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Performance of polycaprolactone-based heterotrophic denitrification for recirculating aquaculture systems with varying hydraulic retention times

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ABSTRACT

In this study, a pilot-scale fixed-film denitrification bio filter was developed using polycaprolactone (PCL) as a carbon source and biofilm carrier to remove nitrate-nitrogen (NO_3^-–N) from the water of a recirculating aquaculture system. As the hydraulic retention time (HRT) increased from 2 to 8 h, NO_3^-–N removal efficiencies increased from 5 to 65%. The highest removal rate of 1.11 ± 0.34 kg NO_3^-–N/m^3·d was achieved at an HRT = 8 h with a flow rate of 0.71 L/m^3·h. Unused dissolved organic carbon (DOC) from PCL degradation for all HRT amounted to less than 10 mg/L, and this value increased with increasing HRT. Observations of the PCL surface indicated that chain scission by hydrolytic degradation and biological utilisation in the reactors did not significantly change its chemical structure. Microbial community analysis showed the predominance of Proteo bacteria and Bacteroidetes in the bio film, with relative abundances of 63.9% and 27.4%, respectively. Six genera namely *Acidovorax, Azospira, Comamonas, Diaphorobacter, Ignavibacterium,* and *Frateuria,* could both degrade PCL and denitrify NO_3^-–N.

Keywords: Polycaprolactone; Recirculating aquaculture system; Nitrate-nitrogen; Denitrification; Organic carbon source

1. Introduction

Aquaculture is one of the fastest growing animal food production sectors [1]. To obtain higher yields, aquaculture was intensified with a high stocking density and artificial food input [2]. Owing to the high protein demand and low digestion capacity of farmed animals, 75% of inputted nitrogen will be discharged [3]. If it is not treated properly before discharge, this nitrogen can detriment the receiving environment (for example, by causing eutrophication) and farmed animals [4]. Recirculating aquaculture systems (RASs) have been explored in response to these concerns [5]. These enable the reuse of water and complete environmental control [6].

In RASs, ammonia and nitrite are generally removed through auto trophic nitrification [5]. Nitrate is the product of auto trophic nitrification, and can accumulate at concentrations of up to 500 mg/L in RASs when nitrifying biofilters are used [5]. Although nitrate is less harmful than ammonia and nitrite, high nitrate concentrations affect the growth of commercially cultured aquatic organisms [7].

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Heterotrophic denitrification has is one of the most feasible processes of removing nitrate from wastewater [8,9]. In this process, heterotrophic bacteria use organic carbon for growth and as an electron source [10]. Solid organic substrates, such as inexpensive waste products (straw, wood, and bamboo fibre) [9,11] or biodegradable polymers (BDPs) [12,13], can be used for denitrification. This is referred to as BDPs-based denitrification.

During BDPs-based denitrification, BDPs can simultaneously serve as a carbon source and biofilm carrier [12]. BDPs are hydrolysed by extracellular enzymes to dissolved organic carbon (DOC), which can be biodegraded further or directly used as an electron donor for heterotrophic bacteria to reduce nitrate [13]. The hydrophobic characteristic of BDP prevent the excessive release of organic carbon to aquaculture water and simplifies dosage control. The rate of BDP biodegradation depends on the properties of the polymers and the environmental conditions [14]. Hydraulic retention time (HRT) is an important factor in the operation of biological removal systems [15]. Determining an appropriate HRT for denitrifying reactors is important as it is directly related to the NO_3^-–N removal efficiency [16].

Of all the BDPs that are currently available for denitrification, low-cost polycaprolactone (PCL) (8–12 $/kg) is the most economically attractive [17]. The feasibility of PCL as a carbon source for denitrification (6 NO_3^-–N + $C_6H_{10}O_2 \rightarrow 3N_2 + 6CO_2 + 2H_2O + 6OH^{-1}$) in the secondary effluent of municipal wastewater treatment plants, groundwater, tap water, and aquaculture water has been investigated previously [12,18–21]. To better document the use of PCL to remove NO_3^-–N in aquaculture water, the effects of HRT (bioreactor pore volume divided by flow rate) on denitrification by PCL were evaluated in a continuous-up flow fixed-bed reactor filled with PCL for treating aquaculture water. Changes in the chemical structure before and after bio film colonisation and the composition of the bacterial community of the bio film were also studied.

