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Application of flow airlift fluidized bed (CAFB) - Aerobic denitrification bioreactor using polycaprolactone (PADR) as organic carbon source and biofilm carrier for synthetic marine aquaculture wastewater treatment

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ABSTRACT

Aerobic denitrification, a novel method for complete nitrate removal process, relies on a constant carbon supply, and carbon availability can be a limiting factor for the process. Three biodegradable polymers - polybutylene succinate (PBS), polycaprolactone (PCL), and polylactic acid (PLA) were used as carbon source of aerobic denitrifying bacteria, *Halomonas venusta* for treating recirculating aquaculture wastewater in batch tests, respectively. The group utilizing PCL displayed the greatest efficiency in nitrate removal. Afterwards, a continuous flow airlift fluidized bed (CAFB) - aerobic denitrification bioreactor using PCL(PADR) as bio-carriers and carbon source was started up. The effect of shifting temperature on the nitrate removal and microbial community of CAFB-PADR was investigated under different hydraulic retention times (HRT). The results showed that no significant difference was detected in denitrification activity at 25 and 30 °C condition, where the denitrification rate was 1.1–1.8 times higher than that at 15 °C. However, the nitrogen removal efficiency was above 50 % and there was barely accumulation of nitrite at 15 °C, indicating the good nitrate removal performance in CAFB-PADR at all three temperature conditions. Microbial composition analyses revealed that as the temperature decreased, the relative abundance of Gammaproteobacteria increased, while those of Alphaproteobacteria and Bacteroidia diminished decreased. The existence of denitrifying function genera (*Marinobacter*, *Pseudomonas*, *Halomonas* and *Hydrogenophaga*) ensured the rapid removal of nitrogen in the biofilter. When the temperature dropped to 15 °C, *Pseudomonas* progressively took the position of *Marinobacter*, both of which had denitrification and degradation activities. The relative abundance of the denitrifying bacteria *Halomonas* that we inoculated has been less than 1 % since phase II. Overall, the PCL-supported CAFB-PADR demonstrated in this study shows potential for removing high concentrations of nitrate from recirculating marine aquaculture wastewater.

1. Introduction

Nitrification biological filters are generally used in recirculating aquaculture systems (RASs) to oxidize total ammonia nitrogen (TAN) to nitrite-N and onward to nitrate-N, as nitrate-N is comparatively innocuous to aquatic organisms (van Rijn, 2013; Zhu et al., 2015; Luo et al., 2016). However, a comprehensive literature reveals that high levels of nitrate-N can have negative effects upon cultured species (Martins et al., 2009; Davidson et al., 2011). For example, Learmonth and Carvalho (2015) showed that 400 mg/L nitrate-N stress led to lower survival and growth rates for juvenile zebrafish *Danio rerio*. Juvenile spotted knifejaw *Oplegnathus punctatus* showed higher mortality and decreased specific growth rate under a 299 mg/L nitrate-N exposure (Yang et al., 2019). Among many negative impacts of nitrate-N are tissue damage, interference with maturation, and abnormal skin coloration (Good et al., 2017; McGurk et al., 2006; Rodrigues et al., 2011). Accumulation of

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400–500 mg/L nitrate-N can occur in RASs due to the lack nitrate removal units, thereby limiting water reuse (Yang et al., 2019). Therefore, it is important to improve technologies to control nitrate-N concentration in RASs. Increasing the water exchange rate is commonly used to maintain the nitrate concentration in RAS, however, that allows for more intaking water (Stevčić et al., 2019).

An alternative is anaerobic denitrification, which is considered the most economically viable and efficient methods of nitrate-N removal from municipal wastewater (Pan et al., 2015). However, it is really costly to perform the anaerobic denitrification in RAS, as we need to remove the dissolved oxygen for denitrification process and then increase the dissolved oxygen again before the water flow back to the culture tank. Nowadays, more and more aerobic denitrifier bacteria were isolated and applied for treating municipal wastewater(Kosar et al., 2023), landfill leachate wastewater(Saxena et al., 2022) and aquaculture wastewater(Gao et al., 2018). In previous study, we isolated an aerobic denitrifier from fish farm, *Halomonas venusta* (Chen et al., 2018b), which exhibited aerobic denitrification capabilities feeding with liquid carbon source (Jiang et al., 2018; Song et al., 2020). However, adding liquid carbon source to the RAS will increase the COD rapidly, might cause other uncertain problems. So, it would be great if we can find some biodegradable solid carbon source. Biodegradable polymers (BDPs), have been developed to provide a solid carbon source and biofilm carrier for anaerobic denitrification, include polyhydroxybutyrate (PHB) (Gutierrez-Wing and Malone, 2006), polycaprolactone (PCL) (Boley et al., 2003), polylactic acid (PLA) (Shen et al., 2013), and poly (butylene succinate) (PBS) (Zhou et al., 2006). By releasing organic carbon constantly and automatically, such polymers might provide a suitable electron donor for aerobic denitrification biofilter in RAS (Zhu et al., 2015).

With the rapid growth of the marine RAS industry, it is important to assess the feasibility of utilizing biodegradable polymers for electron donors and microorganism attachment in saltwater denitrification biofilters. We assessed the performance of three BDPs (polybutylene succinate – PBS, polycaprolactone – PCL, and polylactic acid – PLA) for supporting aerobic denitrification bacteria for treating synthetic RAS wastewater in flask batch tests. PLA, PCL, and PBS were chosen due to their biodegradability and cost-effectiveness (Wang and Chu, 2016), which cause a lower cost for nitrogen removal. After observing that PCL best supported denitrification, we more intensively assessed its utility for treating simulated recirculating marine aquaculture system effluent by evaluating nitrate removal performance and effluent quality of an aerobic-denitrification reactor employing PCL under different temperatures (T). Temporal changes in PCL structure were analyzed with multiple scanning electron microscopy (SEM) images to assess microbial growth and degradation of the PCL. We employed *16S rRNA* bacterial typing to achieve a detailed characterization of the microbial community structure in the aerobic-denitrification reactor. The purpose of this research is to investigate the ability of bacteria to utilize biodegradable polymers for denitrification and to analyze the underlying microbial mechanisms involved in this aerobic denitrification process for removing nitrate from recirculating aquaculture environment.

