Original Article

Single-tank sequencing batch bioreactor with a cellulose substrate for simultaneous nitrification and denitrification of an aquarium

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Abstract

Processing and detoxifying nitrogenous waste in an aquarium is difficult with one tank because nitrifying and denitrifying bacteria need contradictory conditions to grow and survive. Herein, a single-tank sequencing batch bioreactor was used to simultaneously perform aerobic nitrification and denitrification for aquarium filtration. To evaluate its performance at the laboratory-scale, the 8 L bioreactor was connected to a 20 L aquarium with a carbon water filter and containing Oranda goldfish. The bioreactor comprised a bottom layer filled with 2.5 L of polyester sponge, covered with 1 L of carbon pellets and then 2.5 L of cellulose beads or polyester sponge. A submersible pump in the aquarium continuously pumped water into the bioreactor, which created an oxygenated environment with a water filtration rate of 4,608 L/day. Over 117 days, the bioreactor significantly lowered the total ammonia nitrogen, nitrite and nitrate concentrations with minimal water filling to compensate for evaporation. Therefore, the bioreactor can simultaneously perform aerobic nitrification and denitrification in a single-tank and maintain good water quality for the long-term care of ornamental fish. However, aerobic denitrification successfully occurred only in the bioreactor containing cellulose beads. Therefore, metagenomic analysis was performed to examine the microbial communities on the filters and Proteobacteria were identified as the dominant phylum in all samples. The nitrifying and denitrifying bacteria in the filters mainly belonged to Nitrospirota (Nitrospira spp.) and Proteobacteria (Comamonadaceae and Rhodobacteraceae families), respectively. Beta diversity analysis indicated that the differences in bacterial communities could be attributed to changes in the substrate and incubation time. Proteobacteria were the most significant contributors to the physiological activity in the simultaneous nitrification and aerobic denitrification bioreactor.

Keywords: water filtration, sequencing batch reactor system, ornamental fish, nitrogen waste, cellulose beads, water treatment innovation

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Introduction

The main concern for the care of ornamental fish in a closed system is the accumulation of food waste and excretion of nitrogen waste. These negatively affect the growth and health of the fish and thus require periodic water changes, as well as the installation of a water filtration system to reduce the frequency of water changes. Water filtration systems can be classified into three main categories: mechanical, chemical and biological. All three are critical and interrelated and they usually proceed in a set sequence: mechanical, biological and chemical filtration. Mechanical filtration removes solid waste from a recirculating water system and the most convenient method in an aquarium is to use a fibrous sponge to remove particulate substances. Biological filtration uses microorganisms to process and detoxify nitrogenous waste, particularly nitrifying and denitrifying microorganisms (Abakari *et al*., 2020; Sikora *et al*., 2020; Emparanza, 2009; Chen *et al*., 2006). Such microorganisms are spontaneous, grow slowly and live in the water or filter media when appropriate levels of nutrients, pH, oxygen and alkalinity are present. Chemical filtration uses a chemical reaction to bind and remove contaminants and a widely used agent is carbon granules. In general, nitrifying microorganisms perform well in oxygenated environments and denitrifying microorganisms perform well in anoxic environments (Chen *et al*., 2006). Therefore, a conventional single bioreactor usually supports only aerobic nitrification activity to convert ammonia and nitrite to nitrate but it is not suitable for denitrifying microorganisms, which leads to an over-accumulation of nitrates, low alkalinity and low pH over time. A low pH can result in significant bacterial collapse, which can lead to bioreactor failure, anoxic conditions, and eutrophication in the aquarium (Abakari *et al*., 2020; Chen *et al*., 2006). Ammonia then accumulates in the aquarium, which affects the health of the fish. An essential part of aquarium care is reducing nitrate accumulation through periodic water changes and rinsing the filter media, which are generally preferred over adding an additional tank for anaerobic denitrification. The ideal and most convenient case would be if nitrification and denitrification could be performed within the same bioreactor. A possible solution is a sequencing batch bioreactor, but this has never been adapted for use in an aquarium.

The submerged biofilter commonly used for aquarium care accumulates nitrates; removing the nitrates by changing the water stresses the fish and wastes water. In some countries, ornamental fish aquaculture may not be allowed unless it can become more responsive to limited water resources (Asano *et al*., 2003). The selection of the filtration material in the reactor is essential, especially to allow denitrifying microorganisms to develop in an oxygenated environment. A wide variety of materials is in use, most of which are plastic. They are quite good for mechanical filtration but not very good for biological filtration because the hydrophobicity and smoothness of the surface do not support microbial adhesion (Hakim *et al*., 2020). In addition, plastic filters take a long time to decompose and they become hazardous waste. Natural polysaccharides comprising cellulose, chitosan, starch, gum, alginate and pectin offer promising potential for sustainable water treatment owing to their hydrophilicity, hardness, flexibility and porosity. They are biodegradable, non-toxic to fish and leave no residue in the environment (Nasrollahzadeh *et al*., 2021).

We recently introduced a single-tank sequencing batch bioreactor that can be used to care for zebrafish with fewer water changes (Ponpornpisit *et al*., 2022). This type of reactor is beneficial for aerobic microorganisms, prevents anoxic conditions, successfully removes nitrogen waste and maintains the alkalinity level in a single-tank. However, different types of ornamental fish require different levels of filtration. Some ornamental fish eat several times a day; some require live food while others eat dry food. Goldfish have no actual stomach for complete digestion, so they eat and excrete often. They leave much organic matter in their environment, so they need an efficient bioreactor that can keep the water clean without frequent water changes. The objective of this study was to assess the effect of cellulose beads for use in a laboratory-scale single-tank sequencing batch bioreactor for simultaneous nitrification and denitrification. The results of this study may eventually be applied to the long term care of goldfish in aquariums.

Materials and Methods

Laboratory animals: Fifty-four mixed sex juvenile goldfish were obtained from a local ornamental fish farm in Nakornpatom Province, Thailand. Upon arrival, they were screened for good physical condition without the following external appearances: fin deformities, corneal opacity, skin lesions and abnormal swimming. They were acclimated for 2 weeks in a 200 L fiberglass tank. The tank was provided with an air stone connected to a central air pump and filled with water passed through a carbon filter (TMD Co., Ltd., Bangkok, Thailand). They were fed ad libitum once daily with dry shrimp pellets containing 35% protein, 4% fat and 4% fiber (APAC93L; Cargill Siam Ltd., Bangkok, Thailand). During acclimatization, 20 L of water was changed daily.

After acclimatization, the fish were randomly released into nine acrylic aquaria, each containing 20 L of water. They were individually weighed and examined for health conditions on days 0, 35, 70 and 117, which is when the testing period ended. Each tank contained 6 fish, an air stone connected to a central air pump, an 18 W submersible pump (Venus Aqua; Kaixing Electrical Appliance Co., Ltd., Zhongshan, China) and a single-tank sequencing batch bioreactor on top of the tank.

All animal husbandry and experimental conditions were approved by the Chulalongkorn University Animal Care and Use Committee (Protocol number: 2031008).

