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Effect of molasses on the treatment efficiency of fish recycling aquaculture wastewater and microbial community analysis

Na Tong[a,b], Jianqi Yuan[a,b], Hao Xu[a,b], Shaobin Huang[a,b,*], Yongqing Zhang[a,b], Rui Shu[c], Guangwen Tan[d], Jinghua Wu[e]

[a]*School of Environment and Energy, South China University of Technology, Higher Education Mega Centre, Guangzhou 510006, China, emails: chshuang@scut.edu.cn (S. Huang), 1224013722@qq.com (N. Tong), 861157970@qq.com (J. Yuan), Eden_Hao_Xu@163.com (H. Xu), zhangyq@scut.edu.cn (Y. Zhang)*
[b]*Guangdong Provincial Key Laboratory of Atmospheric Environment and Pollution Control, Guangzhou 510006, China*
[c]*Guangzhou Guanxing Agricultural Science and Technology Co. Ltd., Guangzhou 510006, China, email: 13928799130@163.com*
[d]*Guangzhou Pubang Landscape Architecture Co. Ltd., Guangzhou 510006, China, email: 1002871592@qq.com*
[e]*Guangzhou Taihe Water Ecology Technology Co. Ltd., Guangzhou 510006, China, email: 584112007@qq.com*

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ABSTRACT

The study aimed to investigate the effects of molasses on denitrification in an actual aquaculture wastewater through a set of experiments with the addition of an external C source. The difference in pollutant degradation in the aquaculture water was observed through the aerobic denitrification process in the biological aerated filter. Molasses can cause changes in the water environment and increase the bacterial metabolism when added to the reactor as a cosubstrate. High inorganic N bioremoval levels were also due to the different bacterial communities. At the phylum level, the dominant bacteria were Proteobacteria, Firmicutes and Bacteroidetes. At the genus level, molasses addition also stimulated a significant increase in the aerobic denitrifying bacteria, such as *Pseudomonas*, *Comamonas* and *Zoogloea*.

Keywords: Aquaculture wastewater; Molasses; Aerobic denitrification; Microbial community analysis

1. Introduction

Fishery trade has been particularly important for developing countries, with transactions occasionally reaching more than half of the total number of transactions. Aquaculture fishery develops rapidly due to the large demand in the market, but it has also caused some damages to the environment [1,2]. Continuous discharges from untreated culture wastewater will lead to a significant and chronic increase in the total organic matter content. Organic matter decomposition leads to considerable O depletion and affects aquatic organism survival in water [3]. Aquaculture system also produces large amounts of N, P and other organic nutrients (mainly from faeces production and feed consumption) [3,4]. A total of 1/*T* (1,000 kg) of live channel catfish produce 1,190 kg dry matter, 60 kg N and 12 kg P and can be discharged as metabolic waste into the culture water [5]. The accumulation of these substances leads to either eutrophication or algal reproduction which increases the biomass of superficial water and deteriorate water quality [6,7]. At the same time, water quality deterioration also leads to low fishery productivity and loss of biodiversity, even leading to disease outbreak of disease [8]. Therefore, treating the aquaculture wastewater is an urgent concern.

With the rapid development of fisheries, recycled aquaculture system (RAS) technology is increasingly recognised as a sustainable option for modern intensive aquaculture [9]. This system can not only reduce water

* Corresponding author.

consumption and effluent volume but also achieve high yields that are favoured by farmers and have the least impact on the environment [10,11]. In general, fish in RAS is kept at a high density and has a long water retention time and high feeding rates, thereby making the RAS wastewater highly representative and common [12]. In this type of wastewater, N compounds, especially NH_3, tend to accumulate during the cultivation process. When these N compounds exceed a certain concentration, they are potentially toxic to fish and can even lead to fish mortality [13].

Therefore, for the treatment of the wastewater above, a biological filter that has many advantages as a biological treatment method, such as small footprint, low hydraulic retention time (HRT), absence of excess sludge and lack of subsequent purifiers [14], is more effective than physical and chemical methods [15,16]. Biological aeration filter (BAF) is a novel, flexible and efficient biological filter [17,18]. In general, biofilter is a compartment containing a fixed medium for the attachment and growth of microorganisms to form a biofilm [19]. The biofilm on the surface has the function of entrapment and biological flocculation which can not only achieve high organic matter, N compound and suspended matter removal efficiencies at the same time but also the capacity to resist assault [15]. This biofilm can also degrade the intensive aquaculture wastewater well [20].

