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FULL PAPER

Microbial Community Response to Carbon-Nitrogen Ratio Manipulation in Biofloc Culture

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ABSTRACT

Biofloc technology (BFT) is an alternative aquaculture practice that involves the manipulation of carbon-nitrogen ratio (C:N) to manage nitrogenous waste through microbial assimilation. This study aimed to determine the composition of the microbial community present in BFT and describe the microbial community's response to C:N manipulation. The experiment had a complete randomized design with two set-ups (i.e., BFT and control) in triplicates. The experimental unit was a 120-L rectangular tank stocked with 15 mixed-sex tilapia fish, *Oreochromis niloticus*, with an average weight of 15±3 g. The C:N was adjusted to 16:1 using molasses. Illumina MiSeq sequencing platform was used, and bioinformatics was conducted in R using dada2 and phyloseq package. Seventeen (17) phyla were identified, but only seven were remarkably abundant, namely: Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Verrucomicrobia, Planctomycetes, and Fusobacteria. Double Principal Coordinates Analysis (DPCoA), Principal Coordinate Analysis (PCoA) of Weighted Unifrac Distance and Canonical Correspondence Analysis (CCoA) revealed an association of Proteobacteria to low ammonia concentration in BFT treatment. On the other hand, phylum Bacteroidetes was clustered towards the control, characterized by high ammonia. Overall, BFT has increased Shannon and Simpson diversity indices compared to the control. Regime change in the microbial community was not easily caused by organic C supplementation because of community robustness to withstand biotic and abiotic disturbances. Controlled laboratory experiment showed that some bacteria species proliferate as a response to C:N manipulation, but established species remained dominant.

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1. INTRODUCTION

Biofloc technology (BFT) is one of the recent alternative culture technologies in aquaculture that rely on heterotrophic bacteria (Avnimelech 2012). The BFT is based on the manipulation of a microbial community (Asaduzzaman et al. 2008) and encourages the growth of heterotrophic bacteria by increasing the carbon to nitrogen ratio (C:N) (Crab et al. 2012). The technology application offers an opportunity to improve aquaculture production towards sustainable development (Nugroho et al. 2019). The BFT features the reuse of wastewater (Gallardo-Collí et al. 2019) and the proliferation of heterotrophic bacteria that utilize organic carbon and assimilates inorganic waste nitrogen from the water. The benefits of using the BFT in aquaculture are the economy of water use due to rapid microbial removal of ammonia nitrogen from the culture environment (Ebeling et al. 2006) and available natural food of microbial origin (Avnimelech 2007; Crab et al. 2007).

The organic carbon supplement for the BFT can be obtained from downstream by-products of nearby local industry. Still, a preliminary study is

required to assess the effectiveness of the external carbon source (Crab et al. 2012). Bacteria easily assimilate simple sugars compared to carbon sources such as starch; nevertheless, any carbon source can promote microbial metabolism of inorganic waste nitrogen (Hamlin et al. 2008; De Schryver and Verstraete 2009). The capacity of the BFT to control water quality and the nutritional value of flocculation is due to the carbon source profile (Crab et al. 2012), but the underlying factor is the distribution and interaction of microbial community (Souza et al. 2019) that thrive during the carbon-nitrogen manipulation.

At present, the BFT remains novel in the Philippines, and the identification of responsible microbial communities can be facilitated by Next Generation Sequencing (NGS). Preliminary studies reported Proteobacteria and Bacteroidetes were the dominant phyla in *Litopenaeus stylirostris* (Cardona et al. 2016) and *Litopenaeus vannamei* biofloc system (Manan et al. 2016; Pilotto et al. 2018). Cienfuegos et al. (2018) identified a total of 20 phyla from *Oreochromis niloticus* cultured in BFT, of which the most abundant phyla were Proteobacteria, Bacteroidetes, Actinobacteria, Planctomycetes, Verrucomicrobia, and Fusobacteria.

The study's objectives are:
1. to assess microbial community composition and abundance between C:N manipulated (i.e. biofloc) and non-manipulated environment using NGS;
2. to determine microbial community species richness and diversity response to C:N manipulation and temporal change; and
3. to relate microbial community response to water quality.

2. METHOD

2.1 Experimental Design

Two treatments, biofloc (BFT) and non-biofloc/control (CTL), were tested in triplicates. The BFT treatments had molasses supplementation, while CTL had no molasses. The experiment was implemented in a complete randomized design (CRD).

The study was performed in a 120-L rectangular plastic container placed in a climate-controlled room with an evaporative cooling unit to manage the ambient temperature. The water temperature was maintained at 27.5±5°C. Collection of water temperature (°C), total dissolved solids (mg/L), dissolved oxygen (mg/L), pH, and ammonia (mg/L) was performed using a calibrated YSI Professional Plus multi-parameter probe (Xylem-YSI, USA).

Each experimental unit is installed with a submersible pump rated at 800 L/h aside from aeration to ensure uniform mixing of suspended organic particles in the water column. No water change was conducted during the 14-day experiment. Water loss from the culture tank due to evaporation was refilled to maintain the volume of 90 L.

Each tank was stocked with 15 mixed-sex tilapias, *O. niloticus*, obtained from BFAR- Regional Office No. 3 Hanga Technology Outreach Station, Hagonoy, Bulacan. The average body weight (ABW) of fish during stocking was 15±3 grams. Feeds (B-meg, Philippines) with 28.6 percent crude protein content were used, and the feeding rate was set at 5 % of ABW. Feeding was done at 12:00 noon every day.