2. Materials and methods

2.1. Materials

The PBS, PCL and PLA biopolymers used in this study were from the Esun New Material Company (Shenzhen, China). Table S1 shows the physical characteristics of the PBS, PCL and PLA carriers.

Halomonas venusta (Huang et al., 2020) was pre-activated for 48 h at 25 °C and 180 rpm/min, and then inoculated at a volume ratio of 10 %. The inoculation concentration was about 3×10^{10} CFU/mL.

The initial wastewater was from a commercial recirculating marine aquaculture system at Laizhou Mingbo Aquatic Co., Ltd. (Laizhou, China) and the TAN, nitrite-N and nitrate-N concentration of initial wastewater quality was 0.23 ± 0.18, 0.35 ± 0.13 and 10.49 ± 4.23 mg/L. The synthetic wastewater was prepared by dissolving $NaNO_3$ (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) to initial wastewater to produce 50 mg/L and 30 mg/L nitrate-N for trials 1 and 2, respectively. The sum of TAN and nitrite-N concentration in synthetic wastewater was less than 1 mg/L. The salinity and pH were approximately 35 ‰ and 7.9, respectively.

2.2. Batch tests (trial 1)

The batch experiments were conducted in triplicate for each biopolymer carrier in 250 ml Erlenmeyer flasks for 9 days. Each flask contained 35 g biodegradable carrier and 150 mL synthetic wastewater with 50.4 ± 2.73 mg/L nitrate–N (supplied as $NaNO_3$), and were seeded with denitrifying bacterium, *Halomonas venusta* (Huang et al., 2020). The nine flasks were positioned in a thermostatic shaking incubator set at 25.0 °C and 180 rpm. Every 24 h, samples of supernatant water were taken and TAN, nitrite-N, nitrate-N, and total nitrogen were measured immediately as described below.

2.3. CAFB-PADR test (trial 2)

The CAFB-PADR test was conducted in a plexiglass cylindrical bucket (32-cm height, 15-cm diameter) with a working volume of 4.7 L (Fig. S1). The reactor was filled to a depth of 5 cm with 560 g of PCL pellets as both biofilm carrier and carbon source. The reactor was seeded with *Halomonas venusta* (Huang et al., 2020) at 20 % *v/v* with 50 mg/L nitrate-N for 3 days at a temperature of 25 °C. After rinsing the reactor with seawater, synthetic aquaculture wastewater (30 ± 2.73 mg/L nitrate–N) was continuously fed into the bottom of the bioreactor with a peristaltic pump and discharged as overflow. Different temperature (25, 30 and 15 °C) and HRT (4, 6, 8 and10 h) conditions were applied in a series of experimental phases (Table 1). When the effluent nitrate-N concentration for a particular experimental phase (i.e., a particular combination of HRT and T) proved stable (±20 %) for at least for 3 days, the operational mode was changed to meet the requirements of the following experimental phase. Treatment performance was monitored by following TAN, nitrite-N, nitrate-N, total nitrogen and COD concentrations. T, DO, salinity and pH values in the effluent and influent were measured every other day. Dissolved oxygen (DO) was controlled at approximately 6.5 mg/L by an air compressor (V-30, Hailea, Guangdong, China). The pH value was maintained at about 7.8, and salinity was around 35 ‰. A constant temperature was maintained by a water bath.

The volumetric nitrate-N conversion rate (*VRT*; $mg{\cdot}N{\cdot}m^{-3}{\cdot}d^{-1}$), influent nitrate loading rate (*NLR*; $g{\cdot}N{\cdot}L^{-1}{\cdot}d^{-1}$) and nitrate-N removal efficiency (*NRE*; %) were calculated according to Colt et al. (2006), Xu et al. (2018c) and Jiang et al. (2021).

Table 1
Operational parameters for the respective experimental phases assessing the denitrification performance of polycaprolactone. In names for experimental phases, T refers to temperature and HRT to hydraulic retention time.

Phase	HRT (h)	Running time (d)	*Q* (ml/min)
I (*T* = 25 °C)	4	1–30	19.58
	6	31–54	13.06
II (T = 25 °C)	8	55–74	9.79
	10	75–92	7.83
	10	93–112	7.83
III (*T* = 30 °C)	8	113–128	9.79
	6	129–146	13.06
	6	147–164	13.06
IV (T = 15 °C)	8	165–178	9.79
	10	173–193	7.83

2.4. Water sample and quality analysis

TAN, nitrite-N, nitrate-N and total nitrogen concentrations were assayed according to APHA et al. (2005). TOC was performed by TOC analyzer (TOC ASI-L, Shimadzu, Japan). COD was determined via the Chinese SEPA Standard Methods (SEPA, 2002). The DO and pH values were detected by a YSI 85 probe (DO200, YSI, Inc., Yellow Springs, OH, USA) and a pH 100 m (YSI, Inc.).

2.5. Scanning electron microscope (SEM) images of PCL

5 g of PCL medium was sampled at the 30th and 74th days and first prepared in 2.5 % glutaraldehyde for over 4 h and then washed using phosphate buffer. The pellets were dehydrated and transferred according to Chu and Wang (2013). Samples then were dried at 80 °C with liquid CO_2 and scanned in a Philips Model TM-1000 scanning electron microscope.

2.6. Microbial diversity analysis

About 20 g of polycaprolactone (PCL) with biofilm was sampled from the bottom of the bioreactors at the end of the phases. The biomass sample were stored at −80 °C before DNA extraction. The samples were sliced and centrifuged as in Huang et al. (2016) and then submitted to Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The V3-V4 region of the bacterial *16S rRNA* gene was amplified and sequenced according to Lin et al. (2022). For high-throughput sequencing, the PCR products were purified and were performed on a MiSeq platform (Illumina, San Diego, CA). The sequencing data have been uploaded to the NCBI GenBank archive (accession no. PRJNA419809).

In bioinformatic analysis, DNA sequence reads (range 26,336–41,687 per treatment) were normalized to the 26,336 for further analysis (Fig. 4). The alpha diversity indices were computed with Mothur v.1.30.2 (Schloss et al., 2009; Schloss, 2020). Nonmetric multidimensional scaling (NMDS) grounded on the Bray-Curtis distance among OTUs was carried out and visualized using the *vegan* package in R (v. 3.5.1) to illustrate the bacterial community composition similarities across experimental phases (Zhou et al., 2017). The sample sequences were imputed and the module abundance predicted with Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Yin and Wang, 2021). The abundance was estimated with an accuracy of 0.8. The closed OTU table was normalized by *16S rRNA* gene copy number prior to the PICRUSt analysis.