However, the mechanism underlying the increase in the degradation efficiency of BAFs and the removal of pollutants, such as NH_3^-–N, efficiently becomes increasingly important. Insufficient C sources or unstable organic matter is an important influencing factor that can lead to the ineffective biofilter denitrification [21]. In the aquaculture wastewater, the intrinsic C source from water bodies and fish secretions is always insufficient [22,23]. The lack of C source results in incomplete denitrification, thereby causing NO_3^- or NO_2^- accumulation in the wastewater [24,25]. Therefore, the addition of C sources, such as liquid and solid C sources, to increase the C/N ratio facilitates dissolved organic N removal in water and also contribute to the biological utilisation of the dissolved organic N [26] to increase the removal rate of nitrogenous pollutants [22,27]. However, excessive C sources waste expensive electron sources and increase the chemical O demand of wastewater [28]. Liquid C sources, such as methanol and fructose, play important roles in the continuous treatment of the wastewater with low C/N ratio [29,30]. These C sources are generally expensive and prone to biodegradation, thereby limiting its wide application [31]. C source is an important factor to be considered in aquaculture wastewater treatment.

In the aquaculture industry of fish, molasses is an extremely popular feed ingredient and is widely used in some countries in Europe or Asia [32]. Molasses is not only has the advantage of being used as an energy source but also has the advantages of fast digestion and absorption, improved palatability, reduced dust and improved particle quality [33]. However, we are concerned about the promotion of fish growth by molasses addition. The effects of molasses on the water treatment of the recirculating aquaculture wastewater and the changes in microbial communities have not been reported to date. Therefore, in this experiment, cheap molasses was selected as an additional C source to explore the effect of molasses on contaminant removal from the aquaculture wastewater and the change in the microbial communities during biochemical treatment.

2. Material and methods

2.1. Large-scale experiments

Fig. 1 shows a process flow diagram of the large-scale recirculation aquaculture treatment system. The system operated in the recovery mode as follows. First, the aquaculture wastewater entered the treatment tank and was separated by the solid/liquid part of the microfiltration machine. The separated suspended particulates were discharged through the sewage pipe of the microfiltration machine. The filtered water was lifted into the biological filter by the stripping tube. Large numbers of aeration tubes, floor heating tubes and fillers were observed in the biological filter. The fillers were fully suspended, thereby making the dissolved organics and nutrients in the water degrade quickly due to the dense aeration environment. Salt and floor heating pipes were heated centrally in the biological filter to maintain the water

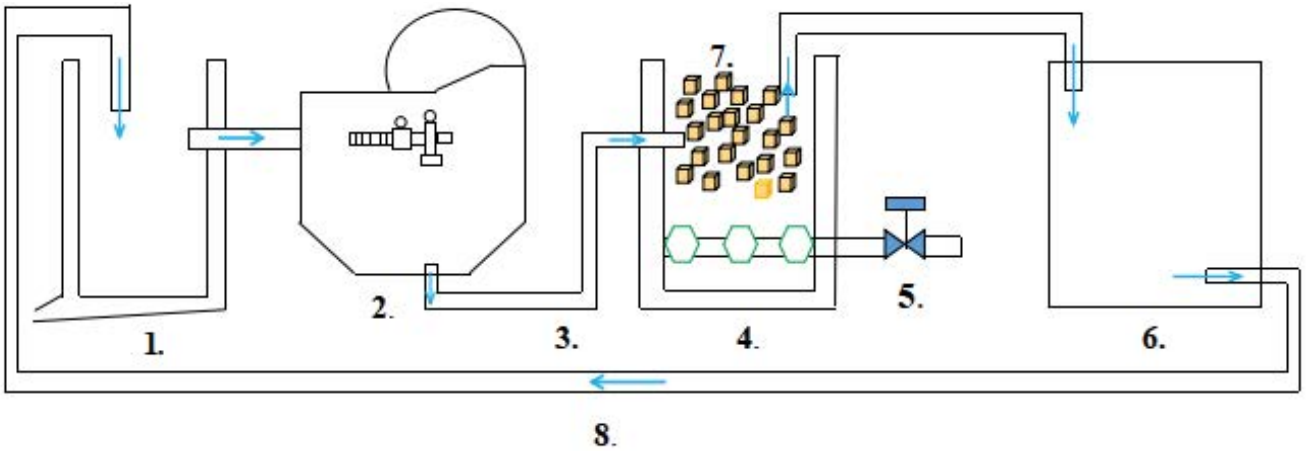

Fig. 1. Flow chart of recycled aquaculture system: (1) aquarium, (2) microfiltration machine, (3) gas lift pipe, (4) biofilter, (5) aeration tube, (6) ozone generator, (7) filler, and (8) tandem tube.