2. Material and methods

2.1. PCL beads

PCL ($[C_6H_{10}O_2]_n$) used in this study had L × D × W dimensions of 2 × 3 × 4 mm, respectively, and a molecular weight of 80000, and was supplied by Guanghuaweiye Ltd. Co. (Shenzhen, China). The main characteristics of the PCL beads were described by Luo et al. [22]. The PCL beads were ultrasonically cleaned (0.1 kW, 40 kHz) and dried at 35°C in a vacuum oven to ensure that they all had the same weight within ± 0.0001 g.

2.2. RAS

Water was obtained from a pilot-scale freshwater RAS stocked with 28 ± 1 kg/m^3 Jadeperch (*Scortumbarcoo*). The RAS was equipped with eight fish tanks (1 m^3), a solid/liquid separator, a gas/liquid mixing device, two nitrification bio filters, a sterilization unit, and a temperature control unit (Fig. 1). The water was continually aerated to maintain the dissolved oxygen (DO) concentration at up to 6 mg/L. The following RAS water parameters were monitored every two days: dissolved oxygen (DO, 6.01–8.21 mg/L), pH (7.25–8.01), total ammonium nitrogen (TAN, 0.08–9.74 mg/L), nitrite-nitrogen (NO_2^-–N, 0.15–3.49 mg/L), nitrate-nitrogen (NO_3^-–N, 89.3–101.68 mg/L), and temperature (23.4–25.9°C).

2.3. Denitrification reactors

Three laboratory-scale up-flow reactors were used in parallel (5.7 L working volume with 70.00 cm high and 10.2 cm diameter; Fig. 1). Each reactor was filled with 12 vol. % PCL beads (750 g).

2.4. HRT effects test

Four different HRT conditions were set: Stage I (HRT = 8.0 h) from day 3 to 26, Stage II (HRT = 6.0 h) from day 27

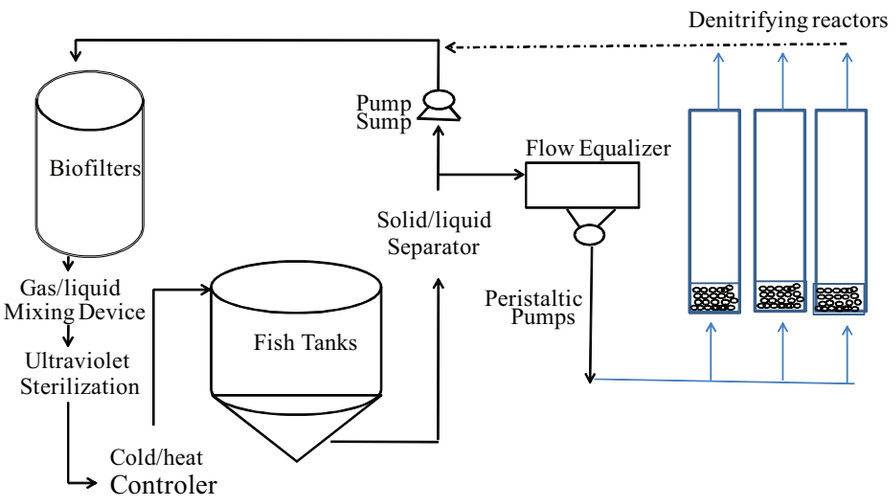

Fig. 1. Schematics of three pilot-scale PCL-packed reactors for treating nitrate-dosed water from a recirculating aquaculture system.

to 42, Stage III (HRT = 4.0 h) from day 43 to 56, and Stage IV (HRT = 2 h) from day 57 to day 67. Continuous experiments were conducted. After a stable NO_3^-–N removal performance was obtained at each HRT, the HRT was shifted to the next stage. The flow rates of the four designed HRT were 0.71 L/h (8 h), 0.95 L/h (6 h), 1.43 L/h (4 h), and 2.85 L/h (2 h). During these periods, the DO concentration of the influent was not controlled [23] and the temperature was maintained at 22 ± 2°C. There was no seed source for denitrification. An acclimation period was required for the denitrification microorganisms to adapt.