Bioreactor setup: Figure 1 shows the bioreactor used in this study, which was modified from a previous study (Ponpornpisit *et al*., 2022). In brief, three pieces of PVC (90 mm diameter, 5 cm length) were placed at the

bottom of the bioreactor for sedimentation and preventing the filter material from obstructing the water outlet of the reactor. The pump was operated continuously in a two-step cycle that had periodic wet and dry phases. The total filtration rate was 24 cycles/h, which was equivalent to approximately 192 L/h.

Three types of filter materials were considered: cellulose beads (WithAQUA Co., Ltd., Tokyo, Japan), polyester sponge (AW103; Qian Hu Co., Ltd., Bangkok, Thailand) and activated carbon pellets (FM131; Qian Hu Co., Ltd.). The cellulose beads were white and oval with an average diameter of 5 mm and density of 0.1 g/mL. The polyester sponge and activated carbon pellets were commercial products sold for general use in aquarium filtration systems. The sponge was blue and had a density of 0.02 g/mL. The carbon pellets were black with a density of 0.5 g/mL.

The experiment was divided into three groups comprising a control and two treatments: bead and no-bead. Each group contained three replicates to ensure repeatability of the results. In the control group, the bioreactor was filled with polyester sponge folded into four layers with a total volume of 2.5 L and a net bag containing 1 L of activated carbon pellets. In the bead group, a net bag containing 2.5 L of cellulose beads was added to the bioreactor. In the no-bead group, another 2.5 L of the folded sponge was added to the bioreactor instead of the cellulose beads.

The experiment was conducted at room temperature (28°C ± 3 °C) for 117 days from March to July at Veterinary aquatic animal research center, Faculty of Veterinary Science, Chulalongkorn university. The fish were fed 3% of their bodyweight every day with shrimp pellets (APAC93L). The fish were weighed on days 0, 35, 70, and 117 and the feeding amount was adjusted according to the weight gain. All aquaria were started up without pre-incubation of the filter material. Each week, 1 L of new carbon-filtered water was added to compensate for vaporization and maintain the same water volume without replacement.

Water quality analysis: Weekly checks were performed for the following parameters. The water temperature and pH (HI98103 checker; HANNA, Woonsocket, RI, USA) and dissolved oxygen (DO) (oxygen meter, YSI550A; YSI Inc., Yellow Springs, OH, USA) were checked at the side of the experimental tanks. A sample of 50 mL of water was taken from each tank and was filtered through a 0.45 µm filter membrane. This sample was then checked for the total ammonia nitrogen (TAN) (salicylate method), nitrites (sulfanilamide NNED method), nitrates (UV screening method) and alkalinity (Alkalinity test kit, Model AL-DT, Hach, Loveland, CO, USA). When the measured alkalinity was less than 100 mg/L, 50 mg/L of sodium hydrogen carbonate solution was added. In total, 16 water samples from each tank were examined; each sample examination was repeated three times.

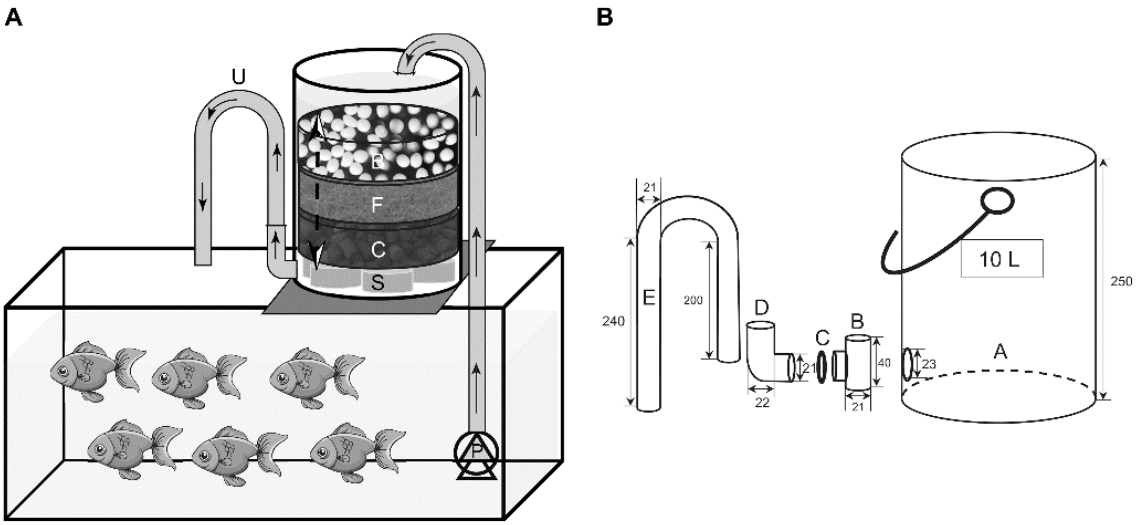

Figure 1 Illustration of the single-tank sequencing batch reactor. PVC pieces are placed at the bottom of the tank (S), which is filled with activated carbon pellets (C), polyester sponge (F) and porous cellulose beads (B). The submersible pump (P) continuously draws water from the aquarium through the water filtration inlet. When the water level is up to the upper part of the U-shaped siphon tube (U), the water is drawn into the tube by differential back air pressure and flows through the water filtration outlet until the water level in the tank is lower than that in the filtration outlet. Subsequently, the water accumulates in the tank again. The water filtration cycle in the tank was continuous over the rearing period. A water container can be constructed by drilling a hole in the side of a bucket (A). A T-shaped PVC joint (B) is inserted inside the water container and the joint is covered with a rubber O-ring (C) to prevent water leakage. An L-shaped PVC (D) is connected to the T-shaped joint outside the water container. The U-shaped pipe (E) is connected to the L-shaped joint on one side while the other side is positioned over the aquarium for water drainage. The total cost of a single-tank sequencing batch bioreactor, including the water container, accessories and filter materials, is approximately 20 USD. This excludes the aquarium and electric water pump. (Measurement unit = mm).

DNA metabarcoding analysis: The biomass retained by the cellulose beads and polyester sponge in the bead group was randomly sampled at three levels from top to bottom and pooled on days 7 and 117. The biomass retained by the polyester sponge from the no-bead group was collected on day 7. The collected biomass underwent microbial community analysis. The total DNA was extracted using DNeasyPowerSoil Pro Kit (QIAGEN, Valencia, CA, USA) and a polymerase chain reaction (PCR) analysis was performed. The 16S rRNA gene was amplified using 341F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTACGGGNGGCWGCAG-3′) and 805R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA GGACTACHVGGGTATCTAATCC-3′) primers to target the V3–V4 variable regions via the 2X sparQ HiFi PCR master mix (Quanta Biosciences, Beverly, MA, USA). The amplification conditions included an initial denaturation step of 2 mins at 98°C, which was followed by 30 cycles of 98°C for 20 s, 60°C for 30 s, and 72°C for 60 s, and a final extension step of 72°C for 1 min. Subsequently, the 16S amplicon was purified using sparQPuremag beads (QuantaBiosciences) and was indexed by using 5 µL of Nextera XT index primer in a 50 µL PCR reaction, followed by 8–10 cycles of the PCR conditions given above. The final PCR products were cleaned, pooled and diluted to a final loading concentration of 4 pM. Cluster generation and 250-bp paired-end read sequencing were performed on an Illumina MiSeq at Chulalongkorn University Omics Sciences and Bioinformatics Center (Chulalongkorn University, Bangkok, Thailand).