2.7. Statistical analyses

Results of assays are presented as mean ± standard deviation. ANOVA WAS applied to calculate statistics, analyze data and assess the significance of diffrences (P-value <0.05) by SPSS v. 22.0 (IBM Corporation, Armonk, NY, USA).

3. Results

3.1. Denitrification ability of polymers (trial 1)

The TOC average concentration by PCL, PBS and PLA were 44.82 ± 1.78 mg/L g, 31.23 ± 3.25 mg/L g and 23.17 ± 3.88 mg/L g. Flask batch test results showed that the nitrate concentrations in the PCL-supported denitrification process rapidly decreased from 50.4 ± 2.73 mg/L to 1.0 ± 0.4 mg/L after 5 days (Fig. 1). Meanwhile, the average total nitrogen removal efficiency of PCL (98.2 ± 0.8 %) was higher than those of PBS (44.4 ± 4.0 %) and PLA (7.1 ± 1.5 %). The amount of nitrite-N in the nine reactors was negligible; however, TAN accumulation (>2 mg/L) in the flasks with PBS (day 6) and PLA (day 2) meant the dissimilatory nitrate reduction to ammonia (DNRA) occurred. The final nitrate concentration in the PLA-supported denitrification reactors was reduced from 50.4 ± 2.73 to 49.6 ± 4.0 mg/L, indicating that PLA did not serve as an effective carbon source for the denitrifier, *Halomonas venusta*. The nitrate concentration of flasks filled with PBS decreased only to 25 mg/L, suggesting that PBS might not provide suitable carbon for denitrification.

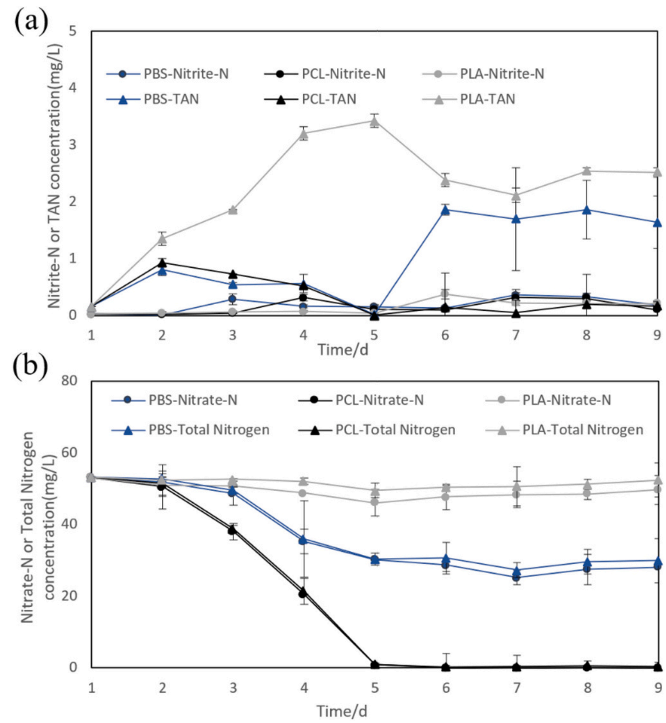

Fig. 1. Denitrification ability of polybutylene succinate (PBS), polycaprolactone (PCL), and polylactic acid (PLA) assessed in terms of dynamics of: TAN and nitrite-N, nitrate-N and total nitrogen.

3.2. Performance and microbial community of reactor (trial 2)

3.2.1. Performance for nitrogen removal

Water quality parameters, including pH (7.7 ± 0.2), dissolved oxygen (6.19 ± 0.24 mg/L), and salinity (33.1 ± 0.7 ‰), remained within acceptable ranges in all CAFB-PADR test treatments. During phase I (start-up), the effluent nitrate concentrations showed fluctuation, and then were stable from days 17 to day 29 (Fig. 2). However, the nitrate removal efficiency (NRE) was quite low, approximately 26.7 %. Obvious accumulation of TAN and nitrite-N was observed for the first 17 days, probably due to the process of DNRA. Afterwards, the effluent TAN and nitrite-N concentrations were under 1 mg/L.

From day 31 (phase II), the HRTs were increased from 6 h to 10 h at a constant temperature of 25 °C. The outlet concentrations of nitrate-N and total nitrogen were quite stable at each HRT condition. The NREs significantly (P < 0.05) increased, and were 61.2 %, 80.6 % and 91.93 % for HRTs of 6 h, 8 h and 10 h, respectively (Table S2).

The operating temperature was slightly increased from 25 °C to 30 °C for Phase III; meanwhile, the 3 HRTs (6 h, 8 h and 10 h) were applied again to determine NRE and VRT. The results showed that the highest NRE (93.5 %) was attained with an HRT of 10 at a temperature of 30 °C. There were no significant (P > 0.05) differences of outlet nitrate-N concentrations at the same HRT between Phases II and III. Hence, the denitrification rates of the reactor did not change under the same HRT when the temperature shifted from 25 °C to 30 °C.

The operation temperature was sharply decreased from 30 °C to 15 °C at day 147 and kept constant during Phase IV (days 147 to 195). The low temperature greatly influenced the efficiency of nitrogen removal. After decreasing the temperature to 15 °C, the effluent nitrate-