The NO_3^-–N, NO_2^-–N, total nitrogen (TN), TAN, DOC, alkalinity (ALK), pH, and DO of the influent and effluent were monitored every two days. At the end of the experiment, the PCL bead weight loss was determined. The biofilm on the PCL surface was stripped so as to analyse its bacterial communities.

2.5. Parameter analysis

2.5.1. Chemical water parameters

The water temperature (°C), pH, ALK, and DO were measured using a YSI 556 meter (YSI Incorporated 1725, Yellow Springs, OH, USA). TAN, NO_2^-–N, NO_3^-–N, and TN levels were analysed following SEPA standard methods [24]. The DOC content was determined using a TOC analyser (TOC-V, CPH, Shimadzu Seisakusho, Japan).

The removal efficiency of the water quality parameters was calculated as the concentration of the parameter in the influent concentration minus that of the effluent. The difference was divided by the influent concentration and multiplied by 100%. The NO_3^-–N, TN, and DOC removal rates, and ALK production rates were based on the change in concentration between the influent and effluent, multiplied by the flow rate (L/d), and divided by the work volume of the used biofilters (5.7 L).

2.5.2. Fourier transform infrared spectrometer (FTIR) and scanning electron microscope (SEM) observations of PCL

Infrared (IR) spectra were collected using a Fourier transform infrared spectrometer (FTIR (Nicolet Nexus 470, Thermo Nicolet Corporation, USA) at a resolution of 2 cm^{-1}. The morphology of the PCL samples was observed using an environmental SEM (Quanta 200 FEG, Holland).

2.5.3. Microbial community analysis

Samples of bio films that were attached to the surfaces of the PCL beads were collected from the middle of the reactor on day 68, which was at the end of the HRT test. DNA was extracted from biofilm samples using a Qubit2.0 DNA Kit, and the concentrations were determined by Sangon Biotech Co., Ltd. (Shanghai, China).

The extracted DNA was amplified following the method presented byJia et al. [25].The polymerase chain reaction products were identified by pyrosequencing using Miseq Illumina by Sangon Biotech Co., Ltd. (Shanghai, China) following standard protocols and software (Data collection software, Illumina). The sequences were clustered into operational taxonomic units (OTUs) by setting a 0.03 distance limit (equivalent to 97% similarity) using UCLUSTv1.1.579 and categorised by phylum, class, and genus. The relative abundance of a given phylogenetic group was the number of sequences affiliated with that group divided by the total number of sequences per sample. The pyrosequencing data reported here have been deposited in the NCBI Sequence Read Archive (SRA) database (accession number SRR5133596).

2.6. Data analysis

All statistical analyses were conducted using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). The effect of HRT was analysed by one-way analysis of variance (ANOVA) for all measured variables. Significant differences between treatments were analysed using Tukey's test at a significance level of 0.05.

3. Results and discussions

3.1. Denitrification performance of PCL-packed bed reactors

The treatment performance of the PCL-packed bed reactors is shown in Fig. 2, in terms of effluent NO_3^-–N, TN, NO_2^-–N, and TAN concentrations, and the removal efficiency of NO_3^-–N. There was a general decrease in the effluent NO_3^-–N and TN concentrations and an increase in the removal efficiency of NO_3^-–N as the HRT increased from 2 h to 8 h. The highest removal rate (1.11 ± 0.34 kg NO_3^-–N/m^3·d) was achieved at an HRT of 8 h and flow rate of 0.71 L/h·m^3. The effluent NO_3^-–N concentrations were below 50 mg/L at a HRT of 8 h. The removal rate of NO_3^-–N was 0.87 ± 0.19 kg NO_3^-–N/m^3·d at an HRT of 6 h, and the effluent NO_3^-–N concentration was below 75 mg/L. The removal efficiency of NO_3^-–N ranged from 5%–20% at HRTs of 4 h and 2 h. The reported nitrate removal efficiency varied widely across different HRTs due to differences in the environmental conditions and bioreactor design. The NO_3^-–N removal rate of this experiment was similar to those reported for denitrification reactors in RAS (0.003–8 kg NO_3^-–N/m^3·d) [26].