Scanning electron microscopy: Cellulose beads from the bead group were sampled on day 117, fixed with 2.5% glutaraldehyde overnight at 4°C, dehydrated in a sequential ethanol series, and dried overnight at 37°C. They were then inspected under a scanning electron microscope (SEM) (JSM IT500HR, JEOL Ltd., Japan).

Statistical analysis: One-way analysis of variance was performed on the water parameters and post hoc analysis was performed with the Bonferroni test using SPSS Version 28.0 (SPSS Inc., Chicago, IL, USA). Results at $p < 0.05$ were considered significant. The goldfish health, survival rate, average daily weight gain and specific growth rate were analyzed via descriptive statistics. Microbiome bioinformatics was performed with QIIME 2 version 2020.8 (Bolyen *et al.*, 2019). Raw sequences of DNA metabarcoding were demultiplexed and filtered for quality by using the q2-demux plugin, which was followed by denoizing with DADA2 (Callahan *et al.*, 2016) (via q2-dada2). The phylogeny was constructed using the SEPP q2-plugin to place short sequences into the sepp-refsgg-13-8.qza reference phylogenetic tree (Janssen *et al.*, 2018). Alpha-diversity metrics (i.e., observed OTUs and Faith's phylogenetic diversity (Faith, 1992)), beta diversity metrics (i.e., weighted and unweighted UniFrac (Lozupone *et al.*, 2007)) and principle coordinate analysis (PCoA) were estimated using the q2-diversity after samples were rarefied (i.e., subsampled without replacement) to 10,203 sequences per sample. Taxonomy was assigned to amplicon sequence variants by using the q2-feature-classifier (Bokulich *et al.*, 2018) and sklearn-based naïve Bayes taxonomy classifier against the SILVA database (version 132) (Quast *et al.*, 2012).

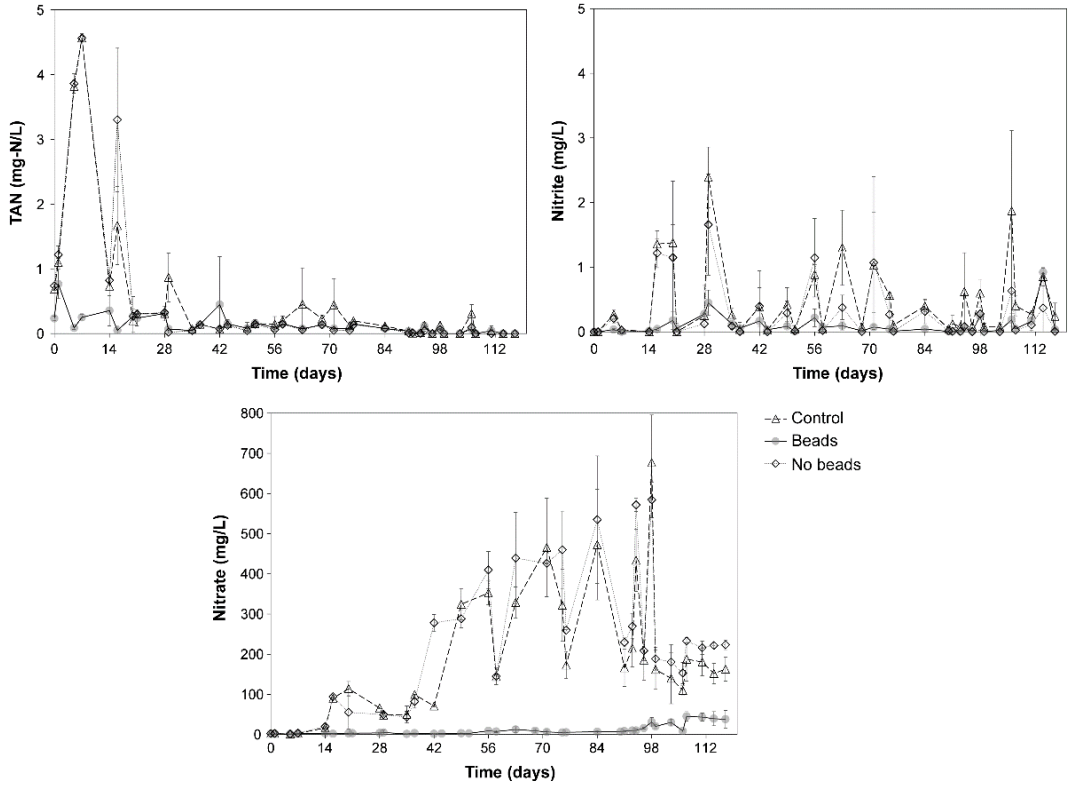

Figure 2 Measured TAN, nitrite and nitrate of bioreactors in the control, bead and no-bead groups. The water parameters were recorded over 117 days.

Results

Water quality: The TAN, nitrite and nitrate levels of the groups were recorded over 117 days (Figure 2 and Supplementary Tables S1–S6). The concentrations in each group were compared for three phases according to the trends of TAN: primary (0–14 days), secondary (16–63 days) and final (68–117 days). During the primary phase, TAN showed significant peaks (>4 mg-N L^{-1}) in the control and no-bead groups and a small peak (<0.54 mg-N L^{-1}) in the bead group. In the secondary phase, the TAN concentration was lower in the bead group than in the control ($p = 0.003$) and no-bead groups ($p = 0.002$). The nitrite and nitrate concentrations did not differ significantly among the groups ($p > 0.05$). The TAN concentration showed no significant intergroup differences. However, the nitrite and nitrate concentrations were significantly lower in the bead group than in the control group ($p < 0.001$ and $p < 0.001$, respectively) and no-bead group ($p < 0.037$ and $p < 0.001$, respectively). In the final phase, the TAN and nitrite concentrations were lower in the bead ($p < 0.025$ and $p < 0.004$, respectively) and no-bead ($p < 0.001$ and $p < 0.013$, respectively) groups than in the control group. The nitrate concentration was lower in the bead group than in the control ($p < 0.001$) and no-bead ($p < 0.001$) groups, respectively. The nitrate removal rate in the bead group in the secondary and final phase was 96% and 77%, respectively (Figure 2).

The alkalinity level of the bead group did not decrease below the initial level and no sharp increase in nitrate was observed; however, in the control and no-bead groups, the depletion of alkalinity levels and high accumulation of nitrate occurred (Figure 3, Table 1, and Supplementary Tables S7–S12).

Scanning electron microscopy: The sponge filter showed strongly disorganized fibers as one continuous sheet (A). The surface was loosely intertwined to form large gaps under a microscope (B). The cellulose beads were oval with a size of approximately 0.5 mm (C). Under SEM, the cellulose beads showed a variable pore size (D, E). The surface was a rough sheet (F). The cross-section revealed cavities of various sizes from outside to inside like a honeycomb (G). At 105 days, the cellulose bead structure had decomposed (H) (Figure 4).