temperature of the culture system. Biochemically treated wastewater was sterilised and decolourised by ozone and finally returned to the breeding container through the series pipe. The central control box controlled the roots blower, microfiltration machine and ozone generators. The central control box also possessed online monitoring and alarm functions. The inlet of the gas flowmeter was connected to the dewar tank, and the outlet was connected to the manifold of the breeding container for O supply. The multisensor can also continuously monitor the temperature and pH, and the data can be recorded by an acquisition system.

2.2. Experimental method

Two sets of breeding systems named A and B were established throughout the experiment. In each breeding box, 800 young fish were placed, and 18 m^3 of water was added. These fish were also fed three times a day, wherein the A system was only added with common fish feed (Hengxing feed), and the B system was added with molasses feed (brown sugar) by 50% of the feed weight after feeding. The amount of molasses was approximately 100 mg/L. As the young fish grew slowly, the amount of molasses increased. However, to control the variables, we maintained the concrete molasses amount during the experiment. The biofilter in the processing tank used a hollow-filled plastic packing with a specific surface area of 3,000 m^2/m^3. The filler volume was 10 m^3, and the HRT was 2.5 h. Water samples were obtained from the outlet of the treatment box at the same time once every 2 d for 2 months to determine the NO_2^-–N and NH_3^-–N contents.

2.3. Analytical methods

Water samples were filtered to remove the biomass. The total NH_3^-–N concentration was determined by the Nessler's reagent colorimetric method (Water Quality Standard for Fisheries; GB11607-89, China), and the NO_2^-–N concentration was determined by the ultraviolet (UV) spectrophotometry method (GB/T 5750.5-2006, China) by using a UV–visible spectrophotometer (JASCO V-550, Japan). The pH value was measured by a pH probe (HQ30d53LDO, Hatch, USA).

2.4. Microbial analysis

The sludge samples obtained from the two reactors were named R1 and R2. The genomic DNA of each sample was extracted using an EZNA™ Mag-Bind Soil DNA kit (Omega Bio-tek, USA). The integrity of the extracted DNA was verified via agarose gel electrophoresis. A Qubit 2.0 DNA kit (Life Technologies, China) was used to quantify the genomic DNA and control the amount of added DNA for polymerase chain reaction (PCR) mixtures accurately. Then, two PCR amplification reactions were performed, in which the primers used for PCR were fused with the universal primers of the MiSeq sequencing platform. After amplification, the PCR product was purified by Agencourt AMPure XP Beads (Beckman Coulter Inc., Brea, California, USA). Then, the DNA concentration in the purified product was measured using a Qubit 2.0 DNA kit (Life Technologies, China). High-throughput gene sequencing was performed by Sangon Biotechnology Co. Ltd., (Shanghai, China) on the Illumina MiSeq platform. The raw sequence data were mass-filtered and analysed using QIIME version 1.8.0. A high-quality representative sequence for each operational taxonomic unit (OTU) was assigned using USEARCH version 5.2.236 with 97% sequence identity.

3. Results and discussion

3.1. Pollutant removal

Figs. 2a and b show that the biological NH_3^-–N and NO_2^-–N removal in the wastewater was degraded by the experimental process. Fig. 2a shows that the wastewater containing molasses and that without molasses possessed similar biological N removal at the early stage, and the degradation was relatively slow. This result may be due to the fact that the biological aerated filter was in the initial natural state at this stage. At this stage, the microorganisms were at the growth stage. Hence, the processing efficiency of the two organisms was close. However, from the 9th day onwards, NH_3^-–N degradation accelerated, and a gradual difference in the removal of NH_3^-–N between the two types of wastewater was observed. After 25 d, the biological NH_3^- removal rate in the biologically aerated filter gradually stabilised. The wastewater containing molasses possessed an advantage gradually, and the removal amount was significantly higher than that without molasses. The maximum removal amount can reach up to 12.04 mg/L. When the aeration biological filter was successfully started, the molasses was used as an additional C source which caused the protein in the feed to ferment, thereby increasing the number of microorganisms. Therefore, the biofilm formation rate is relatively fast, and an increased number of strains participated in the degradation process of contaminants, thereby increasing the removal rate.