NO_3^-–N does not cause acute mortality to farmed animals [4]. New recommendations suggest that NO_3^-–N concentrations of < 75 mg/L are suitable for optimal fish health [27]. Therefore, the <75 mg/L concentration of NO_3^-–N in the effluent of this experiment achieved these standards. Based on this, HRT of 8 h and 6 h were selected for PCL-packed bed reactors that treat aquaculture water containing approximately 100 mg/L of NO_3^-–N. HRT is positively correlated with NO_3^-–N removal over a certain range [28,29]. In this experiment, there was no obvious difference in the performances of HRTs of 2 h and 4 h. However, the performance of the 8-h HRT was better than that of the 6-hHRT, which in turn outperformed the 4-hHRT.

The NO_3^-–N concentration in the current experiment was selected based on the water quality of common recirculating aquaculture systems, and was higher than

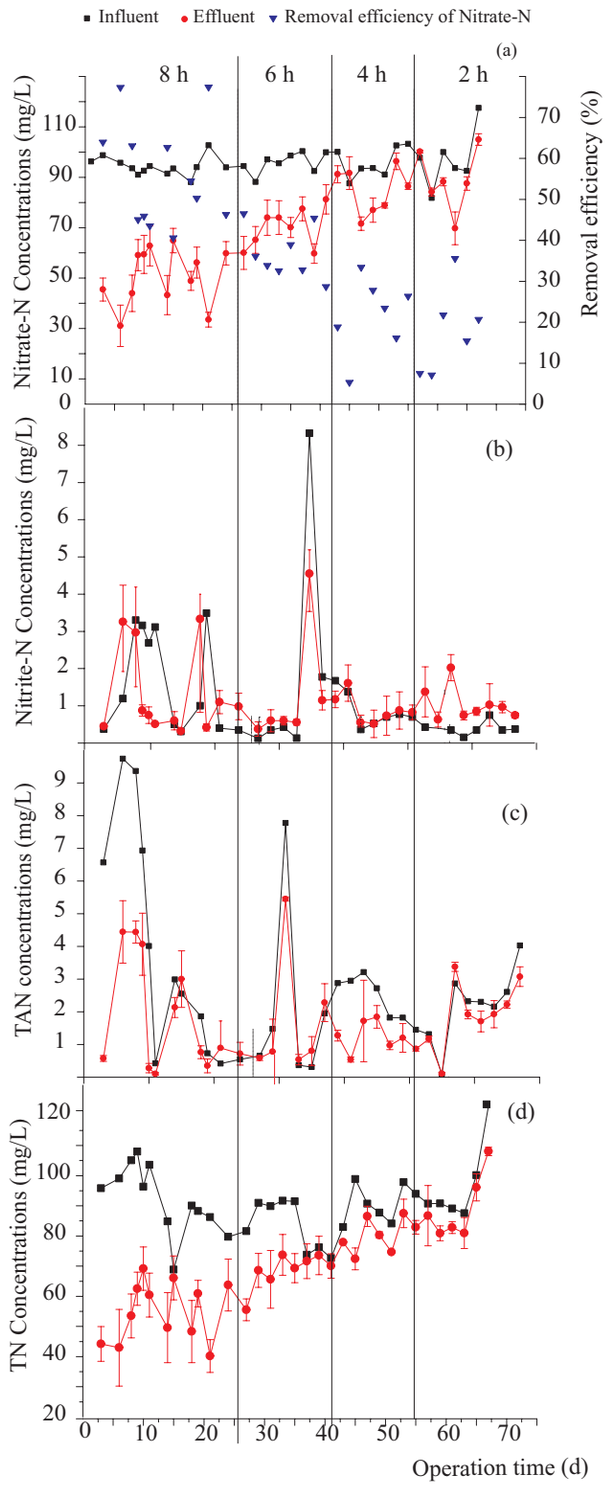

Fig. 2. Influent and effluent NO_3^-–N (a), NO_2^-–N (b), total ammonia nitrogen (TAN, c), and total nitrogen (TN, d) from pilot PCL-packed reactors that treated nitrate-dosed water from a recirculating aquaculture system. The hydraulic retention time (HRT)of the reactors is indicated in the figure in hours.