The cellulose beads in the final phase are shown in Figure 5. Most of the cellulose beads were saturated and they had become gelatinized and decomposed (A). High magnification revealed the diversity of the microorganisms. The intact surfaces were densely covered with filamentous microorganisms (B–E). The matrix was occupied by microorganisms of various shapes including stalk ciliates (F) as well as cocci and bacilli bacteria (D, G, H). A large number of rod-shaped bacteria were observed (G, H), in particular, within the pore surfaces (H).

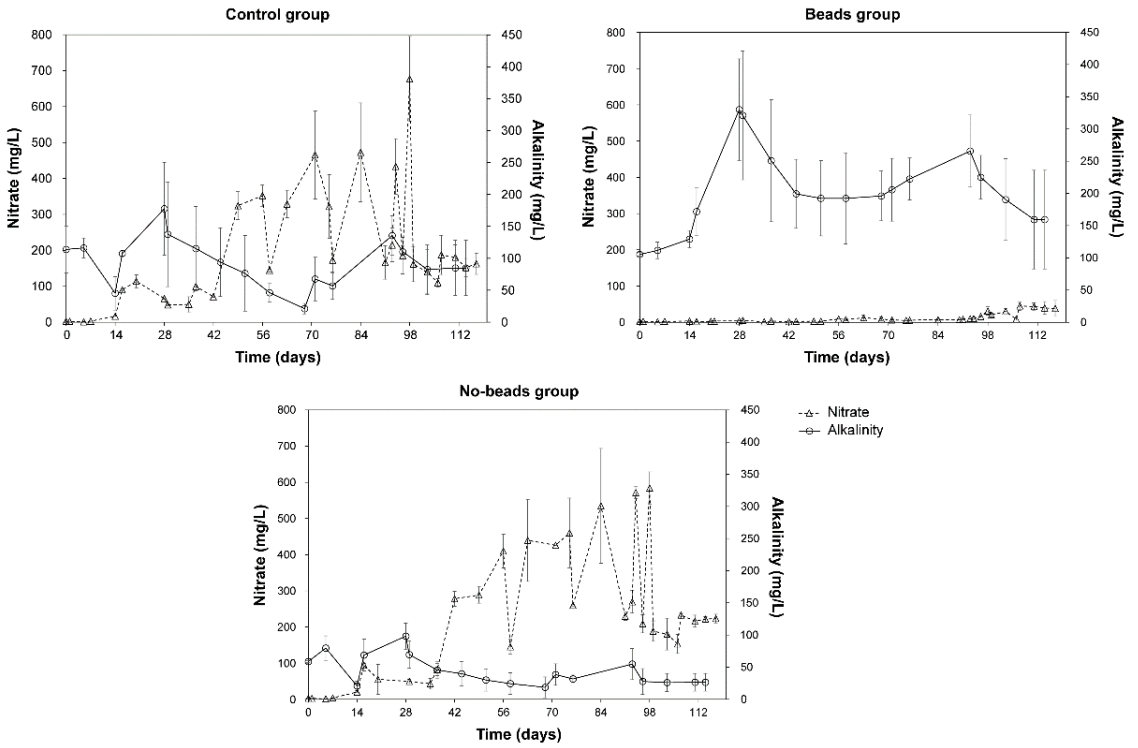

Figure 3 Nitrate and alkalinity levels in the control, bead and no-bead groups.

Table 1 Average water quality parameters in experimental tanks during the study period.

Test parameters	Average ± SD (n = 16)								
	Control			Beads			No-beads		
	1	2	3	1	2	3	1	2	3
pH	7.7 ± 0.4	7.7 ± 0.4	7.6 ± 0.4	8.0^{a} ± 0.3	8.0^{a} ± 0.2	8.0^{a} ± 0.2	7.8 ± 0.3	7.6 ± 0.4	7.5^{b} ± 0.5
Temperature (°C)	29.3^{b} ± 0.7	29.3^{b} ± 0.7	29.3^{b} ± 0.7	29.4^{a} ± 0.7	29.6^{a} ± 0.6	29.5^{ac} ± 0.6	29.4 ± 0.7	29.2 ± 0.7	29.2^{bc} ± 0.7
DO (mg/L)	5.8^{b} ± 0.4	5.9^{b} ± 0.4	5.8^{b} ± 0.5	5.0^{a} ± 0.6	5.0^{a} ± 0.5	5.1^{ac} ± 0.4	5.6 ± 0.5	5.4 ± 0.8	5.6^{bc} ± 0.5
Alkalinity (mg/L)	125^{bd} ± 54	78^{b} ± 32	77^{b} ± 54	235^{a} ± 92	222^{a} ± 62	154^{d} ± 50	100^{bcd} ± 46	71^{bc} ± 47	65^{bc} ± 44

Values with different superscript letters indicate significant difference.