At the same time, NO_2^- degradation in fishery aquaculture wastewater is particularly important because the excessive accumulation of this compound results in fatality due to fish poisoning and reduce fish production [34]. In the experiment, Fig. 2b shows that the difference in the NO_2^- removal from the two water bodies was extremely evident at first, and the degradation amount gradually stabilised after 25 d. During the process of NH_3^-–N degradation, NO_2^- which is an intermediate of aerobic nitrification and denitrification accumulated due to the incomplete reaction process, thereby increasing the NO_2^- concentration. However, adding molasses to the water increased the C/N ratio of the culture wastewater, thereby allowing the microorganisms in the water to have sufficient C sources for improved growth and can be used to dissolve organic N for metabolism. This phenomenon induced the nitration reaction to proceed completely, thereby reducing NO_2^- accumulation, and the removal amount can reach up to 0.479 mg/L.

3.2. Microbial analysis

3.2.1. Microbial community richness, diversity and similarity

Two activated sludge samples of the reactors were collected at the end and named R1 and R2 for differentiation. Then, we used Illumina high-throughput sequencing to analyse and identify the dominant strains and the changes

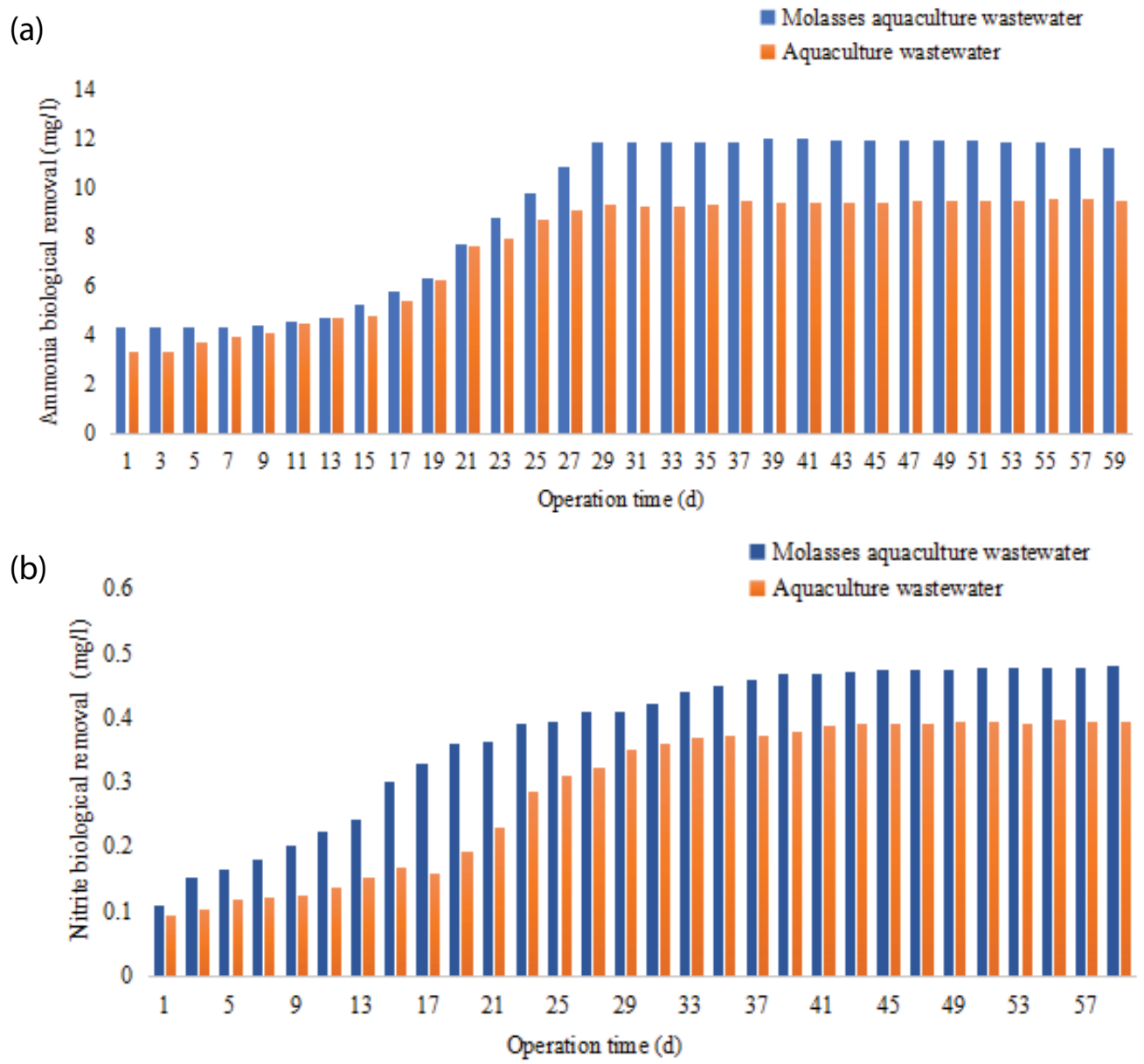

Fig. 2. Effect of molasses on pollutant removal: (a) ammonia nitrogen biological removal comparison chart and (b) nitrite biological removal comparison chart.

in microbial community structure. After removing the low-quality sequences and chimeras, at least 30,000 effective sequences were yielded for each sample. As shown in Table 1, the numbers of OTUs were 3,556 and 2,742 in R1 and R2, respectively. Fig. 3 shows that the number of OTUs shared by