After one month of acclimation to the experimental condition, molasses enrichment was started. The C:N was raised to 16:1 by adding 5 g of molasses one hour after feeding. The recommended C:N range to promote biofloc formation is from 11 to 20:1 (Ebeling et al. 2006; Avnimelech 2012; Pérez-Fuentes et al. 2016). Some studies used 15:1 C:N ratio (Kasan et al. 2018; Cang et al. 2019) while others adapted the maximum recommended ratio of 20:1 (de Souza et al. 2019; Reinoso et al. 2019). In practice, up to 20:1 is the recommended C:N ratio on the first three months of culture followed by a reduction to around 15:1 at the latter part of the culture to manage the decline of pH and production of settled biomass (Pérez-Fuentes et al. 2016). The first and second metagenomic sampling from each tank were conducted 1 and 2 weeks after C:N adjustment. The sampling period coincides with the findings that heterotrophic bacteria become stable 14 days after organic carbon supplementation (Avnimelech 2012).

2.2 Microbial Sampling and Sequencing

Water sampling was conducted along with the collection of water parameters. Suspended particulate matters in the culture media were collected using the installed submersible pump with a nylon bag installed at the discharge end on the pipe. Sample collection was conducted for 1 hour and 15 minutes for a total sampling volume of 1000 L.

Collected suspended particles were placed on sterilized plastic 500 µl microcentrifuge tubes then stored at −80°C. Frozen samples were sent

to Macrogen, Korea, in dry ice (−78ºC), for DNA extraction, amplicon library preparation, and sequencing. The extraction of metagenomic DNA was conducted using a Macrogen extraction protocol. The metagenomic DNA quality control (QC) test used the gel electrophoresis method and the picogreen (Victor 3 fluorometry) method for DNA quantification. The sequencing library targeting the ribosomal DNA 16S at 3 and 4 variable regions was prepared using 16S Metagenomic Sequencing Library Preparation Part #15044223 Rev. B (Macrogen, Korea). Herculase II Fusion DNA Polymerase (Agilent, USA) and Nextera XT Index Kit V2 (Illumina, USA) were used to amplify the region and tagged the resulting amplicons. Sequencing was done using paired-end Illumina MiSeq (Illumina, USA) at 301 read length.

2.3 Bioinformatics Analysis

Raw sequences were trimmed out of primer sequences and indexes using the java-based application Trimmomatic-0.38 (Bolger et al. 2014). Amplicon Sequence Variants (ASVs), which has a 100 percent radius of similarity in contrast to 97 percent of Operational Taxonomic Units (Callahan et al. 2016b), sample inference was conducted in R (R Core Team 2018) using dada2 package (Callahan et al. 2016a). The bioinformatics workflow followed a modified procedure described by Callahan et al. (2016a), which used the phyloseq (McMurdie and Holmes 2013) R package. The change in the procedure was performed on merging similar branches in the phylogenetic tree using the *tax_glom(…)* function. Instead of agglomerating at the genus level, this study combined branches at the species level and disabled the removal of unidentified ASVs.

After using Trimmomatic-0.38, the sequences in fastq format were imported to dada2 for further processing. The forward and reverse sequence reads' quality profile was plotted and inspected to determine the truncation sites. In dada2, *filterAndTrim(…)* function was used to truncate forward and reverse sequences at 290 and 210 base/cycle, respectively. The first 18 reads for both sequences were also trimmed.

Dada2 critical processing steps are to train the algorithm with a parametric model of the errors introduced during PCR amplification and sequencing (Callahan et al. 2017). After modeling the error for forward and reverse sequence with *learnErrors(…)* function, de-replication of sequences was performed using *derepFastq(…)* function. ASVs inference of dereplicated sequences followed using the *dada(…)* function. Forward and reverse ASVs were merged using *mergePairs(…)* function, then chimera removal was conducted with *removeBimeraDenovo(…)* function. Taxonomic assignment of Chimera-free sequences was completed using *assignTaxonomy(…)* and *addSpecies(…)* function with Silva nr 132 fasta file as training sets.

Taxonomically annotated chimera-free ASVs were imported to phyloseq for downstream analysis. Phyloseq pipeline requires ASVs table, taxonomy assignment, phylogenetic tree, and environmental information. Dada2 supplied the ASVs table and taxonomy assignment. De novo construction of a phylogenetic tree was conducted, and a table of environmental data collected during the sampling was also prepared.

Environmental data added to the pipeline included treatment, tank number, date of sampling, and water parameters such as temperature (ºC), pH, ammonia, dissolved oxygen (DO), and total dissolved solids (TDS). Generating the phylogenetic tree was done using DECIPHER (Wright 2016) and phangorn (2.4.0) (Schliep 2011).

2.4 Alpha Diversity

Indices for species richness and diversity were computed and plotted. The species richness (i.e., Chao1 and ACE) and diversity indices (i.e., Shannon and inverse Simpson) were computed using the *estimate_richness(…)* function of phyloseq (McMurdie and Holmes 2013). The above is based on two functions from the community ecology package vegan (Oksanen et al. 2018). To determine changes in Chao1 and Shannon Index from the first week (Week 1) and second week (Week 2) of sampling, a relative change in Chao1 and Shannon Index was computed by dividing Week 2 indices with corresponding Week 1 indices. ANOVA on the relative change in Chao1 and Shannon index was conducted.

2.5 Beta Diversity

Prior to multi-variable projection, the abundance data were log-transformed to approximate variance stabilizing transformation and normalize it (McMurdie and Holmes 2013). Double principal coordinates analysis (DPCoA) (Pavoine et al. 2004; Purdom 2011; Fukuyama et al. 2012) were used to investigate if NH_4^+, DO, TDS, temperature, and pH can be attributed to the observed species abundance and difference between communities. Since DPCoA is not

as robust in dealing with outliers as unweighted and weighted Unifrac (Fukuyama et al. 2012), exploratory ordination and subsequent removal of outlier sample data were conducted. Principal coordinate analysis (PCoA) or classical multidimensional scaling (MDS) of weighted UniFrac distance (Lozupone et al. 2011) was also conducted after DPCoA. Another ordination analysis performed was Canonical Correspondence Analysis (CCpnA) (Braak 1985) using ammonia (NH_4^+), dissolved oxygen (DO), and weeks of sampling as constraints.

