SSU, using primers 3NDf-CS1F (5′ - GGCAAGTCTGGTGCCAG - 3′) and V4-Euk-CS2R (5′ - ACGGTATCTRATCRTCTTCG - 3′) (eukaryotic microbial communities) was performed using a MiSeq PE300 Next Generation system (Illumina) by Genome Quebec, following the company's protocol.

An open-source software package, DADA2 (Callahan et al., 2016), was applied to model and correct Illumina-sequenced amplicon errors. Data were demultiplexed into forward and reverse reads according the barcode sequence into sample identity, and trimming was performed, according to Kokou et al. (2020). For the forward reads and based on the

quality profiles, the first 250 nucleotides were kept and the rest were trimmed, while for the reverse reads, the last 220 nucleotides were kept. DADA2 resolves differences at the single-nucleotide level and the end product is an amplicon sequence variant table, recording the number of times each exact sequence variant (ESV) was observed in each sample (100% sequence identity). Taxonomy was assigned using the Ribosomal Database Project Classifier (Wang et al., 2007) against the 16S gene reference Silva database (138 version) (McLaren, 2020) and the 18S gene reference Silva database (128 version) (Morien and Parfrey, 2018). Owing to the variation in sequence depths between samples, all samples were normalized to the lowest depth by subsampling.

For the alpha-diversity analysis, Shannon H' diversity, richness (observed taxa) and rare taxa abundance were calculated for both the prokaryotic and the eukaryotic microbial communities. Non-parametric tests (Wilcoxon test) and linear mixed-effect models (nlme R package (Pinheiro and Bates, 2007)) were used to assess alpha-diversity. Adonis implementation of Permanova (Anderson, 2001) (non-parametric permutational multivariate analysis of variance) was used for comparison between groups.

2.7. Calculation and data analysis

The specific growth rate (SGR) was calculated as follows, in which $\ln(BW_{initial})$ and $\ln(BW_{final})$ are the natural logarithm of the shrimp individual body weight at the beginning and end of the experiment, respectively:

(1) $$SGR\ (\%BW/day) = \frac{\ln(BW_{final}) - \ln(BW_{initial})}{Days\ of\ culture} \times 100\%$$

The feed conversion ratio was calculated with (FCR-CHO) or without taking into consideration CHO addition (FCR) as follows:

(2) $$FCR + CHO = \frac{Feed\ fed\ (g) + Carbohydrate\ added\ (g)}{Biomass\ gain\ (g)}$$

(3) $$FCR = \frac{Feed\ fed\ (g)}{Biomass\ gain\ (g)}$$

Protein efficiency ratio (PER), nitrogen and energy use efficiencies were calculated as follows:

(4) $$PER = \frac{Gain\ in\ shrimp\ biomass\ (g)}{Protein\ input\ from\ feed\ and\ carbohydrate\ (g)}$$

(5) $$Nitrogen\ use\ efficiency\ (\%) = \frac{Nitrogen\ retained\ in\ shrimp\ (g)}{Nitrogen\ input\ from\ feed\ and\ carbohydrate\ (g)} \times 100\%$$

$$(6)\ Carbon\ use\ efficiency\ (\%) = \frac{Carbon\ retained\ in\ shrimp\ (g)}{Carbon\ input\ from\ feed\ and\ carbohydrate\ (g)} \times 100\%$$

$$(7)\ Energy\ use\ efficiency\ (\%) = \frac{Energy\ retained\ in\ shrimp\ (g)}{Energy\ input\ from\ feed\ and\ carbohydrate\ (g)} \times 100\%$$

The treatment effects on shrimp, water, biofloc, and periphyton were analyzed using IBM SPSS Statistics 23 software (IBM Corporation, NY, USA). Water quality parameters were analyzed using repeated-measure ANOVA to account for sampling dates. Parameters relating to shrimp, biofloc, and periphyton were analyzed using one-way ANOVA. Due to significant difference in initial shrimp body weight among phases, the treatment effects on shrimp growth and nutrient utilization were analyzed with one-way ANCOVA, considering initial biomass as a covariate. The probability (P) value < 0.05 indicates a significant effect, while P value < 0.1 indicated a trend toward a significant effect. Least Significant Difference (LSD) post-hoc test was performed when a significant effect was found.

3. Results

3.1. Shrimp performance

The treatments had significant effects on the harvested biomass, feed conversion ratios, and protein, nitrogen and energy utilization efficiencies (P < 0.05) (Table 5.2). Specifically, the harvested biomass and protein efficiency ratio was highest when carbohydrate (CHO) was added separately (treatment COM-Feed + CHO) (P < 0.05). Treatment COM-Feed had higher harvested biomass than treatment CHO-Feed (P = 0.05). Meanwhile, the energy efficiency was the best when additional carbohydrate was not used (treatment COM-Feed). The FCR and nitrogen efficiency in treatments COM-Feed and COM-Feed + CHO were not different from each other (P > 0.05) but were higher than those in treatment CHO-Feed (P < 0.05). The survival rate was not different among treatments (P > 0.05) but tended to be higher in treatment COM-Feed + CHO than in treatments CHO-Feed, and COM-Feed respectively (P < 0.1). Similarly, the final body weight, weight gain, and growth rate of the shrimp were not different among treatments (P > 0.05) but tended to be higher in treatments COM-Feed and COM-Feed + CHO than in treatment CHO-Feed (P < 0.1).

Table 5.24 Production performance of *L. vannamei* juveniles subjected to different feeding treatments in the 4-week experiment.

Parameters	COM-Feed	COM-Feed + CHO	CHO-Feed	SEM	P values
Feed fed (g/tank)	358	356	708	-	-
Cornstarch added (g/tank)	-	359	-	-	-
Harvested biomass (g)	421[b]	445[a]	403[c]	4.210	**0.011**
Final BW (g/ind)	9.1	9.0	8.4	0.129	0.097
Weight gain (g/ind)	5.8	5.8	5.1	0.875	0.097
Survival rate (%)	93	99	96	0.129	0.089
SGR (% BW/day)	3.5	3.5	3.2	0.051	0.090
FCR	1.4[a]	1.3[a]	3.0[b]	0.051	**0.000**
FCR + CHO	-	2.6[a]	3.0[b]	0.052	**0.000**
Protein efficiency ratio	1.9[b]	2.1[a]	1.8[b]	0.032	**0.010**
Nitrogen efficiency (%)	43[a]	46[a]	38[b]	0.784	**0.009**
Carbon efficiency (%)	21	12	10	1.554	**0.000**
Energy efficiency (%)	21[a]	13[b]	11[c]	0.203	**0.000**

Values are adjusted means of four replicate tanks per treatment and standard error of the means (SEM) when body weight (BW) at the start is considered as a covariate. SGR = specific growth rate; FCR = feed conversion ratio; FCR-CHO = feed conversion ratio accounting carbohydrate as feed. Probability (P) values in bold indicate significant treatment effects ($P < 0.05$). Adjusted means with different superscripts are statistically different. Treatments include COM-Feed (diet only, C/N = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6).

3.2. Water quality

During the experiment, the water temperature, pH, salinity and dissolved oxygen were 27 ± 2°C, 8.2 ± 0.1, 24 ± 1 ppt, and above 6 mg/L, respectively, and similar among treatments ($P > 0.05$). Most measured parameters did not change during the experimental period ($P_{Time} > 0.05$) (Table 5.3). The TN, IN, and NOx changed with time, but the changes were dependent on the treatment effects ($P_{Treatment \times Time} < 0.05$). The fluctuation of TN during the experiment is shown in Figure 5.1. The fluctuation of IN and NOx (Figure not shown) showed similar pattern with that of TN. Orthophosphate and inorganic carbon ($P_{Time} < 0.05$) change with time. In all treatments, TC and OC increased during the experiment.

The treatments had significant effects on most water quality parameters ($P_{Treatment} < 0.05$), except total ammonia nitrogen, organic nitrogen, and inorganic carbon ($P_{Treatment} < 0.05$). The TAN concentration was similarly low in all treatments ($P > 0.05$) but was numerically lower when carbohydrate was added ($P > 0.05$). Total and inorganic nitrogen, nitrite and nitrate nitrogen (NO_x), and phosphate were lower, whereas total and organic carbon were higher when CHO was added ($P < 0.05$), irrespective how. The chlorophyll a and chemical oxygen demand (COD) increased during the experiment ($P_{Time} < 0.05$) and were similar among treatments ($P_{Treatment} > 0.05$) (Figure 5.2).

Table 5.3 Summary of selected water quality parameters among different feeding treatments of the 4-week experiment.

Parameters	COM-Feed	COM-Feed + CHO	CHO-Feed	SEM	P values		
					Treatment	Time	Treatment*Time
TAN (mg/L)	0.4	0.1	0.1	0.1	0.238	0.328	0.173
NO_x (mg/L)	20^a	15^b	15^b	1.0	**0.009**	0.083	**0.046**
PO_4 (mg/L)	2.0^a	1.3^b	1.3^b	0.2	**0.029**	**0.032**	0.231
TC (mg/L)	8^a	11^b	12^b	0.9	**0.019**	0.525	0.224
IC (mg/L)	0.9	0.7	0.8	0.1	0.202	**0.044**	0.197
OC (mg/L)	7^a	10^{ab}	12^b	1.0	**0.032**	0.270	0.121
TN (mg/L)	21^a	17^b	16^b	1.0	**0.018**	0.292	**0.044**
IN (mg/L)	20^a	15^b	15^b	0.9	**0.005**	0.119	**0.030**
ON (mg/L)	0.7	1.6	1.4	0.2	0.062	0.209	0.462

Values are means of four sampling times of four replicate tanks per treatment. Starting values of each parameter were used as covariates in ANCOVA analysis of the respected parameters. TAN = total ammonia nitrogen, NO_x = nitrite and nitrate nitrogen, TC = total carbon, IC = inorganic carbon, OC = organic carbon, TN = total nitrogen, IN = inorganic nitrogen, ON = organic nitrogen. Probability (P) values in bold indicate significant treatment effects ($P < 0.05$). Means with different superscripts are statistically different. Treatments include COM-Feed (diet only, C/N = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6).