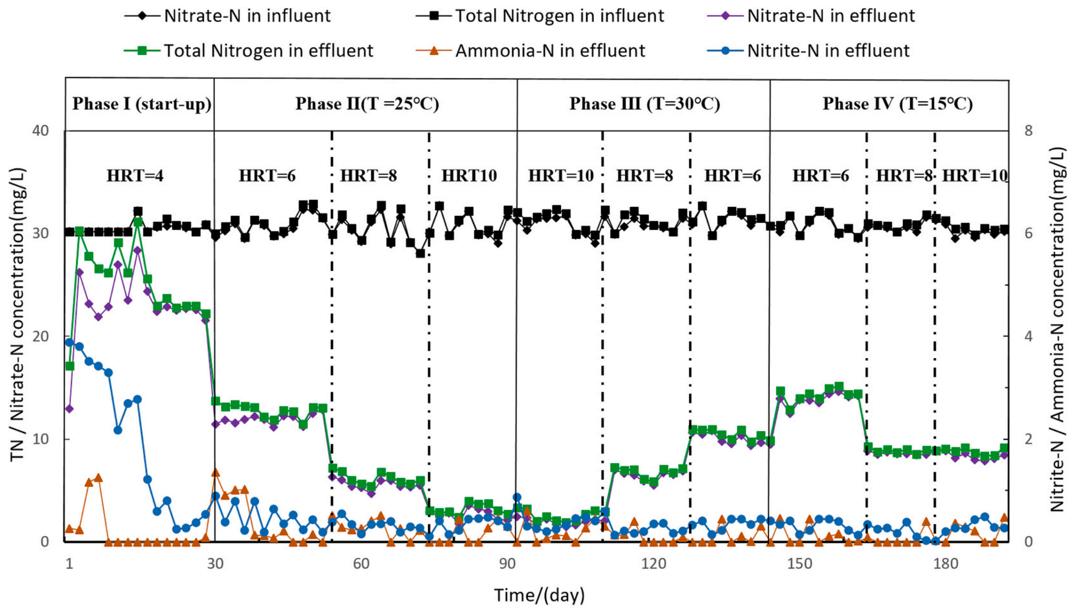

Fig. 2. Long-term nitrogen removal performance of the denitrification reactor using polycaprolactone with different combinations of hydraulic retention time (HRTs) and temperature.

N increased to 14.34 mg/L with a low NRE (52.9 %) and VRT (775.4 mg·N·m^{-3}·d^{-1}) for an HRT of 6 h when the microorganisms could not adapt to the low-temperature environment. The effluent quality recovered gradually by extending HRT to 8 and 10 h, and the average nitrate-N concentration in the effluent dropped to about 8 mg/L. During Phase IV, the low nitrite-N and TAN concentrations remained under 0.54 and 0.45 mg/L, respectively.

3.2.2. Morphology of biofilm carrier

SEM was used to observe the morphology of the biofilm carrier (PCL) at Phases I and II. Observation showed that the appearance of the PCL was comparatively smooth with minor protuberances during the startup (Fig. 3a, b, phase I, day 30). In contrast, SEM images of the carrier surface during phase II (day 74) exhibited obvious roughness and valleys, indicating that the PCL continuously eroded due to microbial activity and carbon release (Fig. 3d, e). Interestingly, more numerous and larger rod-shaped bacteria were observed at day 30 (Fig. 3c) compared to day 74 (Fig. 3f) at the higher magnification (1000×).

3.2.3. Microbial community analysis of the CAFB-PADR

3.2.3.1. Alpha diversity. The ACE and Chao 1 indices showed the richness of the microbial community, and the Simpson and Shannon indices manifested the diversity and evenness of the microbial community. As shown in Fig. 4, the ACE and Chao 1 indices rose from Phase II to Phase III, and then decreased from Phase III to Phase IV, indicating that community richness was positively affected by the higher operating temperature of the biofilter, and also indicating that the biofilm attached to the PCL carrier exhibited greater community richness and diversity at the highest temperature (30 °C). The Shannon index increased, while the Simpson index decreased significantly from Phase II to Phase III ($P < 0.05$), indicating increased diversity and evenness of the community when the temperature was elevated from 25 °C to 30 °C. From Phase III to Phase IV, the Shannon index decreased and the Simpson index increased. There was a significant difference between Phase II ($T = 25$ °C) from Phase IV ($T = 15$ °C) for the Shannon index ($P = 0.0221$); however, the Simpson index at 15 °C was significantly higher than at 25 °C ($P = 0.032$).

3.2.3.2. Dynamics of the microbial community. The composition of the microbial community in the denitrification bioreactor was analyzed to gain insight into the denitrification process and hopefully to provide a foundation for understanding the dynamics of microbial nitrate removal. Gammaproteobacteria, Alphaproteobacteria, Bacteroidia, Gracilibacteria and Deltaproteobacteria were the five dominant classes in the CAFB-PADR denitrification bioreactor in the respective experimental phases, constituting >90 % of the microbial community (Fig. 5a); however, their relative abundances dramatically changed among phases. The abundances of Gammaproteobacteria varied from 39.3 % to 57.6 % to 38.6 % and 72.7 %, respectively, from phase I to phase IV. In contrast, the abundances of Bacteroidia gradually decreased from 22.7 %, 18.2 %, and 14.0 % to 4.9 %. The changes in relative abundance did not always follow the changes of temperature. The bacterial community of phase I (start-up period) showed relative abundances different from other phases; e.g., the abundances of Anaerolineae and Mollicutes were 3.7 % and 3.1 %, respectively, and rather low in the other phases (1.6 ± 0.3 % and 0.1 %).

The genus-level distributions of relative abundance in the bacterial community are shown in Fig. 5b. The most dominant genera among the four phases were *Pseudomonas* (17.8 ± 3.6 %), *Marinobacter* (16.5 ± 2.9 %), *Simplicispira* (11.4 ± 3.6 %), genera belonging to Family Rhodobacteraceae (9.0 ± 2.7 %), *Vitellibacter* (4.3 ± 1.9 %) and *Muricauda* (4.1 ± 1.5 %). The denitrifying bacterium *Halomonas* that we inoculated into the biofilter was common only in phase I (around 5.1 %), and then was not relatively abundant in the remaining phases (all below 1 %).

Using non-metric multidimensional scaling (NMDS) (Fig. 5c) analysis and cluster analysis as shown in a heatmap (Fig. 5b), the bacterial community was clearly divided into four clusters based on the different temperatures related to the respective phases, indicating that temperature had a greater effect upon denitrifying community structure than did hydraulic retention time (HRT). As mentioned in Fig. 6, the similarity of microorganisms across phase I and II was only 41.5 %. However, the microbial community composition similarity was above 50 % over the last three phases, illustrating the development and maturation of biofilms. At 25 °C, the microbial community composition similarity for biofilter biofilm was greatest(about 79.2 %) when HRT was 8 h and 10 h. The microbial community in phase IV was barely changed(similarity >78 %), suggesting the importance of environment temperature.