2.6 Linear Modeling

Microbial community diversity, Shannon index, relationship with the environment was modeled. In the analysis, the effect on the Shannon index of the environmental condition such as C:N manipulation through molasses addition was modeled. The computed Shannon index (H') per tank from the previous section was assigned as the model's response variable. Linear mixed effect (lme) model to study the relationship between microbial community diversity, age of community (i.e., weeks of sampling), and treatment was performed using the package nlme (Pinheiro et al. 2018). The following relationship was used:

$H' = Week \times Treatment + (1|Tank)$

where Week is the week of sampling used as a proxy for the age of the microbial community, Treatment is the use of molasses to manipulate the C:N. The term (1|Tank) instructs the model to assume a different intercept for each tank. After the model was tested using ANOVA, prediction intervals were prepared from the model fitting function *predict(…)* in R.

3. RESULTS

The water parameters during the time of metagenomic sampling were presented in Table 1. During the first week of the experiment, the dissolved oxygen levels range from 6.5 to 7.2 mg/L. After a week, the dissolved oxygen range was from 4.04 to 4.62 mg/L. The temperature increased by 1°C in the second week of the experiment. An increase in the total dissolved solids was also observed in the second week. On the other hand, pH has decreased during the second week of the experiment.

Table 1. Water parameters collected during metagenomic sampling. Week 1 samples were collected May 12, 2018 while Week 2 samples were collected May 19, 2018.

Sample ID	Diss. Oxygen (mg/L)	Temperature (°C)	Total Diss. Solids (mg/L)	pH	NH_4 (mg/L)
Week 1					
BFT1W1	7.20	27.0	245.0	6.62	5.63
BFT2W1	6.52	27.0	199.5	6.95	1.62
BFT3W1	5.51	27.1	191.0	6.74	1.45
CTL1W1	6.60	27.2	267.1	6.29	6.52
CTL2W1	6.50	27.3	197.0	6.83	4.97
CTL3W1	6.51	27.4	192.0	6.5	4.04
Week 2					
BFT1W2	4.31	28.2	300.6	6.28	6.86
BFT2W2	4.39	28.3	239.5	6.51	1.45
BFT3W2	3.74	28.6	267.5	6.17	4.48
CTL1W2	4.24	28.2	312.1	6.04	8.14
CTL2W2	4.04	28.5	252.2	6.11	7.44
CTL3W2	4.62	28.5	248.8	6.11	6.87

The top 12 most abundant taxa from the sample set belong to Phylum Bacteroidetes, Deinococcus-Thermus, Proteobacteria, Fusobacteria, and Verrucomicrobia (Figure 1). Phylum Bacteroidetes is represented by Flavobaterium sp., F. nitratireducens, Pelobium manganitolerans, Lacibacter nakdongensis, and an unnamed taxon belonging to the Order of Chitinophagales. Deinococcus-Thermus was only represented by Deinococcus misasensis. Devosia insulae, Pseudomonas sp., and Acidovorax sp. represents the Phylum Proteobacteria. Phylum Fusobacteria was composed of Cetobacterium somerae and Cetobacterium sp. Verrucomicrobia was composed only of Luteolibacter sp., which was present from all tanks during the experiment (Figure 2).

3.1 Alpha Diversity

The result for α diversity computation for each tank per week is presented in Table 2. An observed or actual count of taxa present is also shown. The highest Chao1 index was from BFT tank 3 on week 1 (BFT3W1) at 532.75 ± 2.610. However, this value was reduced to 422.82 ± 2.645 in the second week of the experiment (BFT3W2). The second tank with high species richness was from BFT2W1 at 489.96 ± 2.198, which showed an approximately 50 percent reduction during the second week at 212.08 ± 1.336 (BFT2W2). In general, biofloc treated tanks showed a decrease in species richness during the second week of the experiment while the control increases.