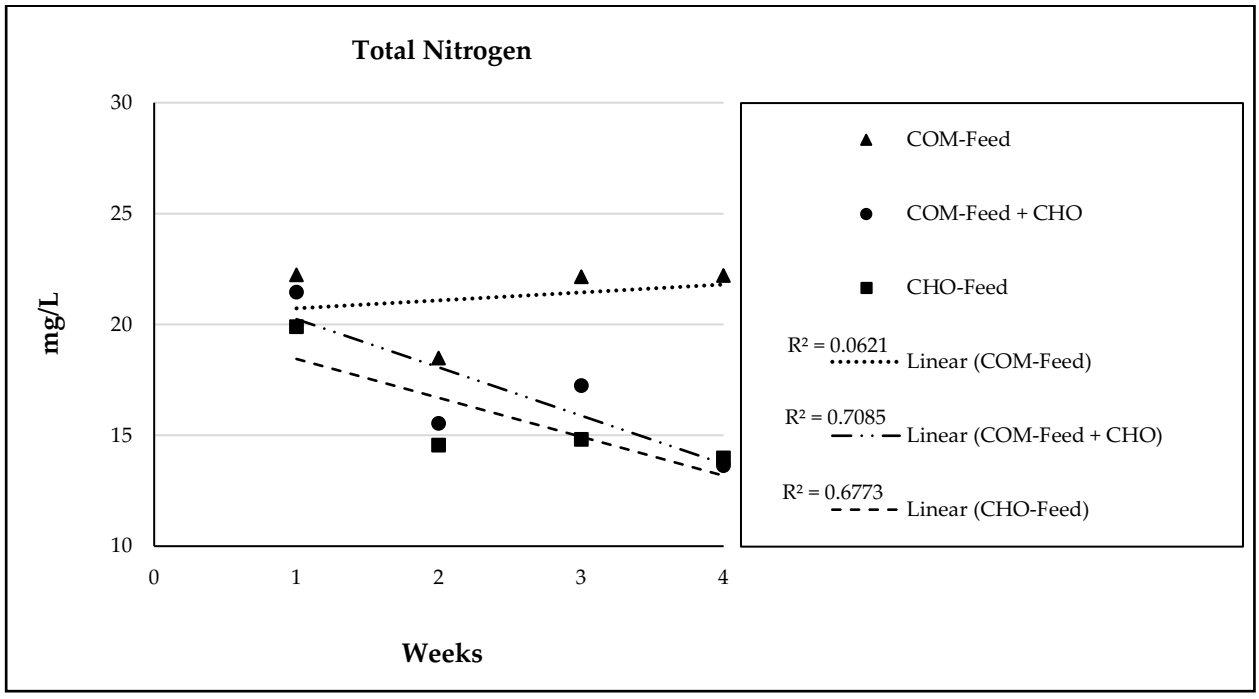

Figure 45.1 Fluctuation of total nitrogen in the water during the 4-week experiment. Data points are means of four replicate tanks per treatment. Treatments include COM-Feed (diet only, C/N = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6).

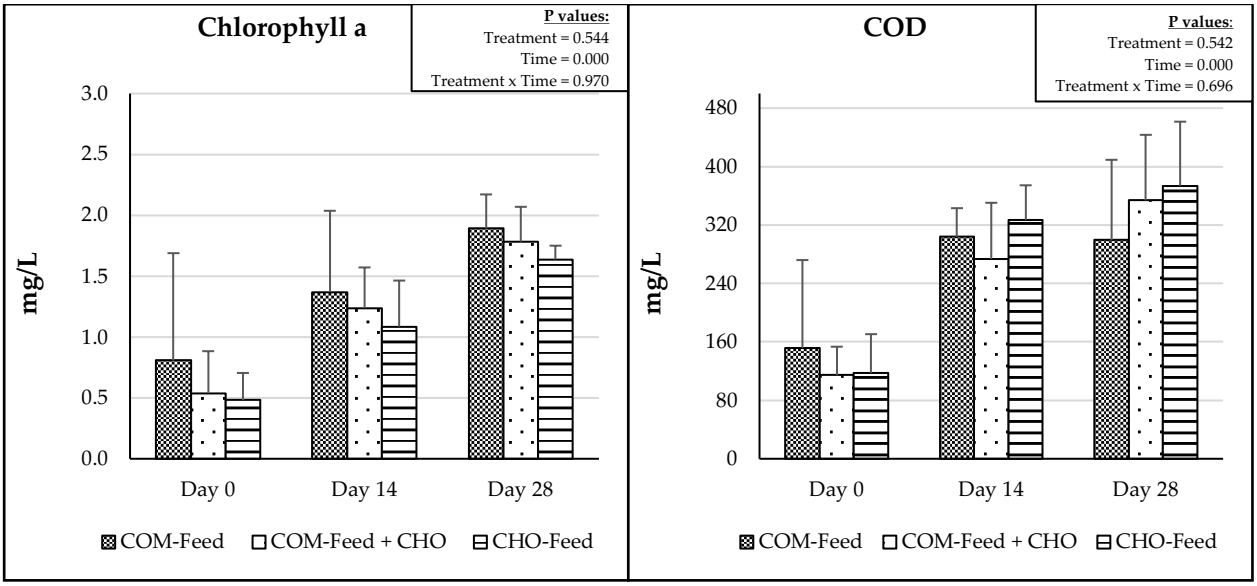

Figure 5.2 Fluctuation of chlorophyll a and chemical oxygen demand (COD) in water among different feeding treatments. Column heights are means with error bars showing standard deviation of four replicate tanks per treatment. Treatments include COM-Feed (diet only, C/N = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6).

3.3. Biofloc growth

Biofloc growth evaluated using total suspended solids (TSS) and volatile suspended solids (VSS) is shown in Figure 5.3. During the experiment, both TSS and VSS increased with time ($P_{Time} < 0.05$). One-way ANOVA with data on each sampling date showed that the TSS and VSS were not statistically different among treatments at any of the sampling times ($P_{Treatment} > 0.05$). However, both parameters were numerically higher in treatment CHO-Feed at the end of the experiment than in other treatments.

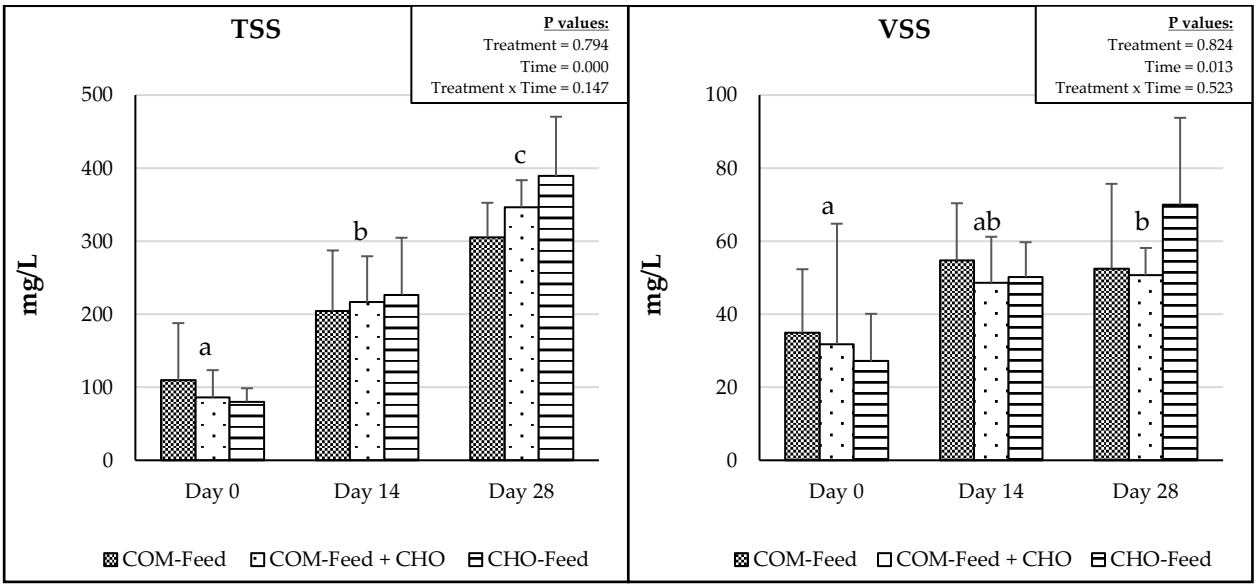

Figure 5.3 Fluctuation of total suspended solids (TSS) and volatile suspended solids (VSS) in water among different feeding treatments. Treatments include COM-Feed (diet only, C/N = 7.6), COM-Feed + CHO (diet

+ separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6). Column heights are means with error bars showing standard deviation of four replicate tanks per treatment. Sampling days with different letters on the top are statistically different ($P < 0.05$).

3.4. Proximate composition of shrimp, biofloc, and periphyton

Shrimp proximate composition was not affected by treatments ($P > 0.05$) (Table 5.4). Similarly, biofloc proximate composition was not different among treatments ($P_{Treatment} > 0.05$) but changed with time ($P_{Time} < 0.05$) except for phosphorus content ($P_{Time} > 0.05$) (Table 5.5). Specifically, the ash, calcium, and magnesium contents of biofloc decreased, whereas the crude protein, energy, total carbon and nitrogen contents increased during the culture period. Regarding periphyton, the total biomass, and dry matter and ash contents were not affected by treatment ($P > 0.05$), although the ash content tended to be higher in treatment COM-Feed than in treatments CHO-Feed, and COM-Feed + CHO respectively ($P < 0.1$) (Table 5.6). The periphyton in treatment COM-Feed + CHO had statistically the highest crude protein, energy, and total carbon and nitrogen contents ($P < 0.05$), while these values are similar among the other two treatments ($P > 0.05$).

Table 5.4 Proximate composition on dry weight basis of *L. vannamei* juveniles at the end of the 4-week experiment.

Parameters	COM-Feed	COM-Feed + CHO	CHO-Feed	SEM	P values
DM (g/kg WW)	271	271	271	0.3	0.524
Ash (g/kg)	131	138	129	2.6	0.429
CP (g/kg)	767	777	763	3.5	0.260
Fat (g/kg)	38	41	42	1.7	0.648
Energy (kJ/g)	20	21	21	0.1	0.474
TC (g/kg)	475	470	470	3.4	0.854
TN (g/kg)	127	126	123	0.9	0.854

Values are means of four replicate tanks per treatments and standard error of the means (SEM). DM = dry matter, WW = wet weight, CP = crude protein, TC = total carbon, TN = total nitrogen. Treatments include COM-Feed (diet only, C/N = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6).

Table 5.5 Proximate composition on dry weight basis of biofloc in different feeding treatments in the 4-week experiment.

Parameters	COM-Feed	COM-Feed + CHO	CHO-Feed	SEM	P values		
					Treatment	Time	Treatment*Time
Ash (g/kg)	263	230	238	48	0.881	**0.018**	0.969
CP (g/kg)	338	382	379	32	0.581	**0.001**	0.966
Energy (kJ/g)	13	16	16	2.2	0.716	**0.012**	0.824
TC (g/kg)	328	338	335	9.1	0.754	**0.004**	0.985
TN (g/kg)	54	54	55	2.7	0.959	**0.001**	0.924
P (g/kg)	16	18	16	3.0	0.850	0.569	0.613
Ca (g/kg)	118	132	119	23	0.888	**0.005**	0.712

Mg (g/kg)	30	31	29	3.7	0.901	**0.001**	0.870

Values are means of three sampling times of four replicate tanks per treatment and standard error of the means (SEM). CP = crude protein TC = total carbon, TN = total nitrogen, P = phosphorus, Ca = calcium, Mg = magnesium. Probability (P) values on bold indicate significant treatment effects. Treatments include COM-Feed (diet only, C/N = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6).

Table 55.6 Proximate composition on dry weight basis of periphyton at the end of the 4-week experiment.

	COM-Feed	COM-Feed + CHO	CHO-Feed	SEM	P values
Biomass (g WW)	85	149	139	23	0.507
DM (g/kg WW	891	860	913	26	0.747
Ash (g/kg)	409	361	384	8.7	0.055
CP (g/kg)	292[a]	327[b]	303[a]	6.1	**0.040**
Energy (kJ/g)	12.7[a]	14.4[b]	13.3[a]	0.2	**0.013**
TC (g/kg)	298[a]	337[b]	315[a]	5.9	**0.005**
TN (g/kg)	47[a]	55[b]	49[a]	1.1	**0.001**

Values are means of four replicate tanks per treatment and standard error of means (SEM). DM = dry matter, WW = wet weight, CP = crude protein; TC = total carbon and TN = total nitrogen. Probability (P) values in bold indicate significant treatment effects. Means with different superscripts are statistically different. Treatments include COM-Feed (diet only, C/N = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6).