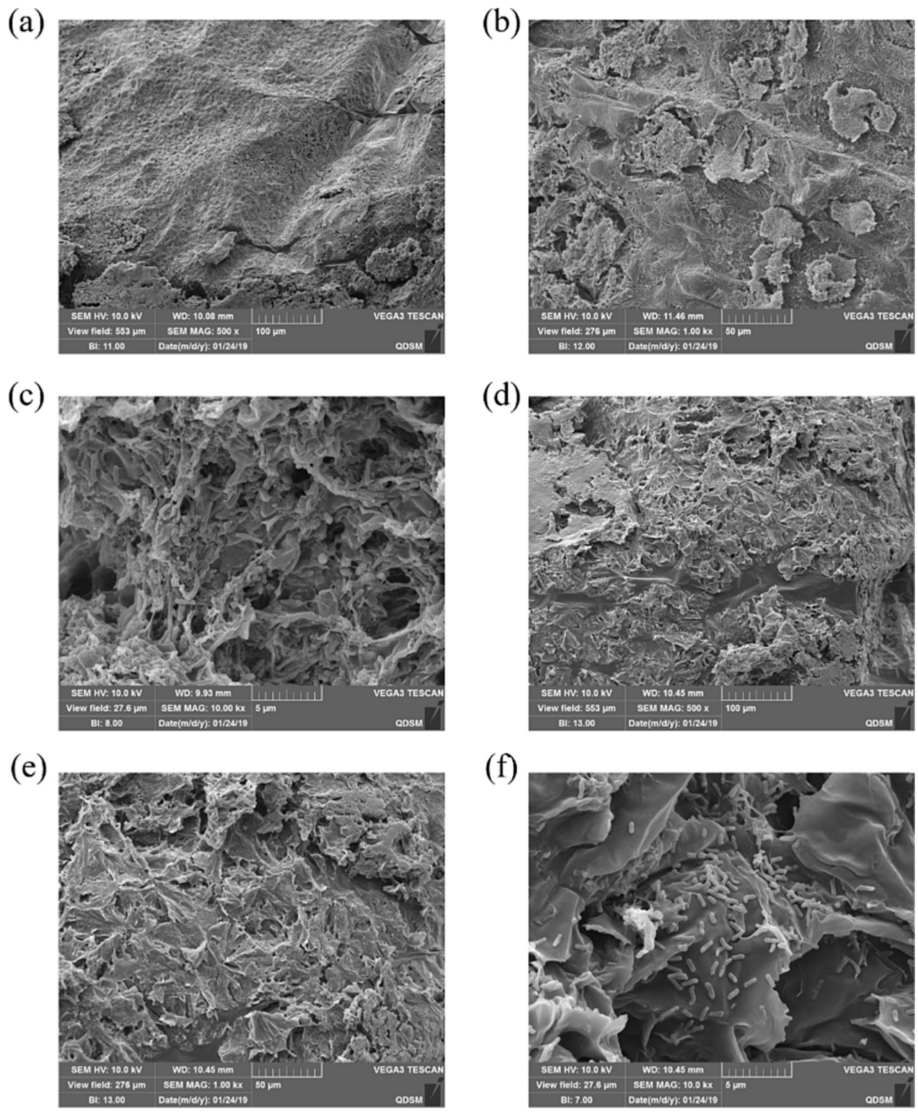

Fig. 3. Surface morphology and elemental composition of the biofilm carrier (PCL): (a) PhaseIwith magnification of 500×, (b) Phase Iwith magnification of 1000 ×, (c) Phase I with magnification of 10,000 ×, (d) Phase IIwith magnification of 500×, (e) Phase II with magnification of 10,000 ×, (f) Phase IIwith magnification of 10,000 ×, using SEM with an acceleration voltage of 10 kV.

3.2.3.3. Effect of different temperature on KEGG module involved in nitrogen metabolism. A search in the KEGG Pathway Database (http://www.kegg.jp/kegg-bin/show_pathway?map00910) showed the activities of various nitrogen prediction modules in the reactor. Regarding the nitrogen cycle, the modules with greatest gene prediction were denitrification M00529 and dissimilatory nitrate reduction M00530. The module with lowest gene prediction was nitrification M00528 (Fig. 7). The denitrification module for nitrate reductases (M00529) was analyzed, and the abundance values for the denitrification module were highest in phase III. This result illustrated that high relative abundance of denitrifying bacteria caused the high denitrification rate (average above 81.2 %) observed. Meanwhile, we observed that dissimilatory nitrate reduction (DNRA) was higher (M00530) as the temperature decreased. In addition, low M00528 abundance further suggested that autotrophic nitrification was not ongoing in our reactor, the same result observed by Ruan et al. (2016).

4. Discussion

4.1. Nitrogen removal performance analysis

The denitrification performance of PBS-, PCL- and PLA-supported marine water filtration systems was assessed in our preliminary experiment. The average total nitrogen removal efficiency for PCL was higher than for PBS and PLA polymers. Hiraishi and Khan (2003) compared polymers biodegradability and indicated that PCL was degraded much faster than other polymers; PLA and PBS were quite hard to degrade in denitrifying environment. Xu and Chai (2017) indicated that PLA could not attach denitrifying bacteria to form biofilms. Obaja et al. (2005) found that carbon sources would significantly impact on nitrate removal and intermediate production. Our results support the view that it is critical to choose a suitable carbon source for achieving an effective aerobic denitrification process.

4.2. Process performance

The nitrogen removal efficiency of a PCL-supported aerobic marine recirculating aquaculture system biofilter under different HRTs and

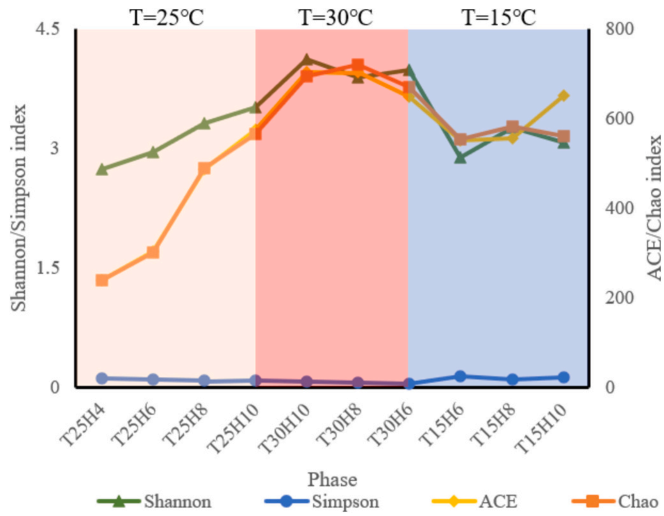

Fig. 4. Richness and diversity indices during different experimental phases. *Different superscripts within a column denote significant differences ($P < 0.05$) among groups based on one-way ANOVA.