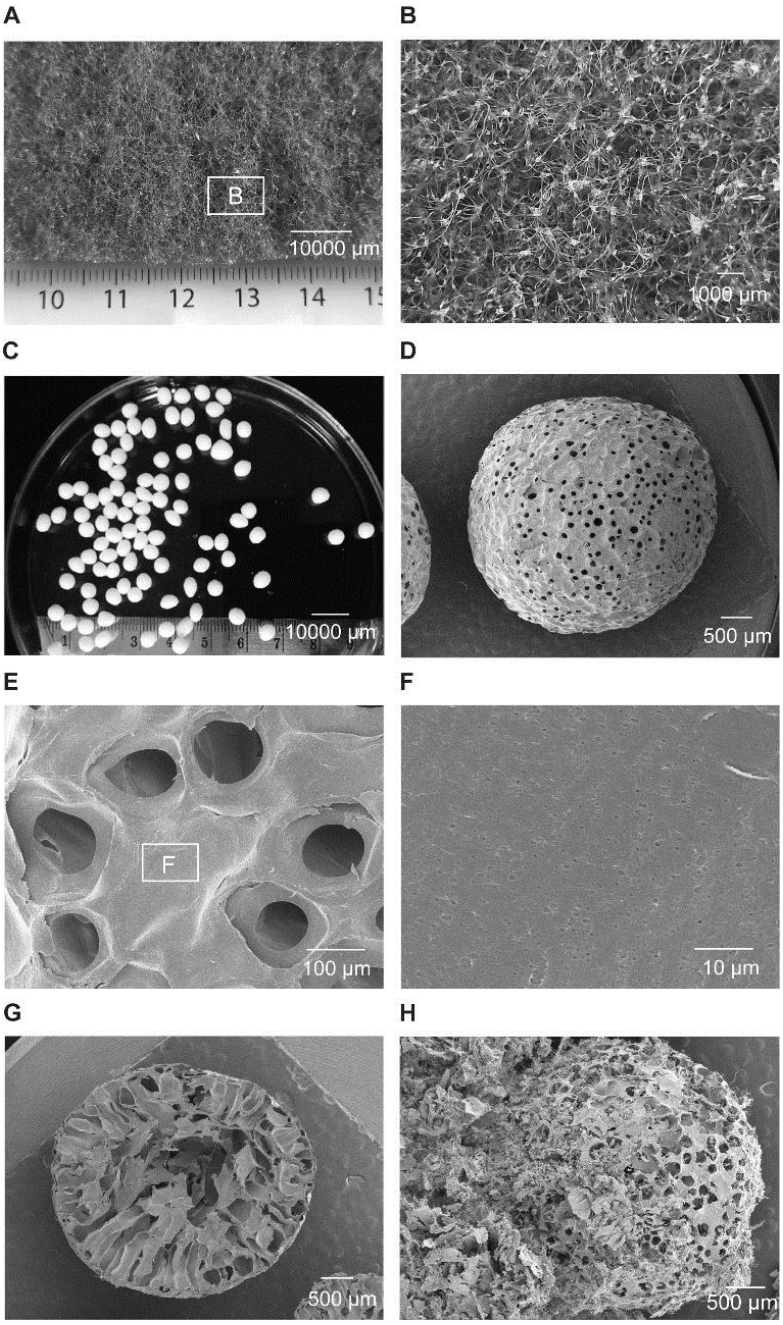

Figure 4 Textures of the sponge filter and cellulose beads. (A) Sponge filter to the naked eye and (B) under a microscope (magnified view of area B in A). (C) Cellulose beads to the naked eye and (D, E, F) under SEM. Cross-section of a cellulose bead: (G) initial and after (H) 105 days.

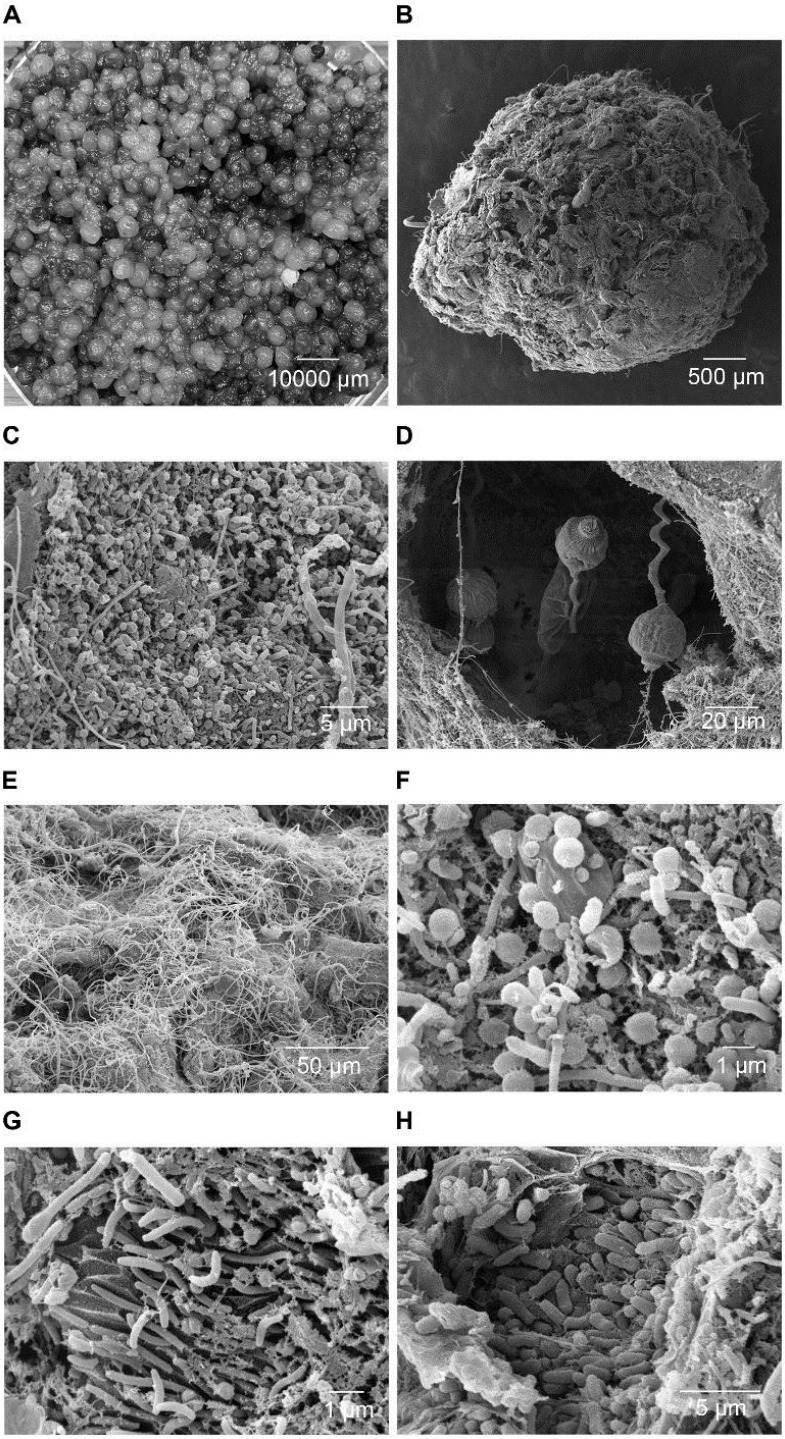

Figure 5 Cellulose beads after 105 days.

Microbial community: Microbial community analysis of the samples was performed. In total, 452 species from 320 genera of 185 families, 129 orders and 56 classes belonging to 26 bacterial phyla were identified. As shown in Figure 6, 15 major groups of bacteria were found at the phylum level, 27 groups at the class level (Figure 6A) and 27 groups at the family level (Figure 6B). Proteobacteria were the dominant phylum in all samples except FL4, for which Bacteroidota was dominant.

High bacterial abundances were detected in the cellulose beads on day 7, especially for Proteobacteria (B4: 65.54%, B5: 64.06%, B6: 79.66%). Of this, the Alphaproteobacteria class made up a much higher percentage than the Gammaproteobacteria class. The family with the highest percentage was Rhizobiaceae (B4: 29.80%, B5: 25.83%, B6: 28.20). The relatively high percentage indicated that Proteobacteria were the most significant contributors to physiological activity within the bioreactor. In contrast, the relative percentage of Proteobacteria was lower in the sponge (F7: 46.25%, F8: 41.70%, F9: 38.51%).

In the final phase, increased bacterial diversity was detected in all samples. Proteobacteria were still the most abundant in the cellulose beads and sponge (BL4: 49.77%, BL5: 30.83%, BL6: 77.25%, FL5: 30.67%, FL6: 38.76%), followed by Bacteriodota (FL4: 26.04%). At the class level, the most abundant bacteria were

Alphaproteobacteria, Gammaproteobacteria, Planctomycetes and Anaerolineae. At the family level, the most abundant bacteria were Gastranaerophilales (BL5: 12.63%), Rhodocyclaceae (BL6: 35.17%), Saprospiraceae (FL4: 24.55%), Caldilineaceae (FL5: 18.68%), Comamonadaceae (FL6: 16.85%) and unclassified family in the class Polyangia (BL4: 30.04%). Figure 7A shows that the nitrifying bacteria *Nitrosomonas* and *Nitrospira* spp. Were found in the bead group. The relative abundance of nitrifying bacteria revealed that a large number of these bacteria preferentially developed in sponge (FL) than in cellulose beads (BL). In addition, *Nitrospira* spp. appeared to be the main nitrifying microorganism in the system with a relative abundance of 2.41%, whereas *Nitrosomonas* spp. had an abundance of only 0.06% (FL5). The relative abundances of the Comamonadaceae and Rhodobacteraceae families, increased on day 117, as shown in Figures 6B and 7B.