3.5. Nutrient balances

The absolute amounts of carbon and nitrogen in each experimental unit at stocking and harvesting of the experiment are shown in Figures 5.4 and 5.5, respectively. The carbon in shrimp, water, and biofloc at stocking and in shrimp, water, biofloc, and periphyton at harvesting were similar among treatments ($P > 0.05$). The total carbon at harvest in COM-Feed + CHO and CHO-Feed treatments was similar ($P > 0.05$), but higher than that in treatment COM-Feed ($P < 0.05$). The carbon in water at harvesting was not different among treatments ($P > 0.05$) but tended to be higher in treatment CHO-Feed than in treatments COM-Feed + CHO, and COM-Feed respectively ($P < 0.1$). Of the total input carbon 58% was lost at the treatments COM-Feed + CHO and CHO-Feed during the experiment, while at the COM-Feed treatment this was only 39%.

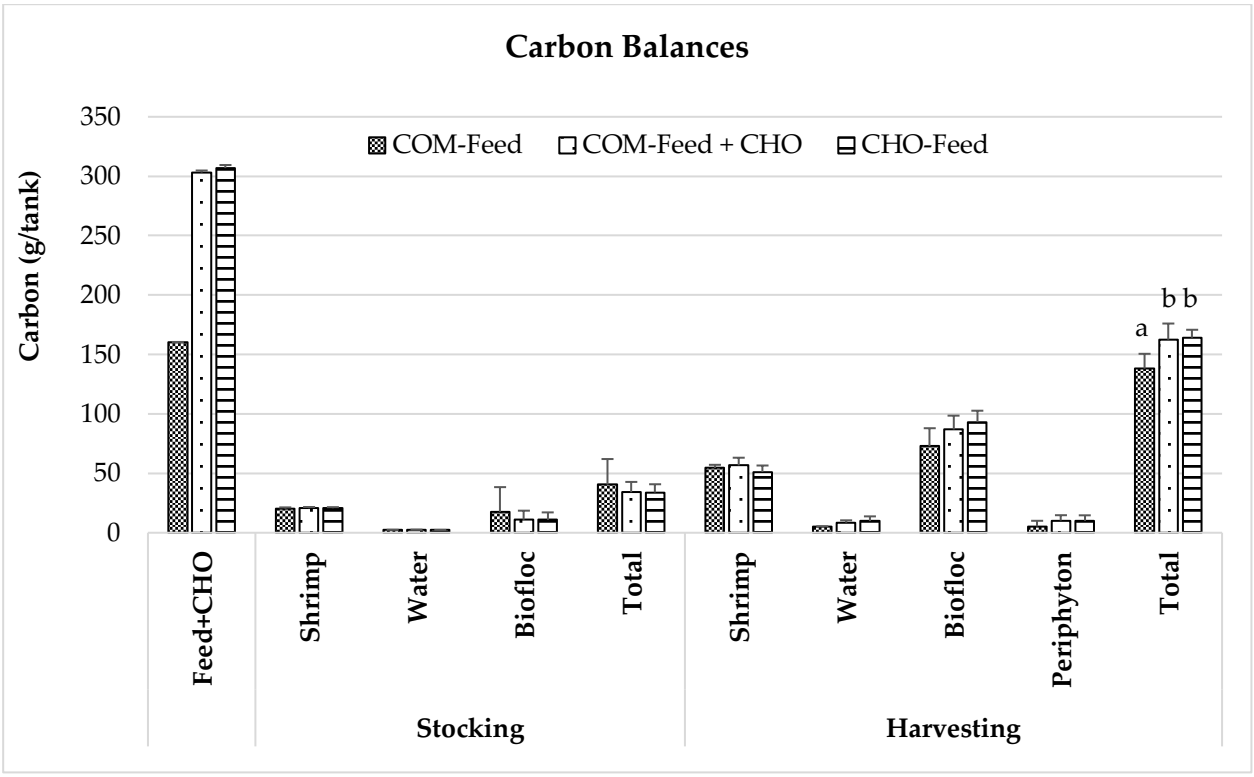

Figure 5.4 Carbon balances among different feeding treatments. Column heights are means with error bars showing standard deviation of four replicate tanks per treatment. Treatments include COM-Feed (diet only, C/N = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6). Bars in the same group with different letters on the top are statistically different among others ($P < 0.05$). The carbon input from Feed+CHO was controlled, and therefore was not statistically analyzed.

The total nitrogen at stocking and harvesting was similar among treatments ($P > 0.05$). The nitrogen from shrimp, water, and biofloc at stocking were similar among treatments ($P > 0.05$). At the end of the experiment, the nitrogen content in shrimp of treatment COM-Feed + CHO was similar to the content in treatment COM-Feed ($P > 0.05$), but higher than that of treatment CHO-Feed ($P < 0.05$). The nitrogen in biofloc at harvesting was not different among treatments ($P > 0.05$) but tended to be higher in treatment CHO-Feed than in treatments COM-Feed + CHO and Feed, respectively ($P < 0.1$). All treatments tanks retained more than 92% of the total input nitrogen.

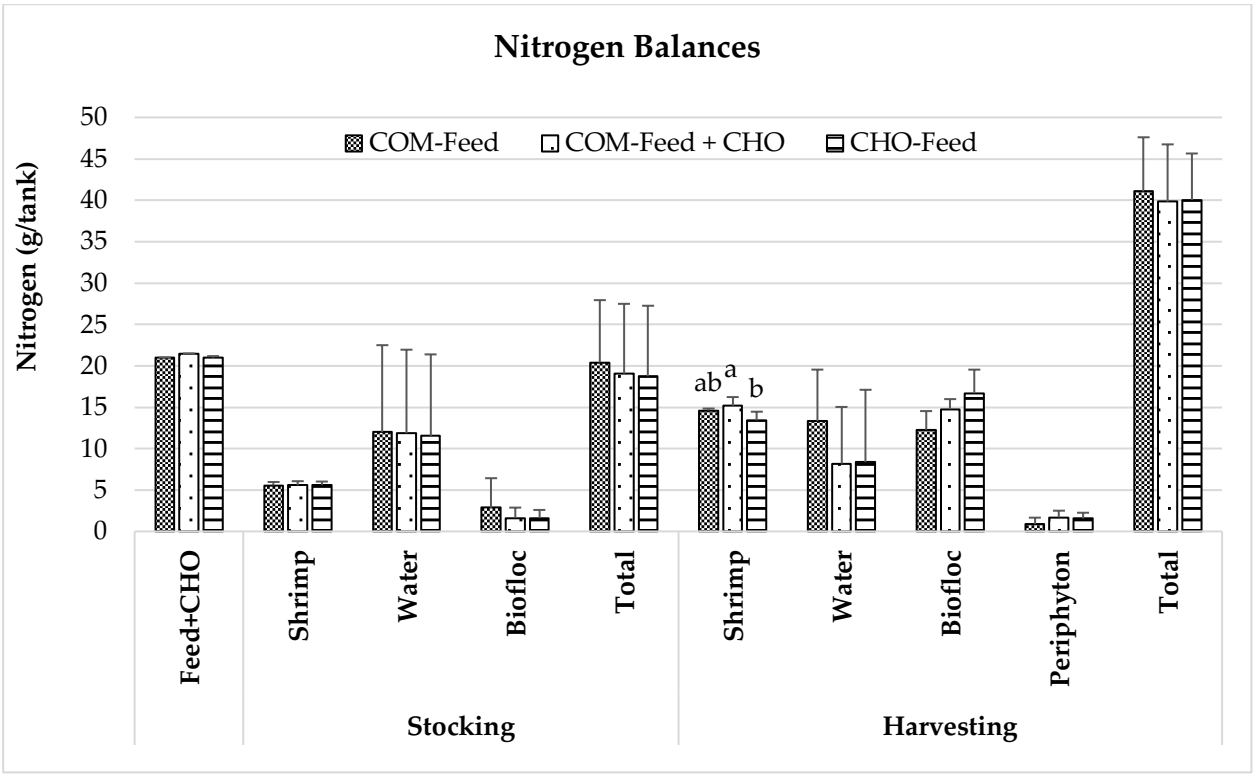

Figure 5.5 Nitrogen balances among different feeding treatments. Column heights are means with error bars showing standard deviation of four replicate tanks per treatment. Treatments include COM-Feed (diet only, C/N = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6). Bars in the same group with different letters on the top are statistically different among others ($P < 0.05$). The nitrogen input from feed and corn starch was controlled, and therefore was not statistically analyzed.

3.6. Microbiota analysis

Diversity analysis (alpha-diversity) of the prokaryotic microbial communities (16S rRNA) in the two types of biofloc samples, the particulate and the seston communities, did not revealed significant changes between the different treatments (Figure 5.6; $P > 0.05$). However, diversity analysis of the eukaryotic microbial communities (18S SSU) indicated significant difference in the richness between treatments between COM-Feed and CHO-Feed, and on the abundance of rare taxa between COM-Feed and COM-Feed + CHO (Figure 5.6; $P<0.05$). Overall, the eukaryotic microbial community diversity and richness was numerically higher in the particulate biofloc samples, while CHO-Feed was found to have higher alpha-diversity compared to the rest of the treatments. This observation was confirmed by linear mixed-effects model analysis, which indicated that treatment and sample type (particulate versus seston) were the major factors shaping eukaryotic microbial community richness and abundance of rare taxa, respectively ($P < 0.05$).

Looking at the beta-diversity (microbial composition), both prokaryotic and eukaryotic microbial communities were affected by time (Permanova analysis; $P<0.001$), while no significant difference were observed by the different sample types or treatments ($P > 0.05$). The relative abundance of the most abundant prokaryotic and eukaryotic phyla and genera are presented in Figure 5.7 and 5.8, respectively. In the prokaryotic communities, the most abundant microbial phyla was Proteobacteria (35% of the relative abundance), followed by Planctomycetote (15%), Bacteroidota (15%), Cyanobacteria (12%) and Chloroflexi (10%). With regard to genera, a large portion of the microbial communities was not assigned to a genus (57% of the relative abundance), while the most abundant genus was *Phaeodactylibacter* (4%), followed by *Maricauda* (2%), *Planctomicrobium* (2%), *Pseudoalteromonas* (1%) and *Ruegeria* (1%). In the eukaryotic communities, Chlorophyta phylum occupied the higher proportion of the relative abundance (45% of the relative abundance), followed by Rotifera (5%), Perkinsidae (4%), Nematoda (2%) and Dinoflagellates (1.2%). Among the Chlorophyta phylum, *Nannochloropsis* (20% of the relative abundance) was the most abundant genera.