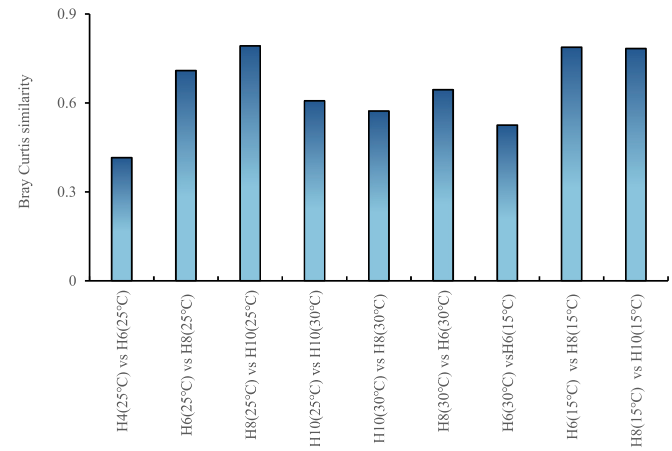

Fig. 6. Moving window analysis for comparing microbial community composition of one sampling time to the following sampling time, based on average Bray-Curtis similarity for biofilter biofilm.

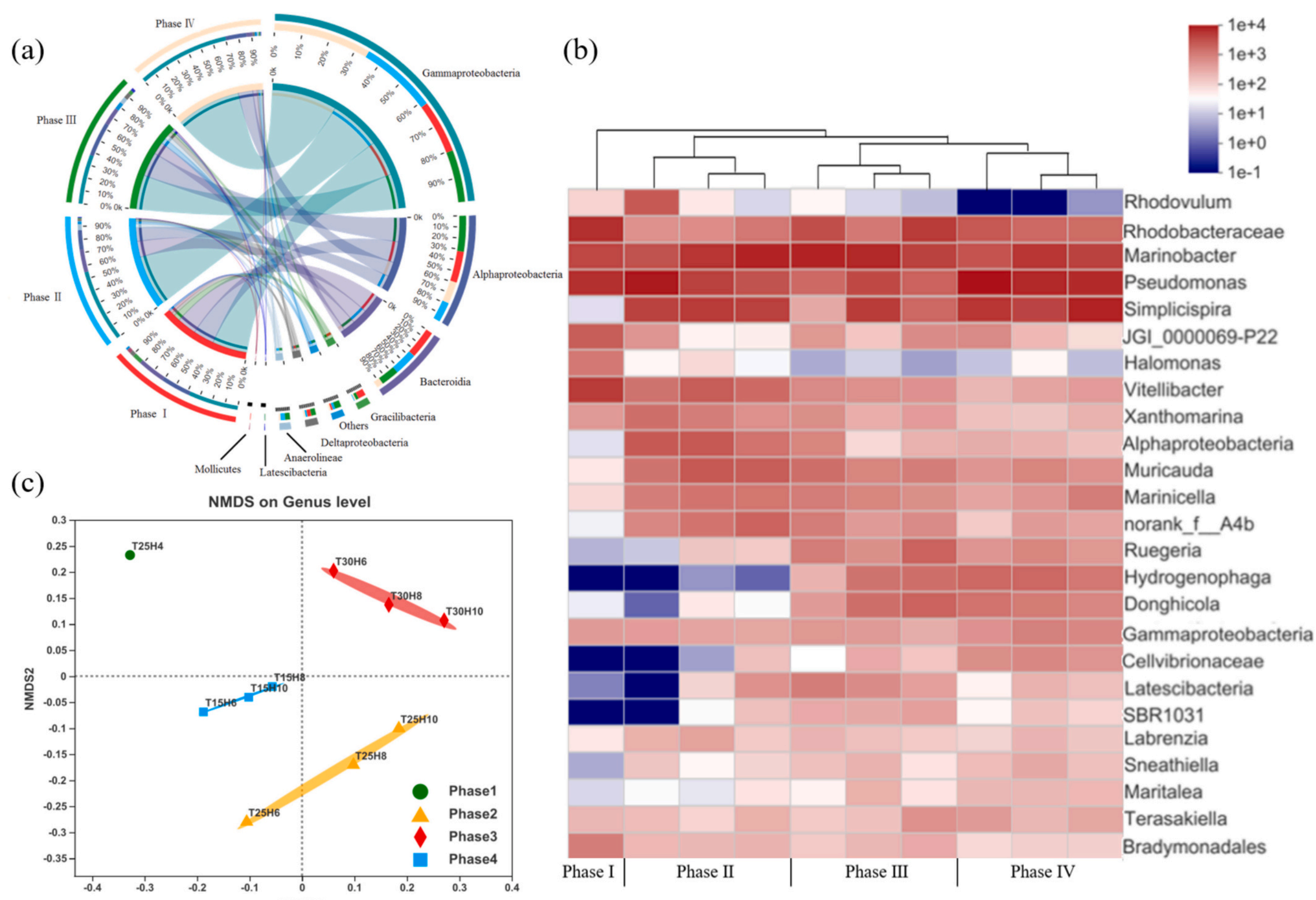

Fig. 5. (a) Distribution of microbial community for phases at the class level. The data were visualized by Circos (Krzywinski et al., 2009). The length of the inner segment and the outer segment represent the read abundance and relative abundance of each taxon, respectively; (b) heatmap of the top 25 genera in each sample, showing their relative abundance in microbial communities among phases. Hierarchical clustering of the bacterial community was performed among the phases. The color code indicates relative abundance, ranging from blue (low abundance) to white to red (high abundance). (c) Non-metric multidimensional scaling analysis (NMDS) of microbial communities of 10 samples. The operational taxonomic units (OTU) were identified by 16S rRNA gene sequencing. Phylogenetic distances between samples were determined using the Bray-Curtis algorithm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