R1 and R2 was only 174, thereby accounting for 2.92% of the total observed OTU number. R1 with added molasses showed increased OTU numbers, thereby indicating that molasses not only increased the microbial abundance but may also reduce sludge specificity. This situation was the same as increasing the amount of OTU caused by an increase in the organic matter concentration and sludge specificity [35]. In addition to the effective readings and OTU, Shannon diversity, ACE, Simpson's index Chao 1 Richness and Good's coverage values are also listed in Table 1. Species richness

Table 1
Diversity indices of bacterial communities in seven sludge samples: R1: reactor with the molasses; R2: reactor without the molasses

Sample-ID	R1	R2
Seq-num	31,886	33,636
OTU-num	3,556	2,742
Shannon index	5.20	3.68
ACE index	40,168.89	40,052.25
Chao 1 index	17,840.65	16,498.25
Coverage	0.92	0.93
Simpson	0.04	0.10

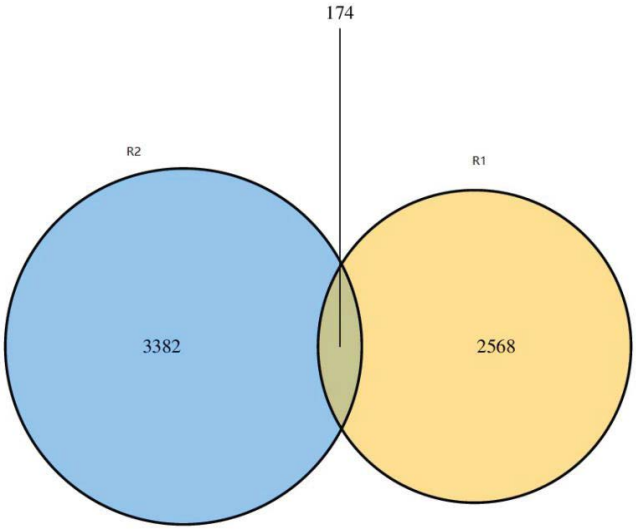

Fig. 3. Bacterial Venn diagram. R1: reactor with the molasses; R2: reactor without the molasses.

was assessed by Chao 1 and ACE estimators and species diversity can be revealed by Shannon and Simpson's indices [36]. In general, the high Simpson index is associated with a decrease in the diversity of the microbial community distribution, whilst the Shannon index value is proportional to the diversity of the community distribution [37]. On the basis of the Shannon and Simpson's indices, the C source concentration was increased due to the addition of molasses which increased the abundance of the community biodiversity. Sufficient C source can provide the nutrients needed for microbial growth to facilitate grow and multiplication of the bacteria [38]. The ecological stability can be increased due to high biodiversity which results in a high capacity to resist environmental stress [39].