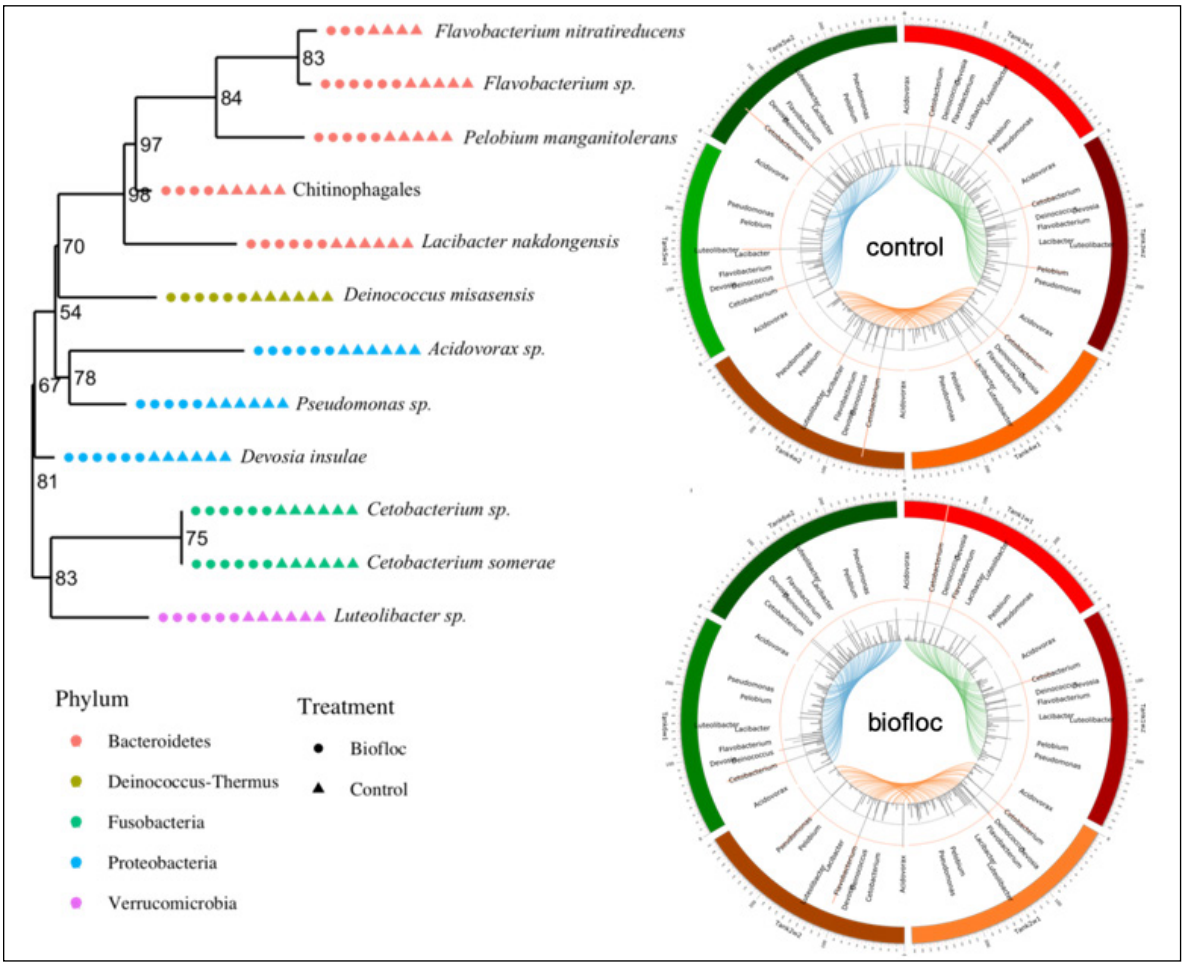

Figure 1. Phylogenetic tree of the top 12 taxa. Treatments used and weeks of sampling were also shown in the tree. The numbers on the nodes are bootstrap values. Circos plot showing the change in abundance and composition of the microbial community is also presented for the control and biofloc treatments. The most abundant genera were annotated. Ribbons connecting week 1 and week 2 marks the genera that are present on both sampling periods. The Gray histogram shows relative abundance below 0.1% and 0.1–1%, while red is 1–5%

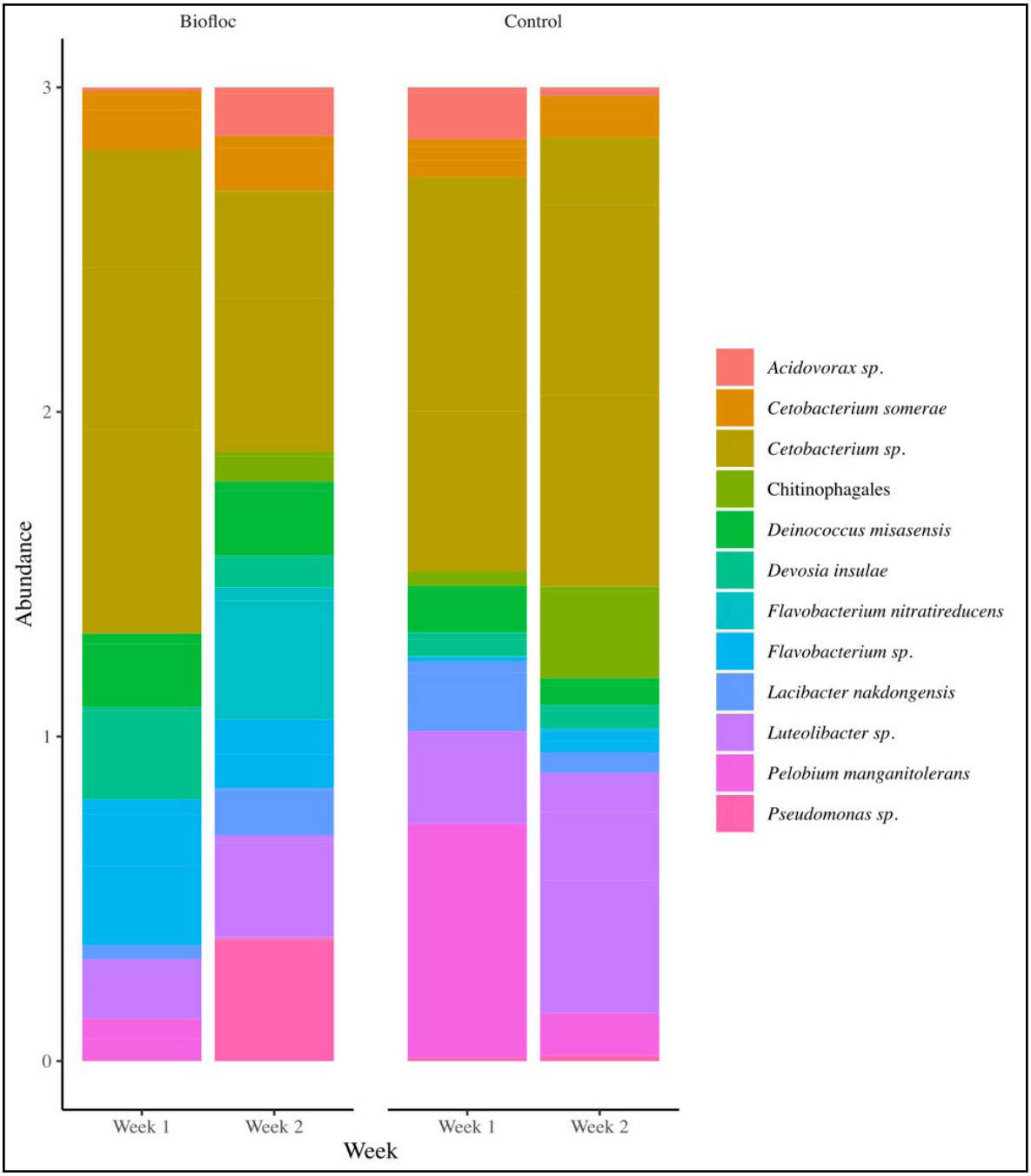

Figure 2. Change in the relative abundance of top 12 taxa from week 1 to week 2 per treatment