Figure 8 shows the differences in bacterial community clusters of the groups according to beta diversity analysis based on either weighted or unweighted UniFrac distance metrics.

Fish growth and health: No mortality was observed throughout the experiment. There was no significant difference in the fish weights between groups (Table S13). The average daily weight gain and specific growth rate did not significantly differ among the groups (p = 0.989) (Table S14). Almost all fish had a good physical appearance, except for a fish in group 2 (control group, Tank no. 2) with tailfin inflammation and a fish in group 7 (no-bead group, Tank no. 7) with right pectoral fin inflammation. Both fish showed no sign of feeding distress.

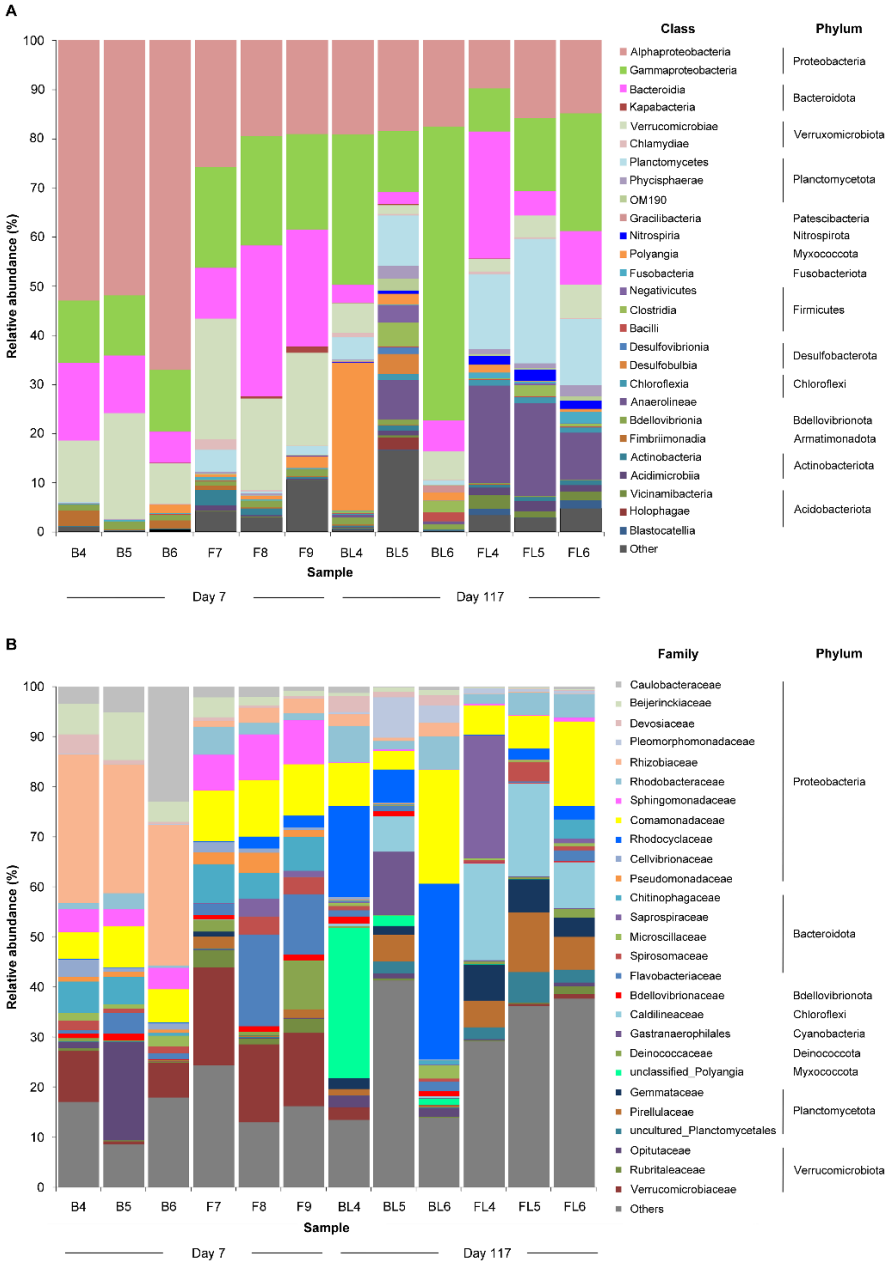

Figure 6 Relative abundances of bacterial communities in the cellulose beads and fiber in the primary phase (B4, B5, B6, F7, F8, F9) and final phase (BL4, BL5, BL6, FL7, FL8, FL9): (A) classes and (B) families.

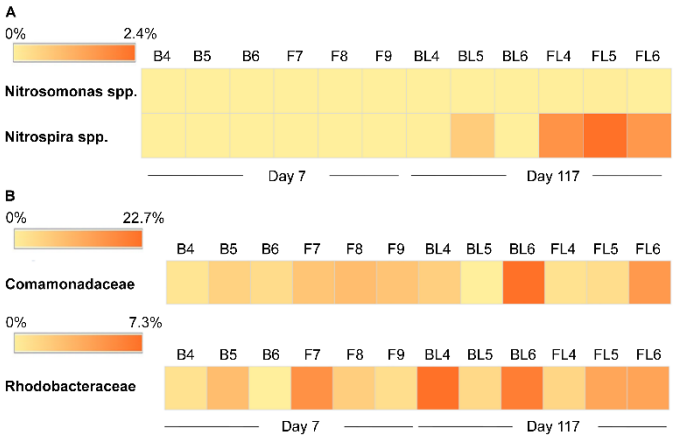

Figure 7 Heat maps of (A) *Nitrosomonas* and *Nitrospira* at the genus level and (B) Comamonadaceae and Rhodobacteraceae at the family level (B) in the cellulose beads and fiber at the primary phase (B4, B5, B6, F7, F8, F9) and final phase (BL4, BL5, BL6, FL7, FL8, FL9).