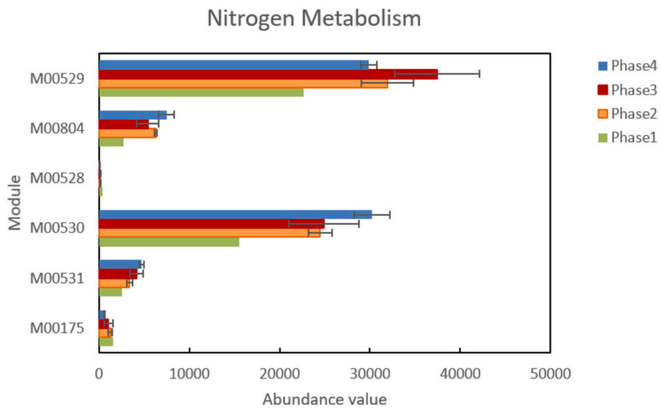

Fig. 7. Module abundance value under different temperatures (M00175: Nitrogen fixation, M00528: Nitrification, M00529: Denitrification, M00530: Dissimilatory nitrate, M00531: Assimilatory nitrate reduction, M00804: Complete nitrification).

temperatures was observed. We observed a decrease of effluent nitrate-N concentration on the first day and an increase on the next day in Phase I (Fig. 2). The decrease likely was caused by the rinsing with seawater, suggesting that the biofilm attached on the surface of the pellets did not adhere well and was lost in the effluent (Xu et al., 2018a). Rapid release of COD occurred during the first 5 days and COD reached a maximum of 35.27 mg/L, suggesting that the energy from PCL polymer degradation was much greater than the consumption for denitrification and bacterial growth. However, COD decreased immediately and constantly, indicating the maturation of the biofilm on PCL granule surfaces. The effluent COD was controlled at 14 mg/L at the end of Phases I-III, but it increased to approximately 17 mg/L in Phase IV with HRTs of 8 h and 10 h due to low carbon utilization and low flow rate (Table S2). Similarly, Shen and Wang (2011) reported that effluent COD concentration peaked at 59.11 mg/L at the 5th day and then dropped to 30 mg/L using starch/PCL blends, a result similar to those of our study. Using SEM, we observed that the biofilm attached to PCL was comprised predominately of rod bacteria and noted a perforated surface on used PCL (Fig. 3). In addition to rod bacteria, coccal bacteria were found by Chu and Wang (2011) on PCL carrier. The same phenomenon was also found by Zhang et al. (2016), where the PCL surface became perforated in their denitrification bioreactor.

DO in the CAFB-PADR reactor was consistently maintained at approximately 6.5 mg/L, but anaerobic conditions may still exist within deeper layers of the biofilm or within the polymer matrix. These microenvironments with limited oxygen availability could support denitrification processes by anaerobic denitrifiers, such as Pseudomonas species, which are facultative anaerobes capable of anaerobic denitrification. Given the high levels of dissolved oxygen in the bulk liquid, it is likely that aerobic denitrification was the dominant mechanism for nitrate nitrogen removal in our system.

In addition to denitrification, nitrate assimilation might also be a key mechanism in nitrate nitrogen removal in the CAFB-PADR. Gregersen and Pedersen (2023) reported that nitrate assimilation likely involves the role of microbial and biological systems in removing nitrate from the water. In our CAFB-PADR, the difference between total nitrogen (TN) and dissolved inorganic nitrogen (DIN) in the effluent is shown in Fig. S2. The results showed that organic nitrogen was present in the effluent, suggesting that nitrate assimilation has been occurring, particularly given the carbon availability provided by the biodegradable polymer (Wang et al., 2015), in addition to aerobic and/or anaerobic denitrification. However, we could not determine the amount of organic nitrogen within the bacteria on the biofilm, making it difficult to speculate on the actual contribution of the nitrate assimilation to nitrate removal.

The activity of the enzymes involved in both nitrate reduction and hydrolysis of the solid substrate can be affected by temperature, indicating a means to control solid-phase denitrification (Wang and Chu, 2016). Xu et al. (2018c) indicated that the lower ambient temperature reduced bacterial activity, causing slower degradation of carbon sources and less efficient nitrogen removal efficiency. Similarly, in our 6.5-month experiment, the denitrification rate was 1.1–1.8 times higher at 30 °Cthan at 15 °C. Shen et al. (2015) observed highest denitrification efficiency of 92.5 % (at 25 °C), compared to 68.7 % (at 15 °C) using a PCL/starch blend. Cameron and Schipper (2010) showed a denitrification rate 1.7-fold greater with temperature increasing (about 10 °C) supported by softwood chips. The volumetric rate of denitrification dropped by about 50 % when temperature was reduced by 5 °C (Chu and Wang, 2013).

HRT plays a critical role in the denitrification process. Xu et al. (2018c) illustrated that a high influent nitrogen concentration could be removed completely under 7.25-h HRT using a denitrification biofilter supported by polyhydroxybutyrate-*co*-valerate (PHBV). Wang and Chu (2016) showed that nitrogen removal rate decreased with prolonged HRT in a PCL-packed bed reactor because of the gradually thickening biofilm and decreasing activity caused by continuous biomass growth. Gibert et al. (2008) reported that denitrification rate and effluent nitrogen concentration were affected by HRT in a woodchip-filled denitrification reactor. In our trial, we also showed that HRT played a critical role in denitrification efficiency.

4.3. Microbial community structure

This experiment showed how environment factors influenced microbial community structure, especially regarding key denitrification-related functional microorganisms. In our study, Class Gammaproteobacteria dominated each sample; its relative abundance was highest at 15 °C and decreased at higher temperatures. Pan et al. (2022) found the relative abundance of Gammaproteobacteria was enhanced due to the increasing COD. This finding coincided with the results of our study, because COD dropped significantly at higher temperature. Alphaproteobacteria play a significant part in the denitrification process (Chen et al., 2018b; Vilar-Sanz et al., 2018). In Phase III, under the highest temperature in our study, the abundance of Alphaproteobacteria increased to 27.1 % while the abundance of Gammaproteobacteria decreased compared with Phase IV, which caused nitrate-N removal efficiency to improve. Using polypropylene (PP), a thermoplastic polymer, as the carbon source, Pan et al. (2022) also showed that sequences belonging to the Betaproteobacteria, Alphaproteobacteria and Gammaproteobacteria were predominant, representing about 70 % of the total bacterial sequences. Bacteroidia was proven as denitrifying bacteria (Tang et al., 2022) with lower denitrification ability and abundance when the temperature was adjusted to 15 °C. The predominant classes in our study were consistent with those observed by Liang et al. (2022), verifying that Gammaproteobacteria, Alphaproteobacteria, and Bacteroidia were related to nitrogen removal. The results showed that temperature changed the microbial community during MBBR operation. Wang et al. (2021) and Yu et al. (2020) regarded denitrifiers as being commonly found among the Proteobacteria. Benbow et al. (2015) and Zuo et al. (2023) indicated that Proteobacteria and Bacteroidetes played significant roles during denitrification in constructed wetlands and sludge bed bioreactors. Wang and Chu (2016) found most of the nitrite reductase (*nirS* and *nirK*) and nitrous-oxide reductase (*nosZ*) clones of the microorganisms in solid-phase bioreactors were derived from members of Class Gammaproteobacteria of Phylum Proteobacteria, suggesting that Gammaproteobacteria played the primary role in denitrification. A few of the Proteobacteria that we found in our study, such as *Acidovorax facilis* and *Brevundimonas bullata*, have been shown capable of both denitrification and degradation of BDPs (Mergaert et al., 2001).