Shannon index of the data set ranges from 2.3 to 3.9, indicating high species diversity or evenness. As Shannon index gives more weight to species richness and having obtained an increased number of Observed species, ACE and Chao1, samples appeared to have a small to moderate values of evenness. Inverse Simpson index, on the other hand, showed that diversity values range from 4.254 to 26.566 for BFT1W1 and BFT3W2, respectively. Although BFT3W1 had the highest observed species at 529, the inverse Simpson index or species diversity is only 12.566. During the second week, the observed species of this tank (BFT3W2) was reduced to 419 but had a marked increase in the Shannon index, which could be due to a few individuals' dominance.

Relative change in Chao1 and Shannon diversity index was not significant at $\alpha = 0.05$. Organic carbon supplementation does not affect species richness and diversity.

Table 2. Computed species richness and diversity indices

Sample ID	Observed	Chao1	Inverse Simpson Diversity Index	Shannon Diversity Index
BFT1 week 1	233	233.39±0.735	4.254	2.302
BFT1 week 2	302	312.00±5.877	11.543	3.269
BFT2 week 1	487	489.96±2.198	10.807	3.401
BFT2 week 2	211	212.08±1.336	6.417	2.519
BFT3 week 1	529	532.75±2.610	12.566	3.736
BFT3 week 2	419	422.82±2.645	26.519	3.998
CTL1 week 1	232	234.12±2.148	11.346	3.102
CTL1 week 2	281	283.06±1.934	8.08	3.014
CTL2 week 1	267	267.66±0.979	6.809	2.722
CTL2 week 2	368	373.37±3.395	5.324	2.774
CTL3 week 1	331	332.72±1.637	7.435	2.951
CTL3 week 2	306	308.68±2.256	6.362	2.901

3.2 Beta Diversity

In DPCoA, only ammonia nitrogen was associated with a cluster of samples with a low concentration (Figure 3). Dissolved oxygen, total dissolved solids, and temperature did not show any discernable association with sampling points.

Unlike the DPCoA ordination, the result for PCoA ordination clearly separates the samples by treatment (Figure 4). PCoA ordination with weighted Unifrac distance showed that the distribution of phylum Bacteroidetes and Proteobacteria, abundant on both biofloc and control samples, had an overall influence on the multidimensional projection of sample points. Actinobacteria, Chlamydiae, Firmicutes, Plantomycetes, and Verrucomicrobia were associated with biofloc treatment.

Canonical or "constrained" correspondence analysis was highly significant with ($P<0.01$) ammonia and dissolved oxygen. Figure 5 shows that the experiment had generally high dissolved oxygen during the first week. Week 2 samples, particularly those belonging to the control, were all characterized by high ammonia concentration. Biofloc samples during week 2 had no defined pattern. However, the lowest ammonia concentration was observed in biofloc treated tanks. This result is expected because molasses addition in biofloc treated samples promotes the proliferation of heterotrophic microbial growth, which has a higher nitrogen-fixing rate than autotrophic groups found in the control. The gradient direction of ammonia and dissolved oxygen was almost orthogonal, showing that these parameters were not collinear.

Among the genera observed, genus *Pelobium* abundantly occurred when DO was high, and NH_4 had moderate concentration. On the other hand, *Flavobacterium* abundance is high at medium to high levels of NH_4. *Cetobacterium*, which was the most abundant genus based on direct plotting, together with *Devosia* and *Luteolibacter*, was observed to thrive in condition with a medium concentration of DO and NH_4. One species of *Deinococcus* thrived on high ammonia concentration, and another was observed to favor medium concentration.