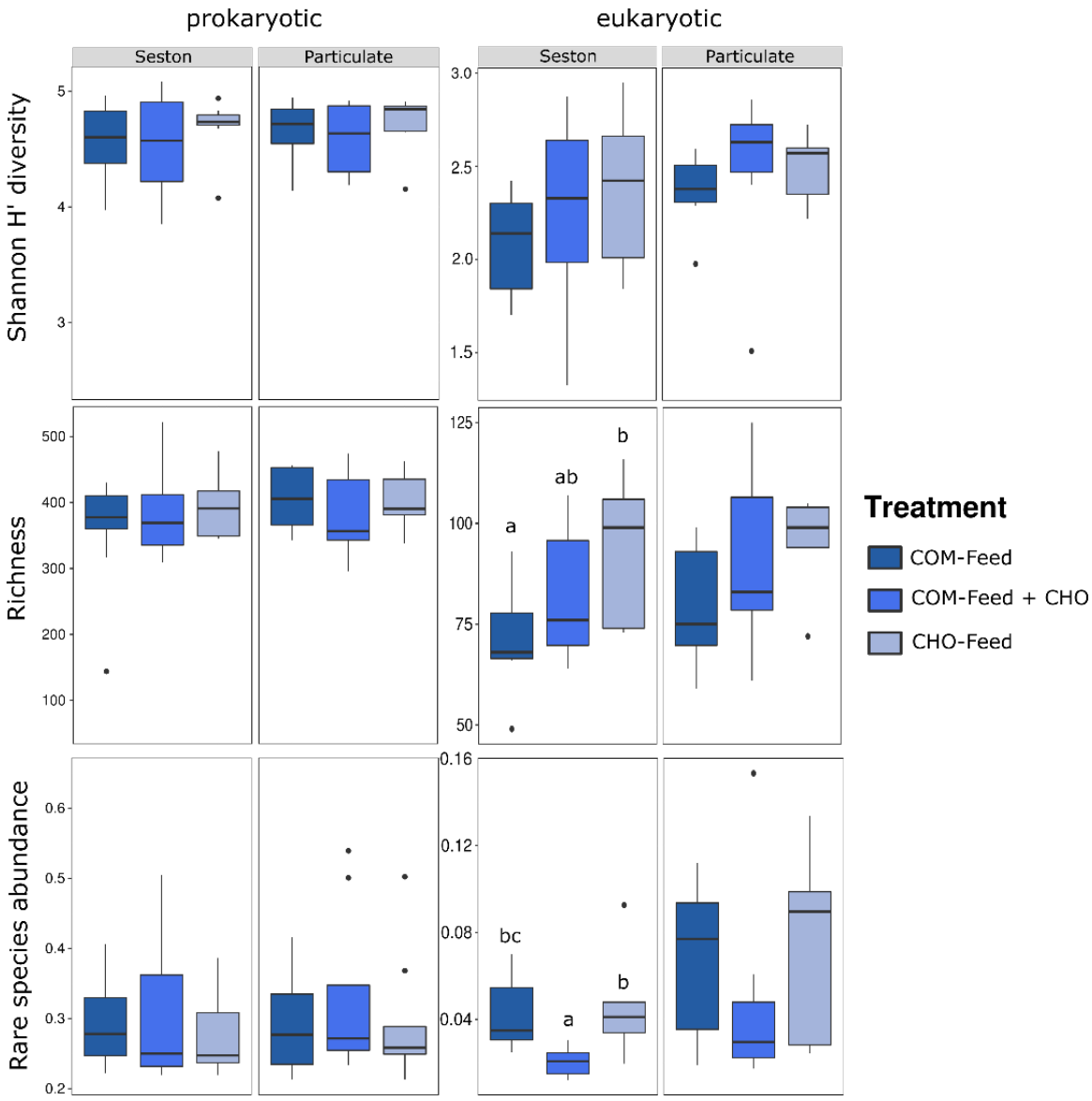

Figure 5.6 Alpha diversity of the prokaryotic and eukaryotic microbial communities in the different treatments. Data are shown as box plots; the horizontal line indicates the median and the whiskers indicate the lowest and highest points within 1.5× the interquartile ranges of the lower or upper quartile, respectively. All data were included in the analysis. Significance was tested with Wilcoxon rank-sum two way test at $P < 0.05$. Treatments include COM-Feed (diet only, C/N = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6)

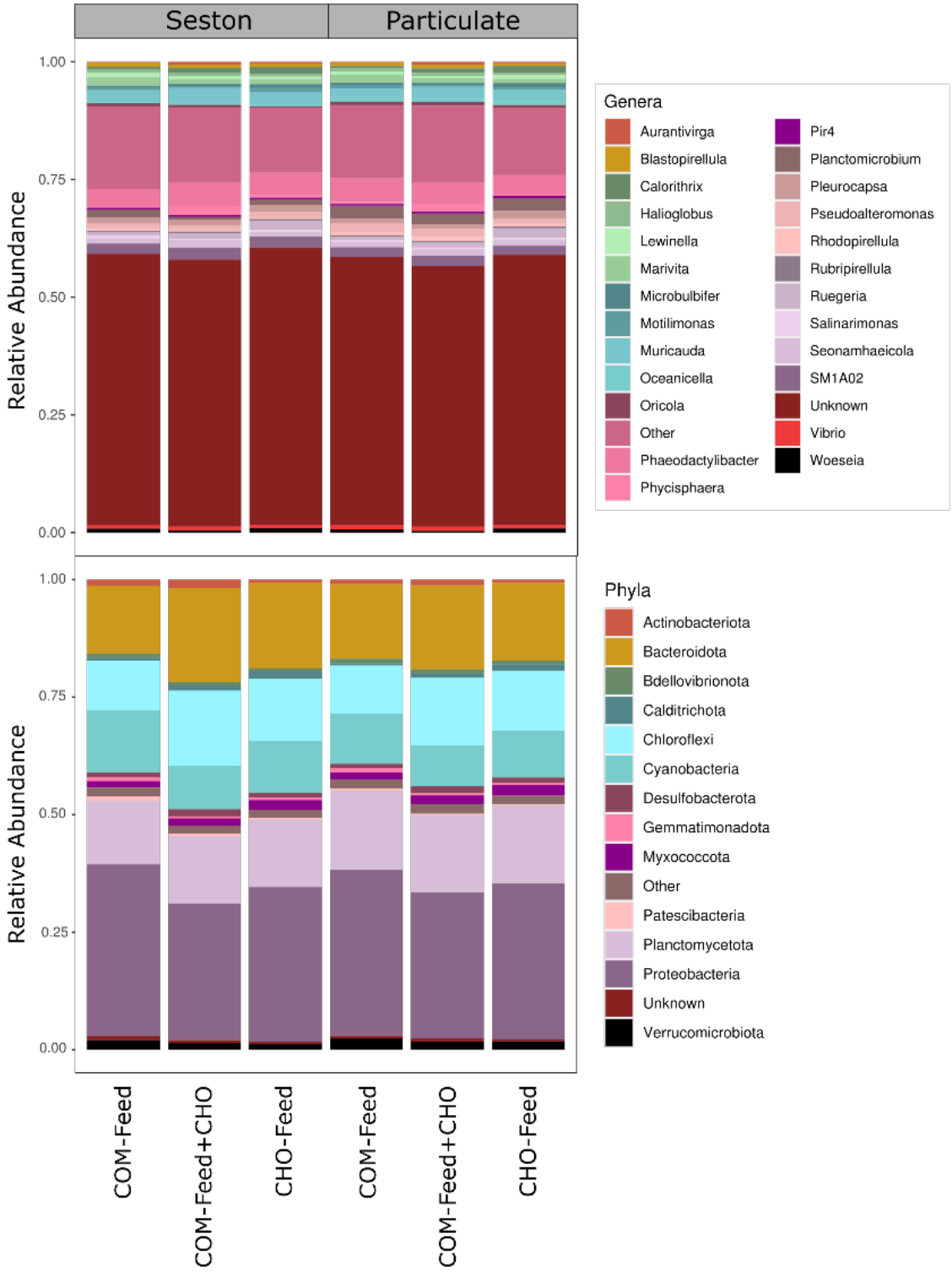

Figure 5.7 Prokaryotic microbial composition of the biofloc samples between the different treatments at the phylum (bottom) and genus level (top). Treatments include COM-Feed (diet only, C/N = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6).

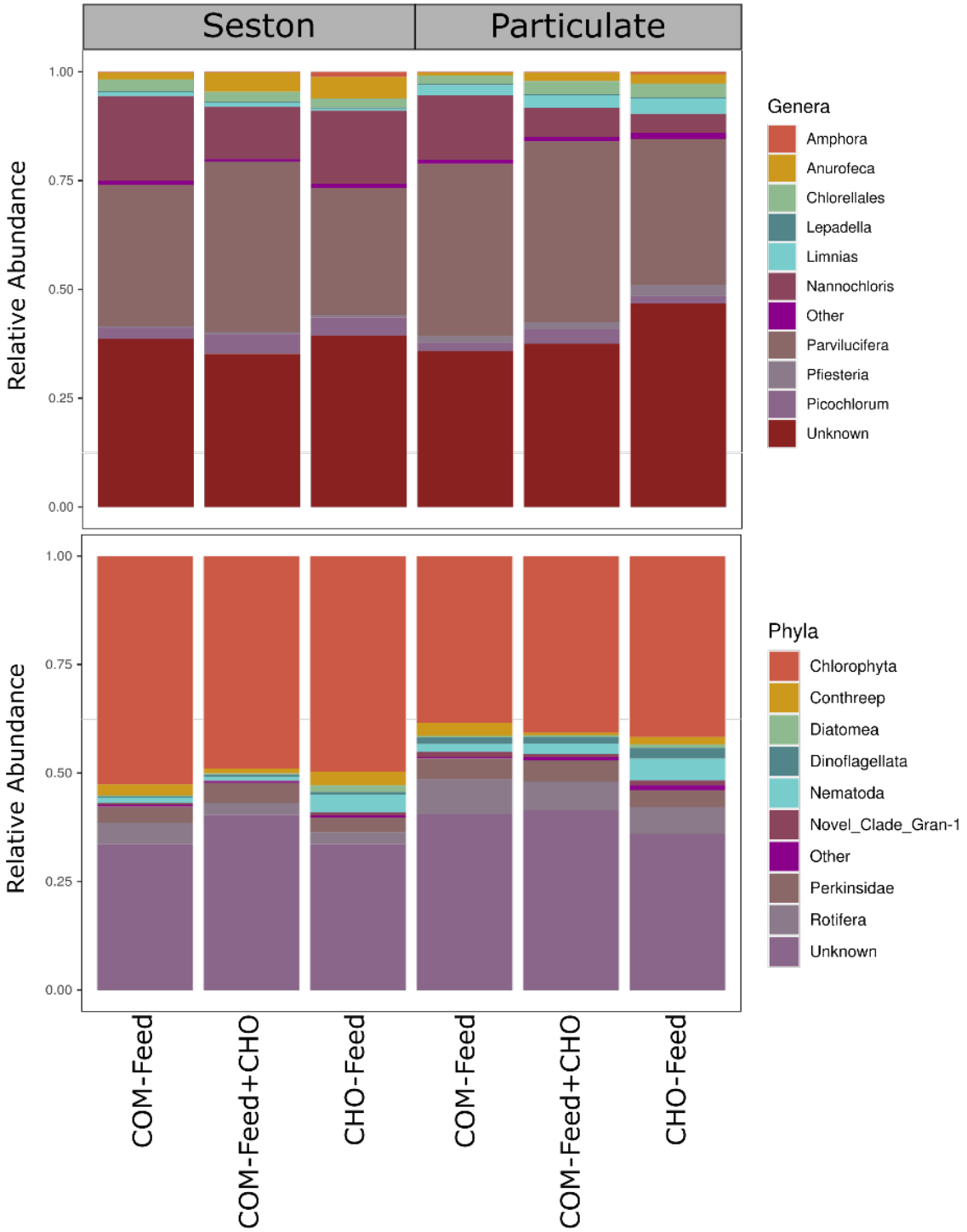

Figure 55.8 Eukaryotic microbial composition of the biofloc samples between the different treatments at the phylum (bottom) and genus level (top). Treatments include COM-Feed (diet only, C/N = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6).