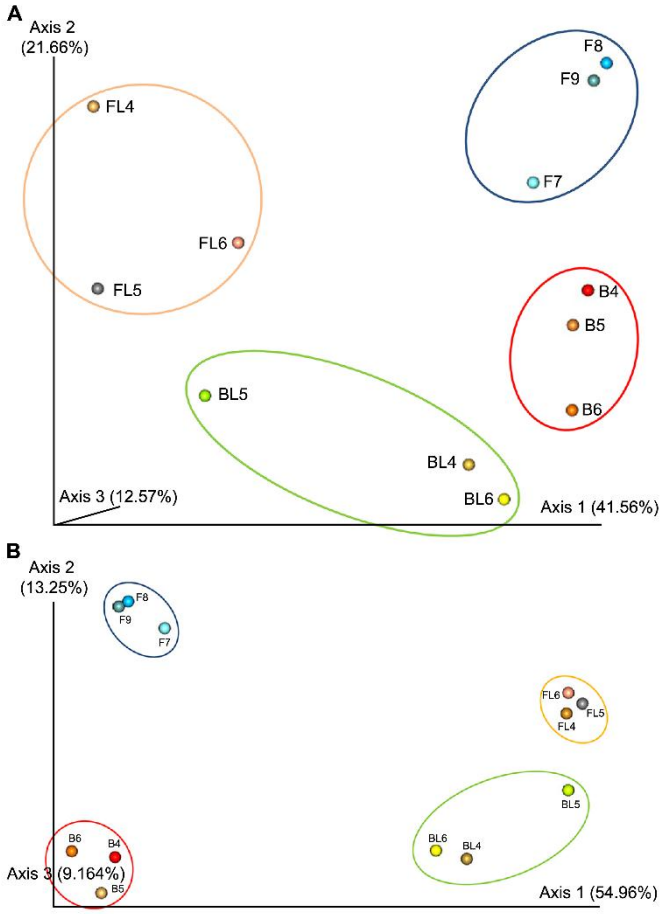

Figure 8 PCoA results for bacterial communities in the cellulose beads and fiber in the primary phase (B4, B5, B6, F7, F8, F9) and final phase (BL4, BL5, BL6, FL7, FL8, FL9): (A) weighted and (B) unweighted UniFrac distance metrics.

Discussion

Water quality: In general, optimizing the functionality of a new bioreactor requires pre-incubation for microbial growth, particularly for heterotrophs that require specific nutrient and environmental conditions (Chen *et al*., 2017; Xin *et al*., 2017; Suneethi and Joseph, 2011). For example, a sewage treatment plant with a 15 L anaerobic membrane bioreactor required 30 days of operation to establish anaerobic bacteria for ammonium oxidation (Suneethi and Joseph, 2011). A one-stage aerobic granular membrane took 40 days to grow in a laboratory-scale sequencing batch reactor treating domestic sewage after denitrifying bacteria were added to a continuous flow reactor (Xin *et al*., 2017). However, aquarium water differs from sewage in terms of quality and quantity of waste. Therefore, operation and incubation of the bioreactor in this study took place concurrently from the beginning. Interestingly, although the bioreactor was operated without incubation, the TAN level in the bead group did not rise while significant TAN peaks were

observed for the control and no-bead groups. Biofilms containing nitrifying microorganisms might gradually develop on the cellulose beads and may have contributed to this. However, in the secondary phase, biofilms successfully developed in the cellulose beads or sponge filter in all tanks. Therefore, the TAN concentration did not differ significantly among all groups. The fluctuating ammonia level in the recirculation water system was mainly related to fish waste and nitrifying bacteria in the bioreactor. The bacteria grew and multiplied in the bioreactor, so they required an appropriate attaching surface or biofloc accumulation (Besen *et al*., 2021; Pungrasmi *et al*., 2016; Ruan *et al*., 2016). The microorganisms in the no-bead group bioreactor found it difficult to grow on the loosely intertwined smooth surface and large gaps in the sponge filter. In contrast, the bead group had cellulose beads that provided a rough surface and pores. Therefore, microorganisms successfully grew from the start of the study period. They consumed inorganic nitrogen to develop; this lowered the levels of ammonia, nitrite, and nitrate in the aquarium, which remained low throughout the experiment. In the no-bead group, the ammonia and nitrite levels decreased after 14 days, which indicates that the nitrifying microorganisms were successfully established more slowly. In the control group, which contained lesser filter material for microorganisms to inhabit, the ammonia and nitrite levels remained higher than in the other groups.

A sequencing batch reactor enhances the diversity of microbial species, which include nitrifying and denitrifying bacteria (Ji *et al*., 2015). Its pattern of intermittent operation is similar to that of tidal flats in coastal ecosystems, where the supplies of DO and organic matter change daily (Miththapala, 2013). This phenomenon supports aerobic and anaerobic microbial remineralization, which is confirmed by the high numbers of microbial cells in the sediments (Schutte *et al*., 2019). To investigate the occurrence of nitrification and/or denitrification in a bioreactor, the change in alkalinity can be used as an indicator (Baikun and Irvin, 2007). Because nitrification and denitrification occur in the same tank for a single-tank sequencing batch bioreactor, the measured alkalinity is the sum of the alkalinity consumed by nitrification and generated by denitrification. Theoretically, the nitrification process consumes approximately 7.14 mg of alkalinity (as calcium carbonate) to oxidize 1 mg of ammonia (Baikun and Irvin, 2007; Lawson, 1995). Although the bioreactor was filled intermittently with bicarbonate, the control and no-bead groups showed depleted levels of alkalinity and an accumulation of nitrate because denitrification did not occur. In contrast, the bead group showed that the alkalinity did not decrease below the initial level, and no sharp increase in nitrate was observed. During denitrification, the pH value increased because of alkali formation (Ji *et al*., 2015). Therefore, the bead group had higher pH and alkalinity than the control and no bead groups, indicating that aerobic denitrification was successful in bioreactors that contained cellulose beads as the substrate; however, this was not observed in the other two groups because of a lack of substrate. However, regulation of the amount of DO is necessary for aerobic denitrification. Therefore, an air stone was used to control the DO, which may lead to non-significant differences in the DO concentration in each tank. However, the DO level was maintained at above 5 mg/L throughout the experiment, based on the optimal level required for aerobic denitrifying bacteria. Aerobic denitrification is a promising method for removing nitrogen in single-tank aerobic reactors such as continuously stirred reactors or sequential reactors (Ji *et al*., 2015).

To achieve efficient nitrification, aeration is a critical control parameter, and the addition of calcium bicarbonate (to produce alkaline conditions) (Abakari *et al*., 2020) and external carbon may be required (Perera *et al*., 2017). However, when high levels of DO are available in the bioreactor, the denitrifying microorganisms use oxygen as an electron acceptor instead of nitrates. They do not perform denitrification and the nitrates remain in the system. However, aerobic denitrification may occur when a particular substrate is provided under optimal conditions for the temperature, pH, and DO. Most denitrifying microorganisms tend to convert nitrate most efficiently at a specific DO concentration (3–5 mg/L) and temperature range (25°C–37°C).

The softened cellulose beads were turned into organic carbon sources of glucose for the denitrifying organisms attached to the sponge underneath, which contributed to aerobic denitrification. The denitrification generated alkalinity, which helped buffer and control the pH in the system. Without cellulose beads, the system lacked a carbon supply; thus, aerobic denitrification was not observed in the control and no-bead groups. Only nitrification was observed, and the alkalinity was consumed in these groups. The reduced alkalinity affected the buffering capacity, which lowered the pH of the control and no-bead groups. To maintain the function of the nitrifying microorganisms in the no-bead bioreactor over the long term, it should be supplemented with methanol and bicarbonate solution when the alkalinity is less than 100 mg/L (Pungrasmi *et al*., 2016). For the bead group, new beads should be added to the bioreactor when most of them have decomposed.