that of research focused on groundwater or drinking water [28]. Nitrite, as an intermediate product of denitrification and nitrification, may have been generated as the result of incomplete denitrification or nitrification. The concentration of NO_2^-–N in the effluent was mainly affected by the level of NO_2^-–N in the influent (Fig. 2b), which appeared to be a minor source of NO_2^-–N for most of the sampling period. The TAN concentrations of the effluent were lower than those of the influent at most of the sampling times (Fig. 2c). The bacterial nitrification response may be influenced by attachment to the surface of the PCL beads. The DO of the influent was not controlled by intentional removal. The nitrification rate decreased with an increase in carbon concentration, but this influence became less pronounced at sufficiently high carbon concentrations [30]. Therefore, TAN removal could be due to nitrification. The increases in the TAN of the effluent may be caused by dissimilatory nitrate reduction to ammonia, which has been observed in several previous studies [29,31,32]. Although increases in TAN and NO_2^-–N were observed, a high TN removal efficiency was achieved at HRTs of 8 h and 6 h, similar NO_3^-–N removal. This indicated that denitrification was responsible for NO_3^-–N removal during the experiment.

3.2. DOC in the effluent and weight loss

There was a DOC concentration range of 10–60 mg/L in the effluent (Fig. 3). More than 50% of the DOC in the effluent was derived from the influent. The remainder was unused DOC from PCL (less than 10 mg/L). At the HRT of 4 and 2 h, DOC increased significantly less than at HRT of 8 h and 6 h. The increase in DOC was highest at a HRT of 8 h.

The accumulation of DOC in the effluent of a BDP-based denitrifying reactor has been reported previously. Effluent

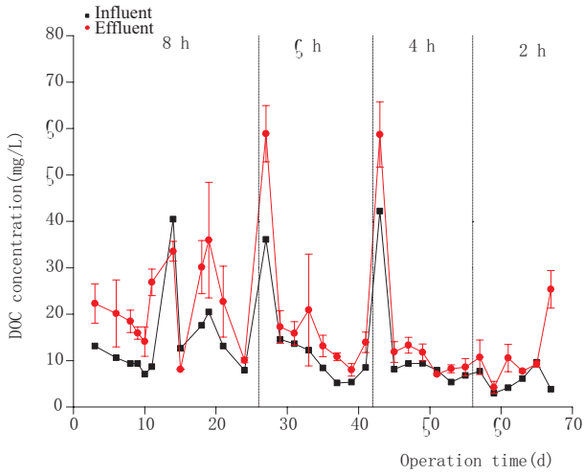

Fig. 3. Influent and effluent dissolved organic carbon (DOC) from the pilot PCL-packed reactors treating nitrate-dosed water from a recirculating aquaculture system. The reactor hydraulic retention time (HRT) is indicated in the figure in hours.

DOC concentrations of 15.46 and 59.11 mg/L have been observed in PCL-based and PCL/starch-based denitrification, respectively [32]. Although conducting physiological and biochemical studies of BDP biodegradation is of primary importance for comprehensively understanding BDP-based denitrification, few studies have reported the physiological and metabolic characteristics of BDPs in denitrifying systems. Little is understood about the metabolic relationship between degradation and denitrification by BDPs.

3.3. ALK in the effluent

There were notable differences between influent and effluent ALK (Fig. 5) due to the reduction of alkalinity by the nitrification and biodegradation of PCL, the regulated addition of sodium bicarbonate to the RAS,and heterotrophic denitrification. During heterotrophic denitrification, ALK was produced ata rate of 3.57 mg $CaCO_3$/mg NO_3^-–N [33]. For the four HRT, there were notable increases in ALK in the effluent from that of the influent (Fig. 4). The increases in ALK at HRT of 8 and 6 h were significantly higher than those at 4 and 2 h. This was similar to the result of NO_3^-–N removal, as discussed above. This suggested that the removal of NO_3^-–N by heterotrophic denitrification can return the alkalinity to the level prior to nitrification [33].

3.4. Microbial community structure of the biofilms formed on the PCL beads

Hydrolysis and denitrification occur simultaneously during PCL-based denitrification. The hydrolysis of PCL is induced by extracellular enzymes excreted by degrading bacteria, therefore, the production of DOC is the first and most important step of PCL-based denitrification. Abou-Zeid et al. [34] reported that *Clostridium* degrade PCL