4. Discussion

The carbohydrate (CHO) addition to the Pacific white shrimp culture systems improves the production, FCR, and protein efficiency. This practice promotes bacterial growth which assimilates inorganic nitrogen and improves water quality (Avnimelech, 1999; Panigrahi et al., 2019; Ren et al., 2019; Tong et al., 2020). Beside this, the bioflocs also serve as supplemental food, and increase the nitrogen retention (Ekasari et al., 2014a; Hari et al., 2004; Schneider et al., 2005). Manipulating C/N ratio increases the abundance of zooplankton, and potentially beneficial bacteria and bioactive metabolites (Gao et al., 2012; Guo et al., 2020). Adding CHO to the culture system has also been related with enhanced digestive enzyme activities (Xu and Pan, 2012), immune cellular response (Xu and Pan, 2013), and disease resistance (Ekasari et al., 2014b; Hostins et al., 2019; Panigrahi et al., 2019) of the Pacific white shrimp. The benefits of adding CHO on water quality and natural food availability were demonstrated in numerous species including Pacific white shrimp (Liu et al., 2014), black tiger prawn (Anand et al., 2013; Hari et al., 2004), giant freshwater prawn (Asaduzzaman et al., 2008), Japanese tiger prawn (Zhao et al., 2012), Nile tilapia (Avnimelech, 1999; Hu et al., 2014) and integrated culture of three carp species (Zhao et al., 2014).

In this research, the inclusion of additional CHO in the feed as one pellet (treatment CHO-Feed) resulted in poorer shrimp production and FCR compared to the separate CHO addition (treatment COM-Feed + CHO) although all treatment tanks were fed similar amounts of protein. This could be a result of reduced feed ingestion due to doubling of feed amount and diluting of feed attractant in treatment CHO-Feed. However, since the feed ingestion was not monitored in any treatment and the water quality was similar among COM-Feed + CHO and CHO-Feed treatments, this hypothesis was not yet validated. According to Xia et al. (2010), increasing the dietary CHO content at the expense of protein may increase amylase activity of the culture animal, and the digestibility of the feed. However, this reduces the protease activity in the digestive system of the culture animal, and possibly the deposition of protein in shrimp body. The CHO-Feed, which had high carbohydrate and low protein composition (Table 5.1), assumingly resulted in lower protein absorption in the shrimp, and therefore lower shrimp growth compared to the COM-Feed. Both assumptions on ingestion and absorption of CHO-Feed remain to be proven. Nevertheless, future research on similar topics should improve the experimental design, for example splitting daily feed ration into more frequent feeding when a low protein feed is used. The Pacific white shrimp

performs best with feed of 39-43% protein (Xia et al., 2010). In the biofloc system where CHO is separately added, lowering the dietary protein level from 35% to 30% or 25% did not significantly affect the growth, FCR, survival, and immune response of the Pacific white shrimp (Xu and Pan, 2014b). The further decrease of dietary protein level to 20% showed negative consequences on shrimp health and growth. Besides, the effects of dietary protein levels and C/N ratios on shrimp growth and water quality are independent of each other (Xu and Pan, 2014a).

The water quality and biofloc growth were similar in both methods of adding CHO. The TAN concentration was low in all treatments of this experiment irrespective of CHO addition. This may stem from the fact that the experiment started with a biofloc concentration of ≥ 100 mg/L TSS, at which an input C/N ratio of 6 was sufficient to maintain good water quality (Emerenciano et al., 2017; Martins et al., 2020). Nevertheless, the nitrite and nitrate (NO_x) concentrations significantly reduced when CHO was added, concurring with observations by Panigrahi et al. (2019) and Ren et al. (2019). Here, we report enhancements in shrimp production at the C/N ratio of 14.6 without negative effects on water quality. However, contradictory results were also reported in which increasing the C/N ratio above 12 showed no significant effects on shrimp growth, TAN and NO_2 (Tong et al., 2020). In some cases, C/N ratios above 12 caused water quality deteriorations, resulting in poorer shrimp growth, yield, and FCR (Xu et al., 2018, 2016).

Both biofloc and periphyton had a protein level above 30% DM (Tables 5.5 and 5.6), and were comparable among treatments and to previous reports (Tinh et al., 2021b; Xu and Pan, 2014a). The proximate composition of biofloc was not affected by the absence or presence of additional carbohydrate in this research (Table 5.5). In other research, adding carbohydrate reduced biofloc ash content (Tinh et al., 2021a; Xu and Pan, 2014a), and increased biofloc protein content (Xu and Pan, 2014a). The contribution of biofloc and periphyton to shrimp growth was not measured in this research, however both biofloc and periphyton are nutritious natural food for cultured shrimp. The biofloc contribution to nitrogen retention in Pacific white shrimp may range between 18% to 29% (Burford et al., 2004). In other shrimp species, the natural food production may contribute up to 37% nitrogen and 40% carbon in the shrimp growth (Cardona et al., 2015). In our study, the biofloc contained a larger amount of carbon and a similar amount of nitrogen compared to shrimp at the end of the research. All this information suggests that the biofloc

utilization efficiency in our study was not yet optimal. In order to increase the nutrient use efficiency, further efforts are required to make use of the remaining biofloc.

The microbial diversity within the bioflocs, composed of heterotrophic bacteria, microalgae, zooplankton and rotifers (De Schryver et al., 2008), has been reported to be affected by the carbon source type and C/N ratio (Deng et al., 2018; Panigrahi et al., 2018). Recent studies using different carbon sources in the water were reported to select for certain phyla, while this could be a way to modulate the developing heterotrophic communities (Gutiérrez et al., 2016; Vargas-Albores et al., 2019). Addition of corn starch as a carbon source either as an external source or in the feed did not significantly affect the biofloc prokaryotic microbial communities in the present study. Despite that the major microbial prokaryotic microbial communities identified were similar to what was previously reported, with Proteobacteria as the most abundant phylum, followed by Bacteroidetes, Planctomycetes and Actinobacteria, and genera such as Maricauda, Ruegeria and Phaeodactilibacter (Gutiérrez et al., 2016; Huang et al., 2020). However, carbon addition increased the eukaryotic microbial community diversity and richness, which increased further when the carbon source was added in the feed. More specifically, diatoms and protozoa were found to be more abundant when carbon was added in the diet. Such an increase may be associated with a higher carbon availability in those treatments. Eukaryotic communities have been reported to alter according to the availability of nitrogenous compounds and suspended solids (Gallardo-Collí et al., 2019). Although not significant, higher TSS and VSS were observed in the carbohydrate diets which could potentially explain such findings. Besides, studies comparing carbon sources with different degradation rates showed significant implication on the microbial communities (Jiang et al., 2020; Serra et al., 2015; Wei et al., 2016). The inclusion of corn starch in the feed which likely went through shrimp digestive tract may have altered corn starch bioavailability, and therefore its effects on the microbial communities compared to separate addition in this research. Further research is necessary to improve knowledge of the eukaryote and prokaryote microbial communities present in biofloc systems.

With an iso-protein feeding level at tank level, the overall nitrogen retention in the system was similarly high in all treatments. A previous report shows that the nitrogen retention was not different between the conventional and biofloc systems, accounting for 77% and 79-87% of the input nitrogen respectively, excluding periphyton (Tinh et al., 2021a). In this experiment, more than 92% of the added nitrogen was retained in the system at the

end of the experiment. However, the addition of extra carbon, either mixed within the feed pellet or added separately, did not significantly improve the nitrogen retention in shrimp, nor the overall retention in the system compared to the control. In fact, most of the additional carbon was lost from the system. The conventional COM-Feed treatment retained 69% of the total carbon input, while in carbon-added treatments this was 48% (Figure 5.4). Tinh et al. (2021a) reported a 62% total carbon retention for conventional non-carbon-added systems, but only 26-31% total carbon retention for biofloc systems. A similar doubling of C loss was reported by Hu et al. (2014) rearing tilapia in a biofloc system when increasing the C/N ratio to 16 compared to the input of feed only (Hu et al., 2014). The carbon retention in biofloc systems seems to be affected by the carbon source used (Tinh et al., 2021b). To reduce the carbon emission from aquaculture systems, and its effects on global warming, research to increase carbon use efficiency in biofloc systems is needed.

5. Conclusions

The separate carbohydrate addition was better than its inclusion in the feed for biofloc culture of Pacific white shrimp as high dietary carbohydrate inclusion may result in poorer feed ingestion and absorption by the shrimp. The additional carbohydrate enhanced the water quality during culture. Adding carbohydrate did not significantly increase the nitrogen retention and resulted in more than 50% carbon loss. Besides, the carbohydrate addition did not affect the microbial composition and prokaryotic microbial community diversity, but increased the eukaryotic microbial diversity.

CHAPTER 6

GENERAL DISCUSSION

The world population is growing at an estimated rate of 2.3% annually, reaching 9 billion in 2050 (World Bank, 2013). To ensure food security, the role of aquaculture activities has become more important than ever. During the last decades, aquaculture production grew 4.9% annually (FAO/FishStatJ, 2021), and is expected to reach 108 million tons of finfish, crustacean and mollusks in 2030 (FAO, 2020). One reason often used to explain why aquaculture production grows faster than terrestrial animal production is due to the more efficient feed use in aquaculture: aquaculture: FCR = 1 – 2.7; livestock production: FCR = 2 - 17 (de Verdal et al., 2018; Fry et al., 2018).

Besides improving feed use efficiency, what we feed aquatic animals also affects sustainability. For each ingredient included in the feed, there are tradeoffs in resource use (e.g. non-sustainable energy, freshwater, greenhouse gas emissions, nitrogen, phosphorous). An example is the use of fishmeal and fish oil in aquaculture diets. Presently, the majority of commercial feed for farmed fish still contains fishmeal and fish oil, even though the industry reduced the dietary inclusion percentages considerably during the last decades. However, the global use of fishmeal remained high due to the growth in total aquaculture production. This causes sustainability concerns about over-fishing of so-called "trash fish" to produce fishmeal and fish oil. Numerous alternatives to fishmeal in aquafeed were proposed, including microalgae (Ju et al., 2012; Vizcaíno et al., 2014), poultry by-product (Amaya et al., 2007; Burr et al., 2012), mealworm (Panini et al., 2017) and plant-based products (Burr et al., 2012). In many cases, animal growth was sustained or even improved when fishmeal was partially or completely replaced (Ju et al., 2012; Panini et al., 2017). These alternatives, however, often lack or are limited in essential amino and fatty acids required for normal growth such as methionine or Ω-3 long chain polyunsaturated fatty acids (Panini et al., 2017). The replacement of fishmeal with plant-based products increases water use 63%, land use 81% and phosphorus use 83% (Malcorps et al., 2019). If the amount of "trash fish" providing fish meal and fish oil to aquaculture would be eaten by humans directly, then the global demand for fish from aquaculture would be reduced by 4-5% in 2030 (FAO, 2020). A careful multi-objective analysis of the advantages and disadvantages of replacing fishmeal and fish oil in aquaculture feed with plant-based ingredients is still needed (Malcorps et al., 2019), especially considering food security and the use of scarce resources.