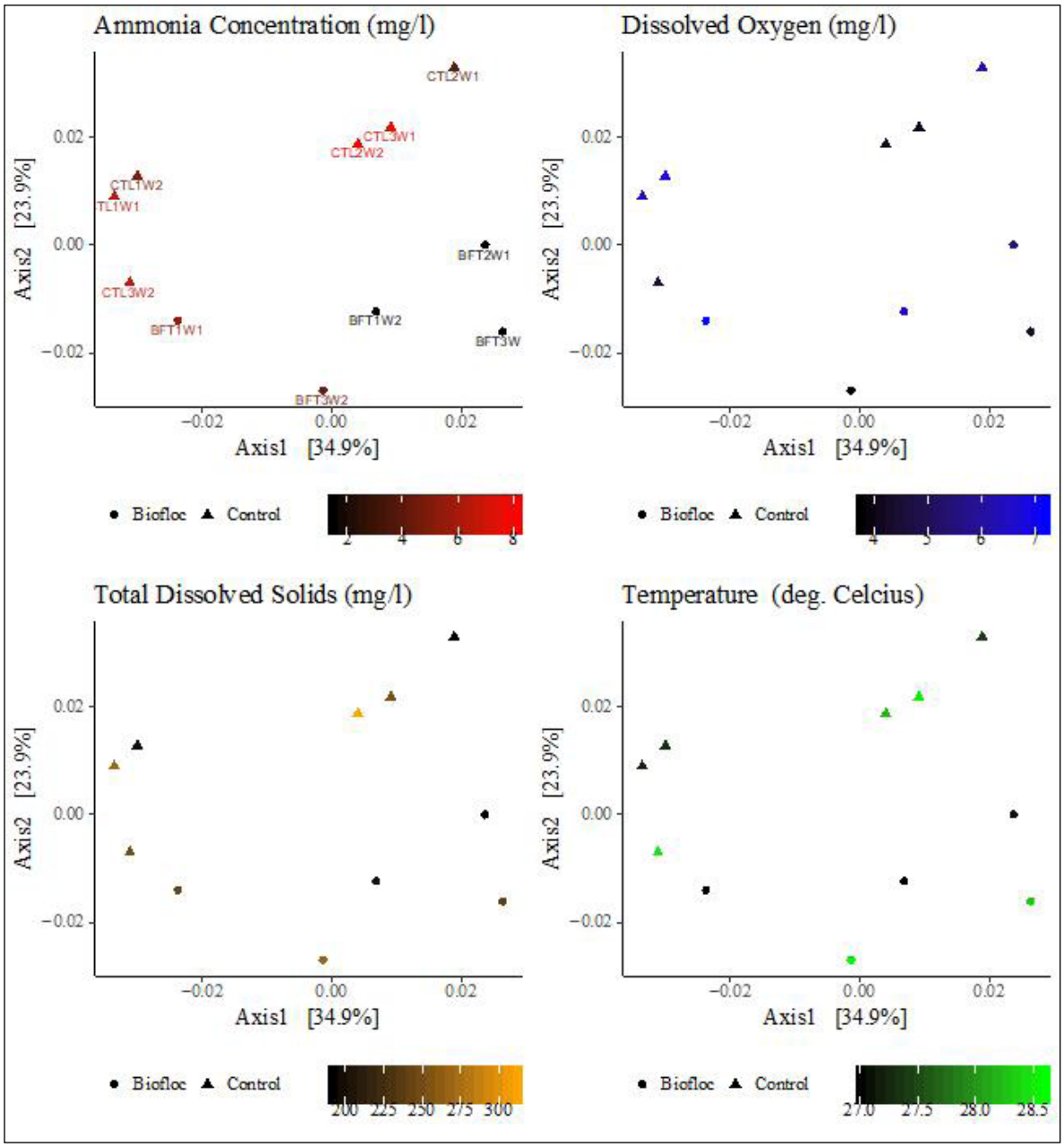

Figure 3. DPCoA plot for each sampling points showing the effect of ammonia concentration. Among the four water quality parameters, only ammonia concentration was associated with the clustering of BFT treatment.

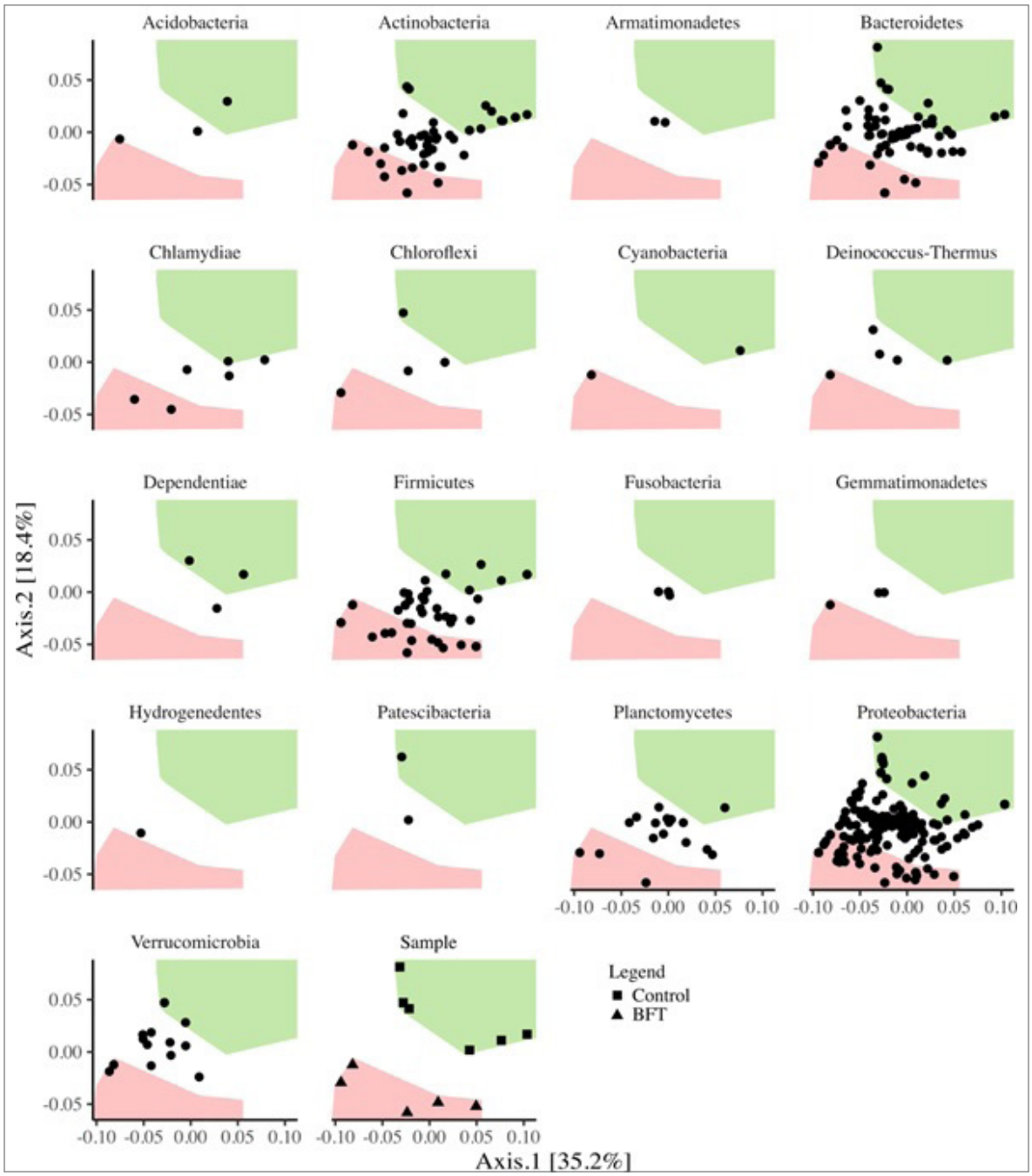

Figure 4. PCoA plot of weighted Unifrac of species separated by phylum rank. The PCoA plot for sample data was also included in the last panel, which shows that the control treatments are aggregated on the upper right quadrant. Phylum Actinobacteria, Firmicutes, and Planctomycetes tend to cluster towards the bottom, dominated by BFT.