Microbial community and SEM: The microbial communities in the filter play an important role because their diversity directly affects the dynamics of organic and inorganic nitrogen in the bioreactor. The finding of Proteobacteria as the main phylum agrees with the results of many other studies. Proteobacteria and Bacteroidota are ubiquitous in soil and sludge and they dominate all community compositions (Qiulai *et al*., 2017).

Although all groups were started without pre-incubation, high abundances of bacteria were detected in the cellulose beads on day 7, especially Proteobacteria. However, the relative abundance of Proteobacteria in the cellulose beads was less on day 117 than on day 7 because of the structural changes in the cellulose beads, the changes in the carbon/nitrogen ratio in the system and the increased species-specific resource requirements (Cardona *et al*., 2016). The intermittent water flow strategy influenced the DO concentration at different heights in the bioreactor,

which can alter bacterial communities in the samples. The relative abundance of nitrifying bacteria *Nitrosomonas* and *Nitrospira* spp. revealed that most preferentially developed in the sponge than in the cellulose beads because the sponge had numerous large gaps that allowed oxygen to pass through more easily. In addition, *Nitrospira* spp. seemed to be the main nitrifying microorganism in the system with a higher relative abundance than *Nitrosomonas* spp. The relative abundance of the Comamonadaceae and Rhodobacteraceae families, which act as denitrifying bacteria, increased on day 117, which may have contributed to the decrease in the nitrate concentration for the bead group.

Weighted or unweighted UniFrac distance metrics indicated that the bacterial communities differed depending on the time and supporting material.

Aerobic denitrifying microorganisms are mainly gram-negative Proteobacteria (Adlin *et al*., 2022; Vinothkumar *et al*., 2021; Zhang *et al*., 2018; Qiulai *et al*., 2017; Ji *et al*., 2015). They tend to be most efficient at a temperature of 25°C–37°C, pH of 7–8, DO concentration of 3–5 mg/L, and C/N load ratio of 5–10 (Ji *et al*., 2015). The relative abundance of Alphaproteobacteria in the cellulose beads during the primary phase indicates that the bead group achieved successful operation without incubation. Alphaproteobacteria are one of the largest groups of bacteria and exhibit a wide spectrum of characteristics in terms of morphology (spiral, rod, stalked), metabolism (phototrophs, heterotrophs, and chemolithotrophs), physiology and cell division mechanisms (Kersters *et al*., 2006; Kainth and Gupta, 2005). They are identifiable by their very low nutrient requirements for growth. Rhizobiaceae was the most abundant bacterial family in the cellulose beads, which agrees with the bacterial populations discovered in sewage treatment ecosystems. Samples from the Feng Huang He Ergou wastewater treatment ecosystem at Chengdu, China showed a dramatic increase in the relative abundances of heterotrophic nitrifiers and aerobic denitrifiers after culturing in a medium (Yang *et al*., 2020). The bacteria in this family are diazotrophs that can fix nitrogen and are symbiotic with plant roots. Cellulose beads derived from wood pulp byproduct can increase the percentage of Alphaproteobacteria (Fu *et al*., 2022; Deng *et al*., 2018).

Many studies have indicated that the type of carbon source can affect the composition of the microbial community (Hollender *et al.*, 2002; Oehmen *et al.*, 2004). Heterotrophic microorganisms require organic carbon for cell growth during the denitrification process. Liquid and solid carbon sources have been considered to carry out nitrification and aerobic denitrification simultaneously. Solid carbon sources are more advantageous because they act not only as electron donors but also as a substrate for biomass growth. Solid carbon sources must be insoluble and biodegradable in nature, and natural and synthetic examples include woodchips, leaf compost, rice straw, polylactic acid, and polybutylene succinate (Fu *et al*., 2022; Rajta *et al*., 2020; Ruan *et al*., 2016). As shown in Figure 5, high magnification of the cellulose beads showed the diversity of the microorganisms. The intact outer surface was covered with filamentous microorganisms that stuck together until almost no gaps were left. The cellulose matrix was occupied by microorganisms of various shapes, including stalk ciliates and bacteria. Within the pore surface, a large number of rod-shaped bacteria were found. However, the bioreactor should be cleaned and new beads be added after 105 days of operation because of the formation of a slime-like material. A wide range of bacteria including *Acetobacter, Pseudomonas, Achromobacter, Alcaligenes, Aerobacter, Azotobacter, Agrobacterium* and *Rhizobium* species produce cellulose extracellular polymer product (Miqueleto *et al.*, 2010). Slime-forming organisms are predominantly aerobic and their oxygen consumption rate is related to the DO concentration of the water (Gray, 1985). In the present study, partial slime clogging in the U-shaped pipelines was observed for the bead group on day 105. Although the pipes were not washed, the bioreactor maintained good functionality until the end of the testing period, at which point that nitrate concentration gradually increased. The water became yellowish, which made it challenging to observe fish health, so the experiment was stopped on day 117.

Fish growth and health: The control, bead and the no-bead groups did not appear to affect the average daily weight gain and specific growth rate. These results agreed with previous work showing that goldfish fed with rations close to maintenance requirements have specific growth rates of 0.443%–0.499% and feed conversion ratios of 2.49–2.83 (Priestley *et al*., 2006). However, the slightly larger fish in the bead group may have resulted in lower dissolved oxygen concentrations in the tank than in the other tanks. In this study, the goldfish were fed shrimp pellets because of their excellent palatability and high stability in water.

The laboratory-scale single-tank sequencing batch bioreactor using a cellulose substrate in this study can support 0.3 fish/L, compared with the industry standard of 0.25 fish/L (Asano *et al*., 2003). Juvenile goldfish should be kept at 0.5 fish/L to maintain good water quality (Shete *et al*., 2013). Goldfish larviculture in a biofloc system proved to have the best results by supporting 10 fish/L (Besen *et al*., 2021). Long-term operation of a recirculation water system can form large amounts of aerobic granular sludge with a high specific gravity, which will clog filters and block the outlet. This is one of the problems that limit the use and promotion of water recirculation systems (Adlin *et al*., 2022). The clogging causes anoxic conditions and filtration system failure and affects fish health. However, a sequencing batch bioreactor prolongs the operation of the filtration system before clogging, helps flush the water outlet, and prevents anoxic conditions because of the intermittent water flow.

In conclusion, the results of this study showed that a single-tank sequencing batch bioreactor with cellulose beads can achieve simultaneous nitrification and aerobic denitrification and maintain good water quality, which are conducive to long term ornamental fish care. Beta diversity analysis identified differences in bacterial communities between groups. Proteobacteria was the dominant phylum in all samples. The results of this study can be used to realize

a practical nitrogen removal system for freshwater aquariums. This will be of great benefit to the aquaculture and fish industries, and the technology presented in this study is applicable everywhere, including areas with water shortages.

Although the bioreactor in this experiment was cheap and easy to use, it was too small and its prolonged use created problems related to high organic load. Therefore, further studies should be performed with bioreactors having a higher capacity to determine if applying sludge removal, cellulose bead reapplication and slime clogging prevention are able to extend the usage time.

Declaration of conflicting interests: The authors declare that there is no conflict of interest.

Data availability: The dataset generated in the experiment is available within the manuscript or in the supplementary information.

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