From a resource use efficiency perspective, animal production is not efficient. For most parameters (e.g. energy, carbon, protein, phosphorous) considered, 50% - 75% of the

amount fed is not assimilated into body mass gain of farmed animals (Alarcón-Silvas et al., 2021; Hardy and Kaushik, 2021; NRC, 2011; Pérez-Fuentes et al., 2016; Thakur and Lin, 2003). A small fraction of the diet is not eaten, non-digested feed is excreted as feces and food digestion leads to the release of metabolic wastes (ammonia, CO_2) into culture water. This deteriorates the water quality in the culture units, especially in intensive systems, increases opportunistic disease incidences, and pollutes surface waters downstream from farms (Ahmad et al., 2017; Cao et al., 2007; Páez-Osuna, 2001; Tovar et al., 2000). Especially in areas with a high density of intensive aquaculture operations, nutrient discharge might degrade environmental quality. Therefore, for aquaculture to grow sustainably, strategies to improve nutrient use efficiency, maintain acceptable water quality during culture and avoid uncontrolled discharge of metabolic wastes from aquaculture systems need to be found and implemented. The above example of fishmeal and fish oil, shows there are no easy solutions.

6.1 Biofloc technology – more complicated than commonly thought

Already more than 30 years ago, biofloc technology caught the attention of the aquaculture industry, as it reduces organic wastes and pollution, maintains water quality and improves the nutrient use efficiency. In doing so, it provides a potential solution for sustainable aquaculture. The first biofloc-based aquaculture systems advocated additional input of carbohydrate. In principle, the addition of carbohydrate sources to aquaculture systems provides energy for heterotrophic assimilation of nitrogen species (ammonia, nitrite, nitrate), improving water quality and providing biofloc as a natural food source for fish and shrimp (Avnimelech, 2009, 1999; Ebeling et al., 2006; Emerenciano et al., 2017). Carbohydrate addition has been demonstrated in theory and practice to effectively control water quality, and to improve fish growth, fish health and nitrogen utilization efficiency. However, our research results indicate that long-term maintenance of water quality in biofloc systems is not easy, yielding often unexpected results for different combinations of C:N ratio and feeding level (Chapter 2), the type of carbohydrate used (Chapter 3), the CHO addition frequency (Chapter 4) and whether the extra carbohydrate applied is mixed in the pelleted diet or not (Chapter 5).

Increasing the C:N ratio benefits aquaculture systems in many ways. A higher C:N ratio reduces the concentrations of ammonium and nitrite-nitrogen, improving the water quality in the rearing environment (Avnimelech, 1999; Ren et al., 2019). It also enhances immune cellular response and antioxidant activities in the plasma and hepatopancreas of

the shrimp (Xu and Pan, 2013). Research results also suggest that biofloc growth reduces the risks of early mortality syndrome outbreak in shrimp, a disease causing significant economic losses since 2009 (Hostins et al., 2019; Tran et al., 2013). Overall, biofloc improves shrimp growth and the feed conversion ratio through consumption of biofloc as natural food, thus reusing otherwise wasted nutrients (Cardona et al., 2015; Khatoon et al., 2016; Xu and Pan, 2012). However, increasing the C:N ratio can also have negative effects. The study by Xu et al. (2016) testing different C:N ratios (9, 10, 12, 15, and 18) in *L. vannamei* culture demonstrated that shrimp growth was the same among C:N ratios ranging from 9 to 12, while C:N ratios above 12 resulted in poorer shrimp growth. In addition, water quality in terms of total ammonia nitrogen and nitrite concentrations was less favorable for shrimp at C:N ratios above 12 (Xu et al., 2016). Pérez-Fuentes et al. (2016) tested different C:N ratios ranging between 10 and 20 for tilapia culture, against a control treatment exchanging 80% of the water in rearing tanks weekly, a common farming method. The control treatment resulted in the highest weight gain. Among the different C:N ratio treatments, the water quality was similar between treatments, the lowest C:N of 10 resulted in the highest production and individual weight gain (Pérez-Fuentes et al., 2016). In our research, both beneficial (Chapters 2 and 5) and non-beneficial effects (Chapter 4) of adding CHO were observed.

In Chapter 2, three different C:N ratios were tested. The benefits of adding CHO on shrimp growth and biomass were consistent, regardless of shrimp stocking density and feeding level. Increasing the C:N ratio consistently resulted in better shrimp growth and biomass gain; on the same time, prokaryotic microbes with antimicrobial properties were enriched with increasing C:N ratio, providing potential beneficial effects for the shrimp. Meanwhile, in Chapter 4, when comparing different CHO addition frequencies to the control treatment receiving no additional CHO, the effects of adding CHO was much less pronounced. The reason for this could be due to low feeding levels used in Chapter 2 where shrimp had to consume biofloc as natural feed, compared to standard feeding levels used in Chapter 4 where shrimp nutrient requirements were met by the formulated pelleted feed. The results show that in biofloc systems, beside using a suitable C:N ratio, other management techniques (e.g. feeding level, water exchange rate) also play important roles.

Numerous types of carbohydrate (including but not limited to corn flour, glycerol, glucose, molasses, starch, and sugar cane) were tested as a carbon source for biofloc

technology (Crab et al., 2010; Guo et al., 2006; Nikodinovic-Runic et al., 2013). All these carbon sources act on the same principle of manipulating C:N ratio in the system, yet their effects on biofloc growth, microbial community, water quality, and performance of culture animals differ significantly (Gutiérrez et al., 2016; Serra et al., 2015). This phenomenon was demonstrated in Chapter 3. Molasses and corn starch were applied to the system at the same C:N ratio, yet their effects on shrimp growth differed significantly: shrimp growth when applying corn starch as CHO source was almost double that when applying molasses. The mechanism of this involves many factors, of which the CHO biodegradability is one of the important factors (Khanjani et al., 2016; Wei et al., 2016). To reach a similar C:N ratio, the amount of molasses given was double to the amount of corn starch. However, the 5-day biological oxygen demand (BOD_5) analysis showed that 1 mg of molasses consumed 0.1 ± 0.02 mg oxygen, while 1 mg of corn starch consumed 0.2 ± 0.4 mg oxygen. In addition, the two CHO sources possessed different mineral profiles in which molasses contained much higher calcium, magnesium, and potassium contents (Table 3.9). The aforementioned minerals play important roles in the culture of aquatic animals, especially in shrimp species due to their high requirements for basal metabolism and growth/molting (Roy et al., 2007). However, how mineral contents in CHO sources affect biofloc functioning remains unclear. Nevertheless, we suggested that beside the CHO chemical structure and degradability, the concentrations of macro and micronutrients in CHO also have important effects on animal performance and biofloc production.

By using cornstarch as a pure CHO source and mixing it in the feed to produce one pellet, we hoped to simplify for the farmer the operation of a biofloc system, making the technology more attractive (Chapter 5). This CHO-included pellet resulted in better water quality than the treatment without extra CHO, and similar water quality to the treatment with separate CHO addition, but also reduced shrimp production. The high inclusion of CHO in the feed may have interfered with the nutrient absorption by the shrimp, resulting in poorer growth. Meanwhile, the nutrients (carbon and nitrogen) released into the culture water still ensured normal microbial functioning, resulting in similar water quality between the two CHO administration methods. Overall, our experiments showed that different diets, CHO sources, feeding levels, and stocking densities all influence biofloc technology performance, and that our present insights in potential interaction among these factors are insufficient to predict farming outcomes.

6.2 Optimal C:N ratio

In many aquaculture systems, culture animals are fed several times daily. However, somewhere in the range of 50% - 75% of fed nutrients are not assimilated (Alarcón-Silvas et al., 2021; Hardy and Kaushik, 2021; NRC, 2011; Pérez-Fuentes et al., 2016; Thakur and Lin, 2003). Uneaten feed, feces and metabolic wastes continuously accumulate, especially in systems with limited or no water exchange. The nitrogen in the waste is consumed by algae, and autotrophic and heterotrophic bacteria (Ebeling et al., 2006). Heterotrophic bacteria have a higher growth rate (5 day^{-1}) than autotrophic bacteria (1 day^{-1}) and dominate over the latter in presence of high loads of organic matter. In general, the metabolic waste accumulating in fed aquaculture systems is rich in nitrogen but low in energy (e.g. carbon). The primary aim of adding organic C to aquaculture systems is therefore to provide heterotrophic bacteria with sufficient energy to immobilize nitrogen in bacterial biomass, and improve water quality (Avnimelech, 1999; Ebeling et al., 2006). The optimal carbon addition for tilapia concurs with a C:N ratio of 10 (Pérez-Fuentes et al., 2016), and for African catfish a C:N ratio of 15 (Abu Bakar et al., 2015). While for shrimp, higher C:N ratios (C:N 12-18) are often recommended (Emerenciano et al., 2017; Hostins et al., 2019). However, other research showed that CHO addition does not always increase microbial production and can even be detrimental (Xu et al., 2016).

Nearly all experiments exploring the effect of CHO addition in biofloc systems were executed with fixed C:N ratios for the full duration of the experiment. This was also the case of the four experiments in this thesis. However, some researchers also suggest to adjust the C:N ratio during culture, recommending a high C:N ratio at the start and to gradually reduce the C:N ratios when the biofloc concentration increases (Emerenciano et al., 2017). To investigate potential trends, data from all four experiments in this thesis were combined. These experiments differed primarily in shrimp stocking density, and biofloc levels at the start of the experiments. Of the 4 experiments in this thesis, two were started with filtered water (TSS levels < 1 mg/L), and two were started with pre-inoculated biofloc water (TSS levels > 80 mg/L). The data from all 4 experiments were categorized based on stocking density (low = 23 ind/m^3, medium = 80 – 120 ind/m^3, and high = 250 – 300 ind/m^3), biofloc level at the start of the experiment (new: TSS < 1 mg/L and old: TSS > 80 mg/L), and the fixed C:N ratio during the experiment (CN<10 = no CHO addition, CN12 and CN≥15). Feed conversion ratio (FCR) and protein efficiency ratio (PER), two important indicators for resource use efficiency (Boyd et al., 2007) were analyzed by 3-

way ANOVA. Because the 3-way interaction was significant, treatment means were compared based on a one-way ANOVA, followed by a Duncan's multiple range test.

Box plots of FCR values of these groups show interesting trends (Figure 6.1). The higher the C:N ratio, the lower the FCR. This improvement in FCR is more pronounced in systems starting with a new biofloc. No differences are observed between systems starting with old biofloc. When looking at PER values, we see similar results. In new systems, the higher the C:N ratio, the higher the protein efficiency ratio, while this is not observed in old systems (Figure 6.2). This suggests that the C:N ratios in biofloc systems should not be fixed for the entire duration of the production cycle, but rather be adjusted according to the prevailing C and N demand during the culture period. Emerenciano et al. (2017) suggested that a C:N ratio of 12 to 20 should be applied when starting the biofloc culture system with a TSS concentration below 1 mg/L. As biofloc matures within 30 to 50 days, reaching a biofloc volume above 5 mL per liter (measured in Imhoff cones), a C:N ratio of 6 is recommended. This is sufficient to maintain the biofloc biomass and system functioning. During this biofloc-maintenance phase, the TAN concentration should be monitored daily to be able to react timely when TAN increases towards toxicity (Emerenciano et al., 2017). This may explain our observation in experiments that started with old biofloc, adding CHO resulted in poorer FCR and PER values (Figure 6.1 and 6.2). However, the old biofloc was not tested for low stocking density. In addition, few treatments started with old biofloc. More research is needed to look into the effect of aging biofloc on water quality and nutrient re-use by fish or shrimp in biofloc systems. This research will also improve insights which biofloc density to maintain, at which stage of the shrimp production cycle and how.