3.2.2. Bacterial community structures

The structures of the bacterial community in the biologically activated sludge systems are closely related to the performance of the treatment system [40]. The taxonomic analysis of species revealed the variation of bacterial community structure in response to the increase in molasses in the bioreactor. Fig. 4a shows the main microbial community dynamics at the phylum level after adding molasses. The most important advantage was the decrease in Proteobacteria from 86.17% to 64.4% after adding molasses. Proteobacteria is the most abundant community in the soil and sewage [41] which was similar to our results. At the same time, many NH_3-oxidising bacteria, NO_2^--oxidising bacteria and N removal agents belong to this category [42,43]. Although the richness of Proteobacteria decreased after the addition of molasses, it did not affect the denitrification efficiency in the wastewater, as shown in Fig. 4b. The figure shows that at the level of the program, γ- and α-Proteobacteria decreased drastically from 25.59% to 6.83% and 17.99% to 6.38%, respectively. However, the β-Proteobacteria abundance showed a slight increase. β-Proteobacteria is important for N removal from the activated sludge [44] and can simultaneously degrade the organic matter and remove N. Many denitrifying bacteria belong to the class β-Proteobacteria, such as *Rhodocyclaceae* sp., *Thauera* sp. and *Azoarcus* sp. This result indicated that the β-Proteobacteria in Proteobacteria played a major role in the denitrification process.

In the microbial community, increasing subdominant phyla was also present. Among the subdominant phyla, Firmicutes and Bacteroidetes increased to 20.13% and 11.92%, respectively. In a previous study, the high Proteobacteria and Bacteroidetes abundances in the system are responsible for N removal [45]. Meanwhile, Firmicutes is a microbial component commonly found in nitrifying biofilters in marine aquaculture systems [46]. This finding was consistent with our results that molasses aided in denitrification. The changes in the abundance of *Chloroflexi* strongly corresponded to the species diversity of the two reactors, with a growth rate of up to 93.47%. *Chloroflexi* bacteria are facultative anaerobic bacteria that are an important factor in granulation and can reduce the potential of biomass pollution by enhancing the particle structure [47]. *Chloroflexi* bacteria are present in highly enriched anaerobic ammonium oxidising biomass [48]. This result can explain why the biological removal of NH_3^-–N in R1 with molasses was high.

Fig. 4c shows the fluctuation of the microbial community at the genus level. The relative abundance of *Zoogloea* decreased from 7.84% of R1 to 0% of R2. *Zoogloea ramigera* is a member of this genus and known as a denitrification agent [49] which contributes to NO_3 degradation, thereby purifying the aquaculture water. Under sufficient C source, the dominant genus in R2 changed from *Acidovorax* (24.8%), *Rhizobium* (15.75%) and *Acinetobacter* (12.41%) to *Pseudomonas* (3.2%) and *Sphaerotilus* (16.98%) and *Comamonas* (3.15%) in R1. In the wastewater treatment system for marine aquaculture, the decline in *Acidovorax* and *Comamonas* caused a decrease in the denitrification rate. Here, *Acidovorax* and *Comamonas* likely participate in partial biodegradation [50]. In our recycling wastewater treatment system, although *Acidovorax* decreased, the read of the *Comamonas* increased significantly, thereby increasing the denitrification rate as well. This result indicated excellent *Comamonas* biodegradation. *Comamonas* was also suitable for a high sugar biological environment.

Another factor that cannot be ignored is the role of *Pseudomonas* which is a typical bacterium for aerobic denitrification and N removal in an O-filled environment to ensure the growth of fish and shrimp. *Stenotrophomonas* also disappeared with the addition of molasses in the reactor. *Stenotrophomonas* is a typical denitrifier that is the keystone species in summer [51]. This result indicated that the molasses inhibits *Stenotrophomonas* growth, which may reduce incomplete denitrification. However, in the actual process, NO_2 accumulation significantly reduced, thereby indicating that *Stenotrophomonas* sp. may not be the dominant species for denitrification after adding molasses water.

Most OTUs in our metagenomic analysis are unrecognised at the species level, thereby limiting our ability to describe other functional components of the biofilter microbial community. Many microbes have never been screened and cultured. Thus, these microbes are informally described in taxonomy which limits our ability to incorporate species analysis into the species levels. Other studies [52,53] have also found a large number of unclassified bacteria in the