At the genus level, the microbial community showed great differences under different temperature conditions in PCL-supported denitrification systems. Four predominant genera (*Marinobacter*, *Pseudomonas*, *Halomonas* and *Hydrogenophaga*) with denitrification function (Xu et al., 2018b; Okamoto et al., 2004; Huang et al., 2020; Wang et al., 2017) were observed. *Pseudomonas* sp. in Family Pseudomonadaceae is capable of aerobic denitrification with soluble organic substances (Chu and Wang, 2016). Both Su et al., (2017) and Chen et al. (2018a) showed that *Pseudomonas* had autotrophic-heterotrophic denitrification capacity. Many studies (e.g., Zhang et al., 2015; Li et al., 2016) have proven its excellent nitrogen removal ability under aerobic conditions. Li et al. (2016) reported oxidation of ammonium to nitrite and then direct reduction to gaseous nitrogen. The high average abundance of *Pseudomonas* at 15 °C reached 26.7 % with increasing COD. Wang et al. (2017) proposed that the abundance of *Pseudomonas* becomes predominant as carbon concentration increases. Timmis et al. (2010) observed that the *Marinobacter* sp. could degrade aliphatic and polycyclic aromatic hydrocarbons as well as acyclic isoprenoid compounds. In our results, the *Marinobacter* accounted for a large proportion in phases II and III with HRT = 10 h, suggesting the rapid hydrolyzation of PCL and release of COD. The high concentration of carbon might be conducive to the rapid propagation of bacteria, driving the rapid removal of nitrogen (*NRE* was 91.9 % in phase II and 93.5 % in phase III with HRT = 10 h). *Simplicispira* and *Pseudomonas* are considered important denitrifying bacteria (Wang and Chu, 2016; Zuo et al., 2023). As the dominant bacteria in marine environment, the Rhodobacteraceae play an essential part in biogeochemical cycling (Simon et al., 2017; Wemheuer et al., 2015). *Simplicispira* was a dominant denitrifier in both our reactor and other poly-supported denitrification membrane biofilm reactors (Liu et al., 2019; Ruan et al., 2016). The results from Xu et al. (2018b) showed that genera *Thauera* (13 %), *Brevinema* (13 %), and *Dechloromonas* (6 %) could be highly involved in the denitrification process in PHBV/PLA polymer-mediated systems. The microbial community of an airlift biofilter based on poly(butylene succinate) showed the denitrifiers *Azoarcus* and *Simplicispira* were dominant and accounted for high nitrate removal (Ruan et al., 2016). *Dechloromonas*, *Desulfovibrio*, and *Flavobacterium* were the main denitrifiers discovered in a PHBV/PLA-based reactor (Xu and Chai, 2017). Some common genera – such as *Thauera*, *Azoarcus*, *Desulfovibrio*, and *Dechloromonas* (Li et al., 2016) – were not found in our study, suggesting that the biofilm carrier may affect microbial community composition.

4.4. KEGG modules in nitrogen metabolism

Regarding the nitrogen cycle, all module categories were found, and we detected high prediction of denitrification (M00529) accounting for 46 %–56 % of the nitrate reduction. Results shown in Fig. 7 showed that higher temperature can enhance the denitrification process, causing the higher denitrification efficiency observed in Phase III. Although dissimilatory nitrate reduction to ammonium (DNRA, M00530) exhibited wide variation among the phases, it was second among more abundant modules. As an important pathway for the formation of ammonia in the effluent, DNRA may correspond to the greatest effluent TAN concentration (Xu et al., 2018a, 2018b, 2018c). However, we didn't find enhancement of assimilatory nitrate reduction (M00531) among different phases. In our experiment, the PCL polymer released electron donors consistently, which may cause the competition between heterotrophic and autotrophic denitrifying bacteria (Zou et al., 2022).

5. Conclusions

The PCL-supported denitrification system is a promising technology for treating recirculating marine aquaculture wastewater with high nitrate concentration. PCL was more favorable for microbial attachment as a denitrification electron donor than PBS or PLA. In our study, with temperatures exceeding 25 °C and HRT of 10 h, the effluent average nitrate-N was below 2.5 mg/L, and more than 90 % of total nitrogen was removed. Meanwhile, Illumina DNA sequencing analyses revealed complex microbial community composition and showed the presence of key functional microorganisms that explained the high denitrification performance of the system. DNA sequences belonging to the Alphaproteobacteria, an important class in denitrification, were most abundant (over 27.1 %) at a temperature of 30 °C. The higher temperature led to more active denitrification and inhibited nitrate from transforming to TAN for nitrate reduction. Additionally, nitrate assimilation was indicated by the presence of organic nitrogen in the effluent, suggesting its contribution alongside denitrification to overall nitrogen removal. Further investigation and a better understanding of PCL-supported denitrification systems are crucial for achieving efficient nitrate removal in practical aquaculture systems. More studies are needed to quantify the relative contributions of nitrate assimilation, aerobic and anaerobic denitrification, especially within biofilm microenvironments.

CRediT authorship contribution statement

Zhitao Huang: Writing – review & editing, Validation, Project administration. **Xiefa Song:** Project administration, Data curation. **Yue Sun:** Data curation. **Fotini Kokou:** Writing – review & editing. **Eric Hallerman:** Data curation. **Paulo Fernandes:** Writing – review & editing. **Zheng Zhou:** Data curation. **Xiaohan Yang:** Writing – review & editing, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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