For a fixed C:N ratio, optimal ratios were recommended for specific species (Abu Bakar et al., 2015; Hostins et al., 2019; Pérez-Fuentes et al., 2016). Meanwhile, although adaptive CHO input-management provides more options to maintain water quality, it requires a high knowledge level from the farmer, the latter depending on training opportunities and technical support from extension services or companies. Farmers need to learn to make quick and reliable calculations of CHO requirements in biofloc systems. Conventionally, Imhoff cones are used to measure the suspended solids volume settling within 15 to 20 minutes from one liter of culture water to estimate biofloc volume (Avnimelech, 2009). However, accuracy of this method depends on how well the culture water is mixed. Besides, it provides a snapshot of one moment in time, as the particle size distribution

and biofloc size and settling properties change over time. In nearly all treatments investigated during this thesis, the algal content decreased, and the concentration of volatile suspended solids increased. While taking care of our experiments, we got the impression that when the water is green and the chlorophyll concentration high, the biofloc settles faster, and have a looser structure, concurring with a larger volume collected in Imhoff cones. The settling velocity of biofloc is affected by the types of carbohydrate used (Mendez et al., 2021). More research is needed to verify this impression.

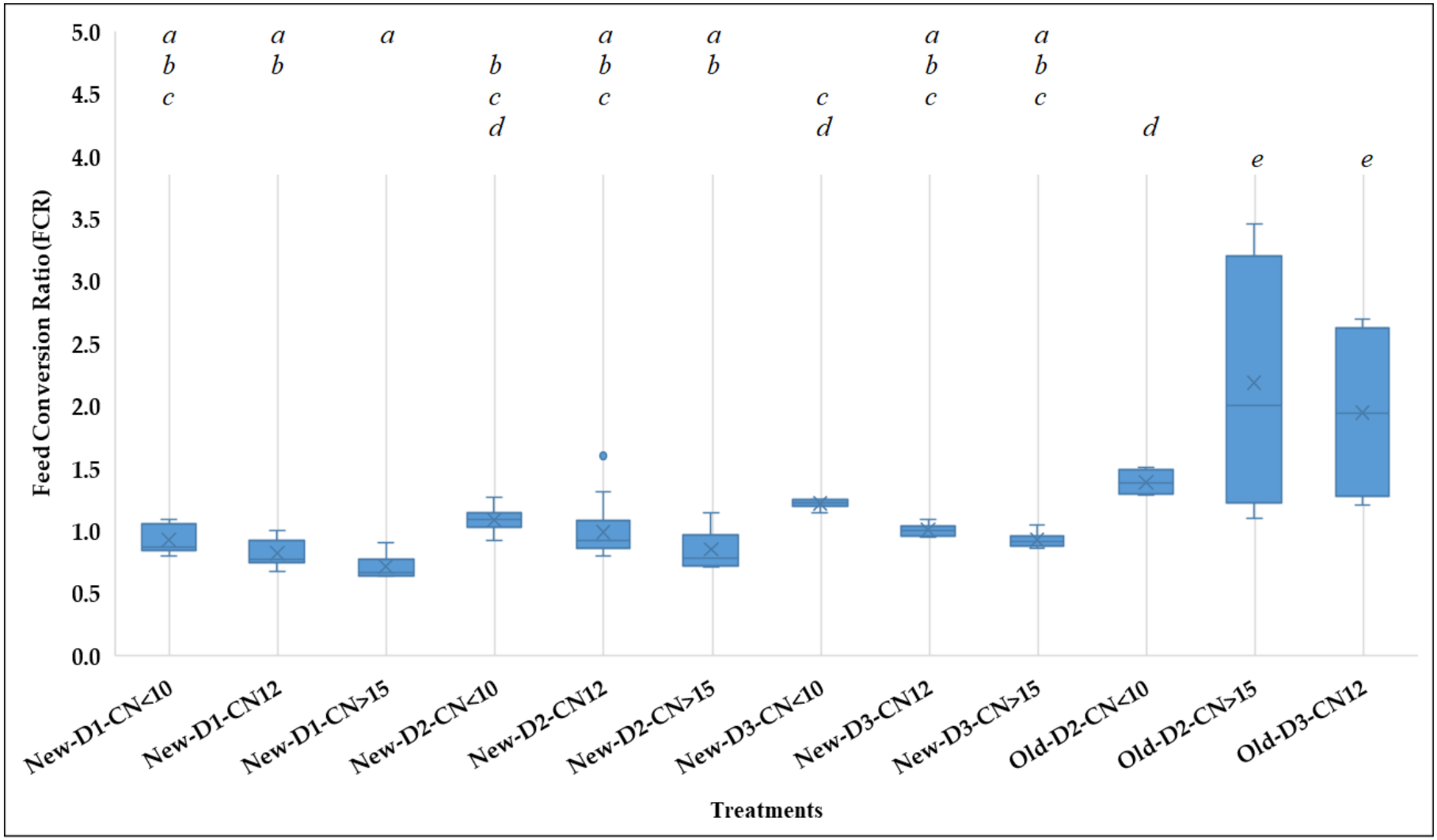

Figure 6.1 Feed conversion ratios among different treatment groups: System starting conditions (New, Old); Stocking density (D1: low; D2: medium; D3: high); and C:N ratios (CN<10; CN12, CN>15). The desired C:N ratios were reached by supplying additional carbohydrate besides the feed. Boxes on the top with no (*italic*) letter in common are statistically different (one-way ANOVA, 3 replicates per treatment, $P < 0.05$).

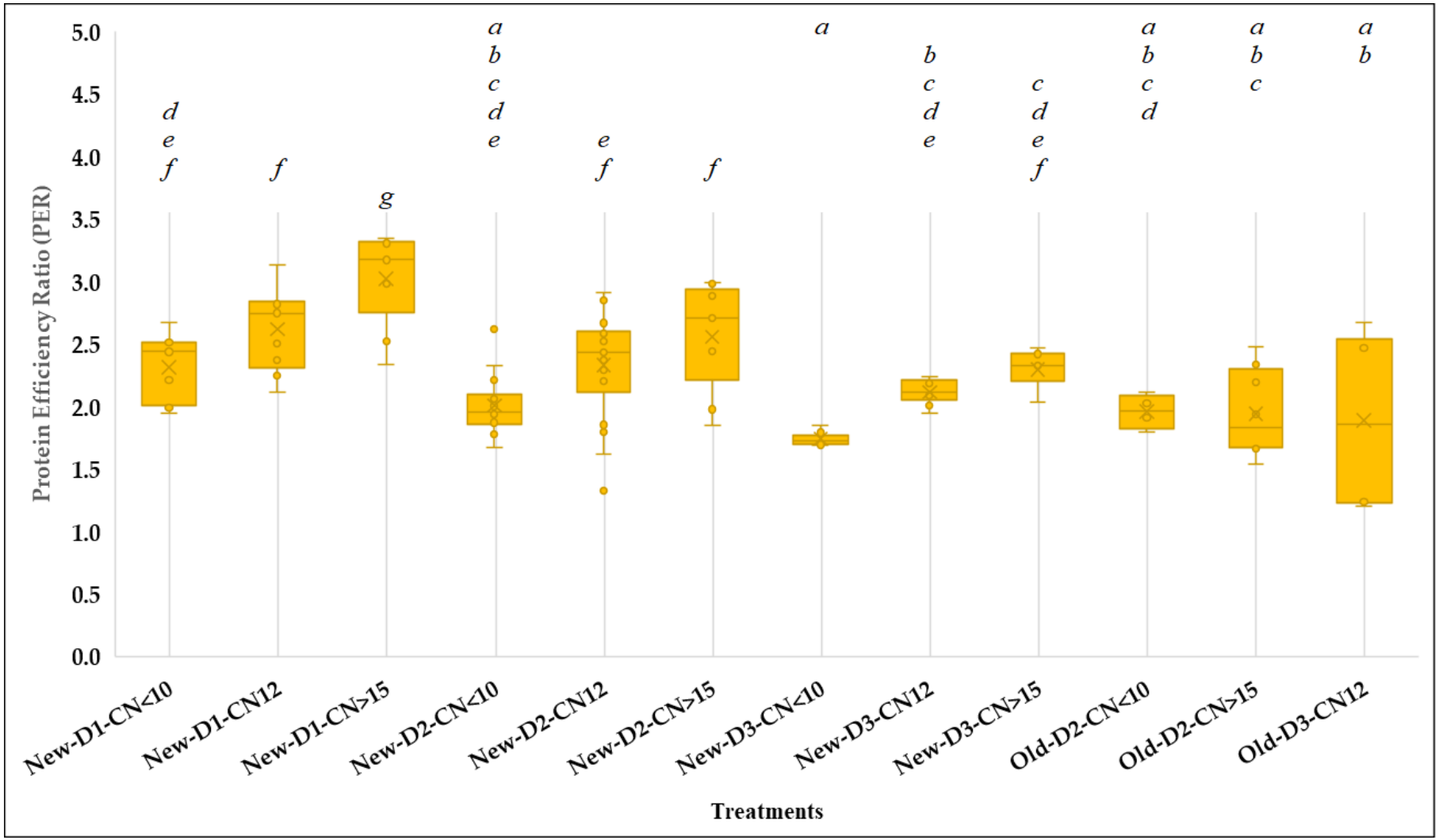

Figure 6.2 Protein efficiency ratios among different treatment groups: System starting conditions (New; Old); Stocking density (D1: low; D2: medium; D3: high); and C:N ratios (CN<10; CN12, CN>15). The desired C:N ratios were reached by supplying additional carbohydrate besides the feed. Boxes on the top with no (*italic*) letter in common are statistically different (one-way ANOVA, 3 replicates per treatment, $P < 0.05$).

6.3 Biofloc developments and contributions to shrimp production

In practice, intensive shrimp culture systems are started up with clean, often disinfected and filtered water, containing a low or even negligible TSS load. As feed and CHO are daily applied during the culture period, biofloc keeps developing, increasing the TSS concentration, especially in closed or limited water exchange systems. When biofloc reaches maturity is not well defined; the 5 mL/L reached after 30-50 days of culture (Emerenciano et al., 2017) mentioned in section 6.2 is normally referred to as a mature biofloc concentration. Although measuring biofloc volume in Imhoff cones is quick and easy, the measurement is highly variable because the 3-D structure of biofloc changes over time and sampling location, and not always correlates well with TSS measurements. Other system maturity indicators often used by farmers include the water color as an indicator of algal presence and turbidity as an indicator of the TSS concentration (personal communication with farmers). TSS should be maintained below 200 mg/L for shrimp and 400 mg/L in fish culture systems (Avnimelech, 2009). Other research recommended 400 – 600 mg/L TSS levels for shrimp, as TSS levels below 200 mg/L are often linked to unstable total ammonia and nitrite concentrations, while TSS levels above 800 – 1000 mg/L may result in shrimp gill clogging (Schveitzer et al., 2013b). High biofloc levels also have greater biological oxygen demands, and therefore impose risks of oxygen depletion (Rajkumar et al., 2016).