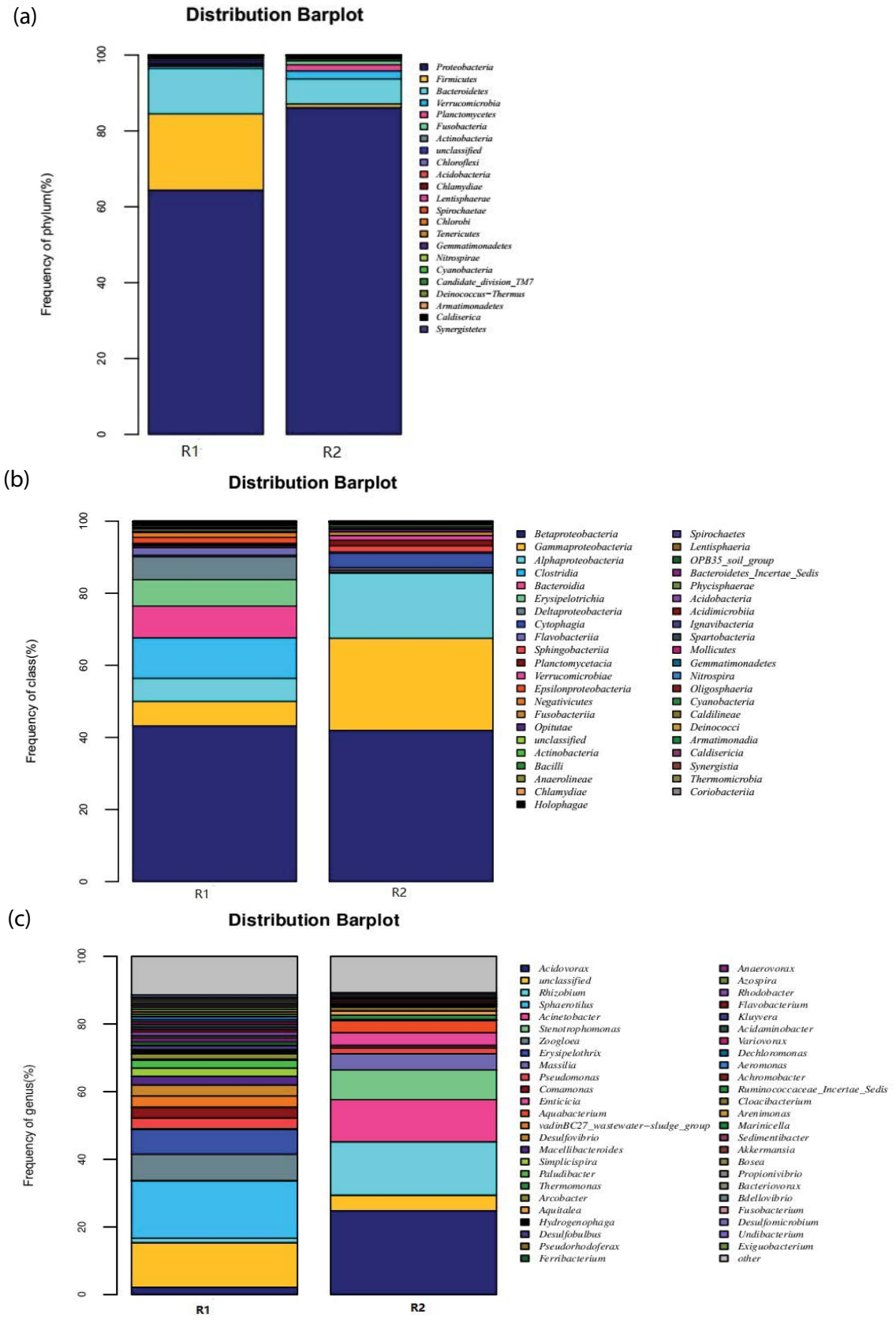

Fig. 4. Relative abundances of major bacterial groups at phylum (a), class (b) and genus, and (c) levels of the two reactors; "Other" represents all classified taxa that were <1% in all samples. R1: reactor with the molasses; R2: reactor without the molasses.

aquaculture systems. Hence, many bacteria may belong to degrading pollutants which will be the topic of our future research in the field of aquaculture water.

4. Conclusion

In the aerated biological filter reactor, the aerobic denitrification process is used to treat the actual circulating aquaculture wastewater. Increased biological N (NH_3^-–N and NO_2^-–N) removal which affects the bacterial community composition is obtained by adding molasses as the cosubstrate. The dominant bacterial phyla in the reactor after adding molasses are Proteobacteria, Firmicutes and Bacteroidetes. At the same time, at the genus level, the functional strains for aerobic denitrification, such as *Pseudomonas*, *Comamonas* and *Zoogloea*, are also selectively enriched.

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