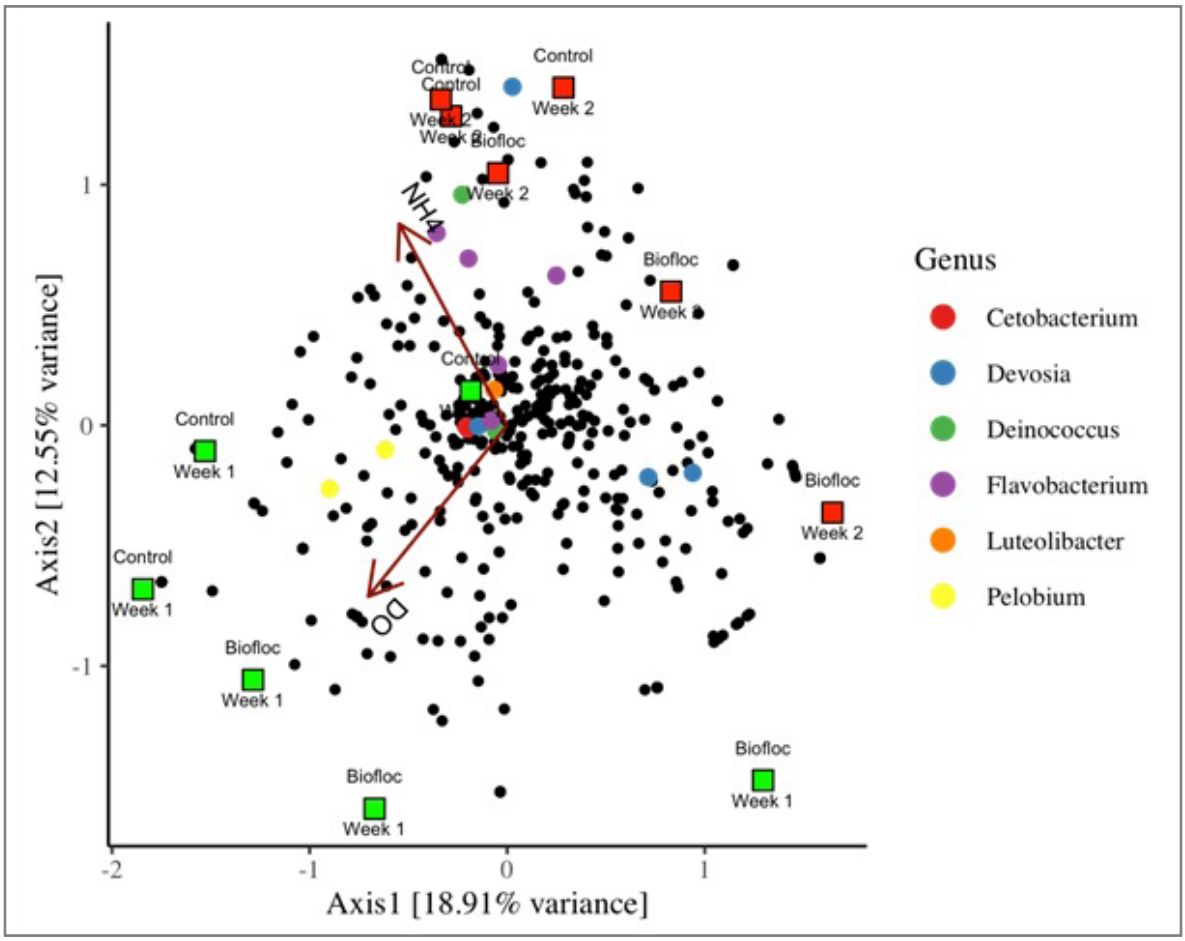

Figure 5. CCoA triplot showing the six (6) genera belonging to the 12 taxa with high abundance in Figure 1. Dark circles are taxa other than the annotated six genera. Squares correspond to the sample points. The sample collected during Week 1 was colored green while Week 2 was coded red. The arrow shows the direction DO and NH_4 concentration gradient.

3.3 Linear Modeling

Linear mixed-effect modeling showed a significant effect on the Shannon diversity index with Treatment and Week (P=0.05). As depicted in Figure 6, the Shannon index generally increases with C:N manipulated environment while the control had a decreasing diversity index. Tank 1, a biofloc treatment, had a slight increase from week 1 to week 2. Tank 5, a control treatment, had an increased diversity while the two control treatments decreased in the second week. Biofloc treated tanks 2 and 6 increased significantly in the second week of the experiment. The computed prediction intervals from mixed-effects modeling in Figure 6 clearly showed that the diversity generally increased in biofloc treated tanks during the second week, while tanks belonging to the control decreased.

4. DISCUSSION

There is a diversity of responses among microbial communities to C:N manipulation. Even within the same Phylum, microbial species response to organic carbon addition and NH_4^+ accumulation varies. In Phylum Bacteroidetes from the top 12 abundant taxa determined by direct plotting, among the five representative species (Figure 1), the response of *Lacibacter nakdongensis* was neutral, *Flavobacterium nitratireducens* was positive as depicted by a marked increase on week 2, while the rest responded by reduction of abundance. *F. nitratireducens*, which could reduce nitrate, was positive for starch hydrolysis (Nupur et al. 2013). The genus *Flavobacterium* reported in the CCoA also showed a positive response to the increased ammonia concentration.