The experiment described in Chapter 2 started with clean water with nearly no suspended solids. Because no water was exchanged during culture, the TSS concentration gradually increased. In the treatment with the highest nutrient input (i.e. highest stocking density, feeding and carbohydrate addition rate), the maximum TSS concentration reached was 298 mg/L, still below the recommended safe level of 400 – 600 mg/L (Schveitzer et al., 2013b). In this experiment, the lowest feeding rate applied aimed to stimulate shrimp to graze on biofloc to complement the feed input. In doing so, the importance of carbohydrate addition to stimulate biofloc development to maintain water quality and provide microbial additional food to the shrimp was clearly demonstrated. When applying a higher feeding rate, close to the recommended feeding level by feed companies (Chapter 4), CHO addition still improved water quality (Table 4.3) but demonstrated less pronounced effects on shrimp production (Table 4.2).

The contributions of biofloc to shrimp production were not measured in this thesis, as these were already estimated using stable isotope analysis. Burford et al. (2004) reported

that less than 3% of biofloc were consumed by Pacific white shrimp and that natural foods, including biofloc, plankton and detritus, contributed 18-29% of the nitrogen retained in shrimp biomass gain. Ray et al. (2017) reported that biofloc contributed 18-60% of carbon and 1-18% of nitrogen deposited in Pacific white shrimp growth. Blue shrimp (*Litopenaeus stylirostris*), grown in a biofloc system, obtained 40% and 37% of respectively fed C and N deposited in shrimp growth by grazing on natural food present in the culture system (Cardona et al., 2015). Differences in the reported contribution percentages of fed nutrients to shrimp growth are influenced by multiple factors, including the nutritional quality and quantity of natural foods, husbandry practices (i.e. stocking density, feeding level, biofloc concentrations, and water exchange), and the accessibility of these natural foods to the culture animals.

6.4 Carbohydrate addition and global warming

Greenhouse gases (GHG) (e.g. CO_2, CH_4, and N_2O) resulting from human activity cause global warming. Energy use (electricity, heat, and transport), industry (cement and chemicals), and waste (landfills and waste water) contributed 82% to GHG emissions in 2016 (Ritchie and Roser, 2020). In the same year, agriculture, forestry, and land use accounted for 18% of GHG emissions. The contribution of aquaculture activities to GHG emissions was neither included nor separately mentioned in the report of Ritchie and Roser (2020).

In 2017, global aquaculture contributed an estimated 0.49% to anthropogenic GHG emissions, which was lower than those from livestock (MacLeod et al., 2020). Intensive aquaculture systems rely on pelleted feed, which accounts for more than 50% to the production cost (Cruz-Suárez et al., 1994) and has a large impact on the carbon emissions from aquaculture. In general, across the aquaculture sector, per kg seafood the CO_2 is 2 to 7 kg (Boyd, 2013). Measured carbon emission on shrimp farms are in the same range from 2.5 to 7 kg CO_2/kg shrimp produced. In farming systems relying on heavy aeration, non-renewable electricity use contributes close to 30% of CO_2 emissions, followed by 24% due to feeding. In addition, additive use (22%), waste and pollution control (15%) and processing, storage and transport, (8%), are the major contributors to GHG emissions (Chang et al., 2017).

The continuous growth of aquaculture requires methods to mitigate its contributions to global warming. One way is to use culture systems with a high resource (e.g. land, water, and feed) use efficiency. Through addition of external CHO sources to aquaculture

systems, nitrogen species, which are often wasted, can be assimilated into microbial biomass. This practice improves water quality and increases natural food production and availability, reducing the need for water exchange, and improving feed use efficiency (Burford et al., 2004; Emerenciano et al., 2012; Gao et al., 2012; Hu et al., 2014). Hu et al. (2014) demonstrated that CHO addition reduced N_2O emission by 83%, while the CO_2 emission from the culture system increased 91%. This is in agreement with our observation in Chapter 3, where only 15-17% of total added carbon stayed in the system. Meanwhile in Chapter 5, carbon losses increased from 39% in conventional systems (without additional CHO) to 58% in CHO-supplemented systems, regardless of CHO addition methods. In these systems, aeration is continuously provided to keep bioflocs in suspension. The dependence on continuous aeration minimizes the development of anaerobic conditions and reduces methane emission (Yuan et al., 2019). With regard to GHG emission from biofloc systems, reducing the release of methane and nitrous oxides, has a higher effect on global warming than feed digestion and associated release of CO_2. The organic carbon fed, is already part of the global carbon cycle, and therefore not considered to contribute to global warming by Ritchie and Roser (2020).

As for biofloc technology, choosing the right CHO source is important, as each type of carbohydrate demonstrates different effects on biofloc (Chapter 4). For example, molasses, a by-product of sugar processing not consumable to human, is suggested as an effective CHO for biofloc systems (de Souza et al., 2014; Khanjani and Sharifinia, 2021; Samocha et al., 2007). The use of by-products for farm animal helps increase the circularity of farming systems (Van Zanten et al., 2019). Results in Chapter 3, however, showed that molasses was less effective compared to corn starch in promoting shrimp performance. The research shows, that the type and dose of the CHO source used should be effective (i.e. improving water quality, natural food availability and shrimp performance) and should be tested before wide scale application.

6.5 Technical constraints in implementing biofloc technology

Biofloc technology has been considered a promising technology for more than 30 years (Avnimelech et al., 1986; Schroeder and Serfling, 1989). Its re-emergence in recent years offers solutions to reduce water use through in-situ water purification. It recycles a fraction of the metabolic wastes resulting from feeding into natural food. In addition, biofloc acts as a probiotic (Ahmad et al., 2017; Ferreira et al., 2015). It can be applied in any production facility, ranging from extensive to intensive. It allows to use cheaper

feeds, containing crop residues, otherwise wasted (De Schryver et al., 2008). Some of these beneficial effects of biofloc were also demonstrated in Chapters 2, 4, and 5 of this thesis.

Until today, little attention has been given to the effect of the type of carbohydrate used. There are many different types, each type demonstrating different effects and making it difficult to standardize the technique. Cheap, but efficient carbohydrate sources must be year-round locally available. Nevertheless, large amounts of carbon are needed to provide energy, which leads to the release of additional CO_2 from biofloc systems, but also re-uses waste streams and reduces N and P losses. In Chapter 4, we suggested that better ways to increase the carbon use efficiency in biofloc systems should be explored. Direct application of carbohydrates is laborious while simply incorporating the carbohydrate in pelleted feed proved to be less effective. A major disadvantage to biofloc technology is the energy required to keep biofloc in suspension and the system oxygenated, which in many locations is costly, and if non-sustainable energy sources are used, is the major on-farm contributor to global warming (Chang et al., 2017; Ngoc et al., 2021).

6.6 Recommendations

To make biofloc technology a predictable and reliable sustainable farming system and improve circularity, more research-based insight is needed into how the type of carbohydrate administrated in biofloc systems influences biofloc development, considering:

- Composition (e.g. proximate composition, microbiota species richness and functionality),
- Amount (e.g. total and volatile suspended solids),
- Water purification (e.g. water quality, waste reduction), and
- Fish or shrimp nutrition (e.g. nutrient use efficiency).

SUMMARY

Aquaculture production is increasing, mainly through intensification, to meet human food demand. Intensification imposes the risks of environmental pollution and deterioration, both of the rearing water and the surrounding areas. By applying carbohydrate to aquaculture systems, the culturing water quality is improved through microbial processes, and production is improved through reutilization of excreted nutrients assimilated in bioflocs. The current thesis investigated some practical aspects of carbohydrate supplementation, in which **Chapter 1** provides a short introduction to the importance of aquaculture and the role and scope of biofloc technology.

Chapter 2 investigated the combined effects of stocking density, feeding level, and carbohydrate application level to shrimp culture tanks. A 3 by 3 factorial design was applied. Shrimp growth correlated positively with feeding levels and carbohydrate application rate, but negatively with stocking density, while shrimp production positively correlated with all 3 factors. Similarly, microbial community positively correlated with feeding levels and carbohydrate application rate. Water quality and biofloc biomass were affected by two- and three-way interactions between stocking density, feeding level, and carbohydrate application level. Biofloc proximate composition was mainly affected by stocking density, and to a lesser extent by C:N ratio.

Chapter 3 compared the effects of two carbohydrate sources, cornstarch versus molasses, representing pure sources and waste product, respectively. Both carbohydrate types were applied at the same C:N ratio of 12. Cornstarch, however, yielded higher shrimp production, growth rate, and better FCR compared to molasses. Biofloc and periphyton qualities in terms of protein content were higher in molasses treatment. Both treatments demonstrated similar effects on water quality, and biofloc quantity.

Chapter 4 examined splitting the daily dosage of carbohydrate into 1, 3, 6 applications per day compared to zero application. Carbohydrate-added systems showed better shrimp performance, but poorer carbon retention efficiency. Biofloc and water quality were not affected by different application frequencies.

Chapter 5 investigated the potential of mixing carbohydrate directly in pelleted feed to simplify the application practice. The treatments included no addition of carbohydrate, administration of carbohydrate besides the feed, and mixing carbohydrate directly in

pelleted feed. Shrimp grew best when carbohydrate was added besides the feed. The additional carbohydrate reduced the inorganic nitrogen and orthophosphate concentrations. Carbohydrate addition also lowered the dietary energy and carbon utilization and increased the microbial diversity in biofloc. Biofloc and periphyton growth and proximate composition were not different among treatments.

Chapter 6 discusses our findings on biofloc and the effect of C:N ratio, the contribution of biofloc to shrimp growth and the effect of biofloc systems on global warming. Some technical constraints to biofloc implementation, and recommendations for further research were also provided.

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ABOUT THE AUTHOR

Tran Huu Tinh was born in 1990 in Can Tho, a province in the Mekong Delta of Vietnam. The area is abundant with rivers and coastal lines, which stimulated him to pursue Bachelor Degree in Aquaculture Engineering (English-taught, commonly referred to as Advanced Program) at Can Tho University. He completed his Bachelor in 2013, working on isolation and characterization of bacteria from seabass, and conducting antimicrobial tests. This project got him a scholarship to pursue Master Degree in Veterinary Microbiology, at Chulalongkorn University in Thailand, where he continued with isolation, characterization of and performing antimicrobial tests with bacteria from marine shrimp, and well as testing plant-based extracts as potential alternatives for antibiotics. “Prevention is better than cure”. After obtaining his Master Degree in Microbiology in 2015, Tinh participated in “Nutritious Pond” project as a PhD candidate at Wageningen University in The Netherlands. In this project, he explored different aspects of Biofloc technology, trying to steer the microbial community in shrimp tanks to create good water quality and increase nutrient use efficiency.

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1. Tinh, T.H., Hai, T.N., Verreth, J.A.J., Verdegem, M.C.J., 2021a. Effects of carbohydrate addition frequencies on biofloc culture of Pacific white shrimp (Litopenaeus vannamei). Aquaculture. https://doi.org/10.1016/j.aquaculture.2020.736271
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Colophon

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