Among Phylum Proteobacteria, *Acidovorax* sp. and *Pseudomonas* sp. responded positively to C:N manipulation while *Devosia insulae* appeared neutral. However, *Acidovora* sp. and *D. insulae* abundance decreased in the second week in control treatments, which was indicative that these species also benefited in the presence of organic C supplement. These types of microbial community variations in different periods of BFT were also reported for *O. niloticus* by Cienfuegos et al. (2018).

Some microorganisms were highly adaptable that they were robust enough to withstand disturbances such as C:N manipulation. The majority of the 12 most abundant genera belonged to this group of microorganisms. This group was not sensitive to a high C:N environment and maintained its abundance throughout the experiment. *Cetobacterium* abundance throughout the experiment suggested that this genus belonged to a robust community of microbes.

There were species of microbes, however that responded positively to C:N manipulation. Cardona et al. (2016) reported a high level of variability among key bacterial communities in biofloc, attributed to changes in biotic and abiotic factors such as organic carbon addition and ammonia accumulation.

Cienfuegos et al. (2018) identified 20 phyla from his study on the effect of biofloc in *O. niloticus* culture, of which the most abundant phyla were Proteobacteria, Bacteroidetes, Actinobacteria, Planctomycetes, Verrucomicrobia, and Fusobacteria. In this study, 17 were identified, and the abundant phyla in descending order were Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Verrucomicrobia, Planctomycetes, and Fusobacteria. Even on *Litopenaeus stylirostris* cultured in a marine biofloc environment, Proteobacteria was the most abundant phylum followed by Bacteroidetes (Cardona et al. 2016). Pilotto et al. (2018) also reported Proteobacteria's predominance, followed by the phyla Bacteroidetes, Actinobacteria, and Firmicutes on their biofloc experiment.

The C:N manipulation through molasses supplements increases the Shannon diversity index as depicted by linear mixed-effect modeling in Figure 6. Shannon-Weaver index relies heavily on species richness; hence, this index rose as the community's number and evenness increase (Kim et al. 2017). The Inverse Simpson diversity index (D2) obtained from this study was from 4.25 to 26.52 or 0.77 to 0.96 when converted to the original Simpson index (D1). The Simpson index (D1) obtained from this study indicated high diversity both on the control and biofloc treatment compared to 0.32 ± 0.19 in the clear water (CW) treatment and 0.26 ± 0.15 in the BFT treatment obtained by Cardona et al. (2016). Kim et al. (2017) reported that the Simpson index gives more weight to evenness than the Shannon index. The Simpson index computed in this study corroborates the review of Kim et al. (2017). Evenness increased due to environmental disruption leading to other species to proliferate among the dominant groups.

When under disturbances such as C:N manipulation, a microbial community promotes heterotrophic bacteria's proliferation and facilitates structural and functional maintenance through robustness (Stenuit and Agathos 2015). Microbial community robustness, which is defined as the resistance and resilience of community towards system perturbations, arises from the assembly and network interactions of heterogenous communities in a particular ecological niche (Stenuit and Agathos 2015). However, a microbial community species composition is rarely fixed and often experiences fluctuations of varying degrees and frequencies (Eng and Borenstein 2018). This change in the microbial community is due to a delicate balance between community resistance and resilience, or the capacity to withstand disturbances and the ability to return to *status quo*, respectively (Stenuit and Agathos 2015). In gut metagenomic studies, the autochthonous microbial community's composition might account for the effects of supplements containing allochthonous microbes (Zhang et al. 2016). But the resident bacteria's susceptibility is increased due to the persistence of transient bacteria (Zhang et al. 2016). Moreover, community robustness cannot solely be attributed to existing microbial composition (Eng and Borenstein 2018).

In summary, some *status quo* among dominant or abundant groups is maintained because of community robustness. Still, intervention such as C:N manipulation supports other less abundant microbial groups to proliferate.

5. CONCLUSION

Microbial response to C:N manipulation was observed. Seventeen phyla were identified, but only seven were remarkably abundant: Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Verrucomicrobia, Planctomycetes, and Fusobacteria. Some genera were observed to be resilient to C:N manipulation and remain dominant during the study. The addition of molasses increases the Shannon

diversity and Simpson indices, which were attributed to increased evenness as some microbial species proliferate with the presence of organic C and inorganic N substrate. DPCoA and CCoA ordination showed that some taxa belonging to Phylum Bacteroidetes, especially from the genus *Flavobacterium*, respond positively to increased ammonia concentration in the water. Hierarchical multiple testing confirmed that phylum Bacteroidetes and Proteobacteria abundance had a significant relationship to temporal change and C:N manipulation. Overall, linear mixed-effect modeling predicted that the Shannon diversity index decreased from microbial community belonging to the control, but increases on BFT-treated microbial community.

Regime change in microbial community was not easily caused by organic C supplementation because of community robustness to withstand biotic and abiotic disturbances. Controlled laboratory experiment showed that some bacteria species would proliferate as a response to C:N manipulation, but established species remained dominant.

The results recommend consistent and regular application of organic carbon source supplements in culture systems aiming to practice BFT. Sporadic organic carbon supply could result in delayed, if not failure, of heterotrophic bacteria to dominate the culture system. Long term solution for BFT is to develop feeds with a high C:N ratio (above 10:1) since feeds are significant inputs in an aquaculture operation. This could be achieved by either increasing the carbon source or reducing the nitrogen source in formulated feeds. The reduction of nitrogen sources could be translated into reduced fish meal ingredients, a critical issue in feed formulation and aquaculture.

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