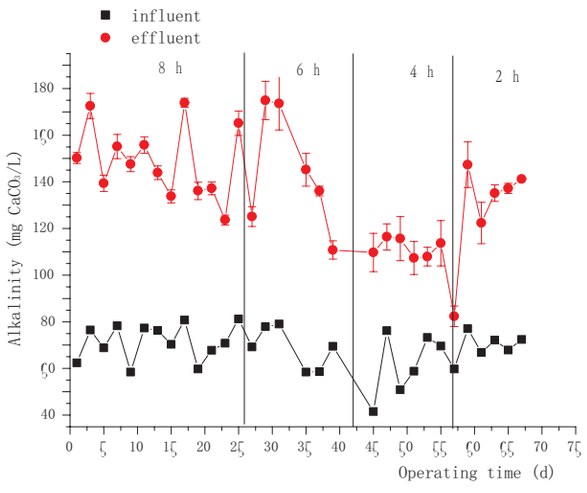

Fig. 4. Alkalinity (ALK) of the influent and effluent of pilot PCL-packed reactors treating nitrate-dosed water from a recirculating aquaculture system. The reactor hydraulic retention time (HRT) is indicated in the figure in hours.

under anaerobic conditions. The phylogenetic structures of the microbial biofilm community structure on PCL beads were characterised at the phylum, class, order, family, and genus levels by Illumina high-throughput sequencing for 16SrRNA genes (Fig. 5 in the supplemental file). Over 99% of the sequences for attached bacteria were identified at the phylum level. Proteo bacteria and Bacteroidetes were predominant in the inoculum, with relative abundances of 63.9% and 27.4%, respectively. Both have been widely reported as major denitrifying bacteriaphyla [19]. Our recent study showed that 75% of the bacteria species identified in PCL-based denitrifying reactors filled with synthetic wastewater were β-proteobacteria [23].

Comamona and *Acidovorax* degrade polymers [18,35], and *Comamonas* has been reported in RASs [36]. Other groups, such as *Thermomonas* and *Diaphorobacter*, have also been widely detected in wastewater denitrification systems [32,37]. The genus *Acidovorax*, belonging to the *Comamonadaceae* family, have commonly been identified as predominant denitrifying bacteria that use biopolymers as a carbon source [12,35,38]. *Acidovorax* was the dominant degrading bacteria in freshwater [39].

Interestingly, *Bdellovibrio* was the most abundant genus (19.4%, Table 1). *Bdellovibrio* are predatory and attach to Gram-negative bacteria, penetrating through the cell wall to form a cello plast and multiply [51]. These bacteria play important roles in reducing microbial density and altering microbial communities through predation [52]. Owing to their intrinsic ability to lyse prey cells, they can act as a potential antibiotic for controlling pathogenic bacteria in aquatic systems [53].The genus *Sedimini* has been isolated from aquatic environments, such as environmental water samples and sediments [54]. *Fluviicola* has been found in suspended and sessile sludge samples, and was one of the most abundant biofilm community members [55]. *Frateuria* is a common microbial group in agricultural soils and plays a role in denitrification [56].

Mergaert et al. [57] found that only two groups of the isolates, the *Acidovoraxfacilis* and *Brevundimonas*-like strains, can both degrade PHBV and conduct denitrification. In this experiment, at least six groups—*Acidovorax, Azospira, Comamonas, Diaphorobacter, Ignavibacterium,* and *Frateuria*—were capable of both degrading PCL and denitrification (Table 1).

3.5. Changes in FT-IR spectra and SEM of PCL

There was little change in the FT-IR spectra of PCL before and after use (Fig. 6a in the supplemental file). The strong absorption peaks at 1,731 cm^{-1} and 1,168 cm^{-1} for fresh PCL, and 1,732 cm^{-1} and 1171 cm^{-1} for used PCL were assigned to the carbonyl (C=O) and –C–O–C– stretching of the ester group [35], respectively. The absorption bands at 2,949 cm^{-1}, 2,948 cm^{-1}, 2,867 cm^{-1}, and 2,866 cm^{-1} represent the symmetric-stretching vibration and anti-symmetric stretching vibration of –CH. The hydroxyl (–OH) stretching peak was that at 3,444 cm^{-1}. There was a free hydroxyl group in the fresh PCL [35].

There were notable differences in the shape and position of the characteristics of fresh and used PCL beads (Figs. 6c and d in the supplemental file). The surface of the fresh PCL beads was relatively smooth, while the surface of the used PCL without a biofilm had cavities, which could be due to corrosion by

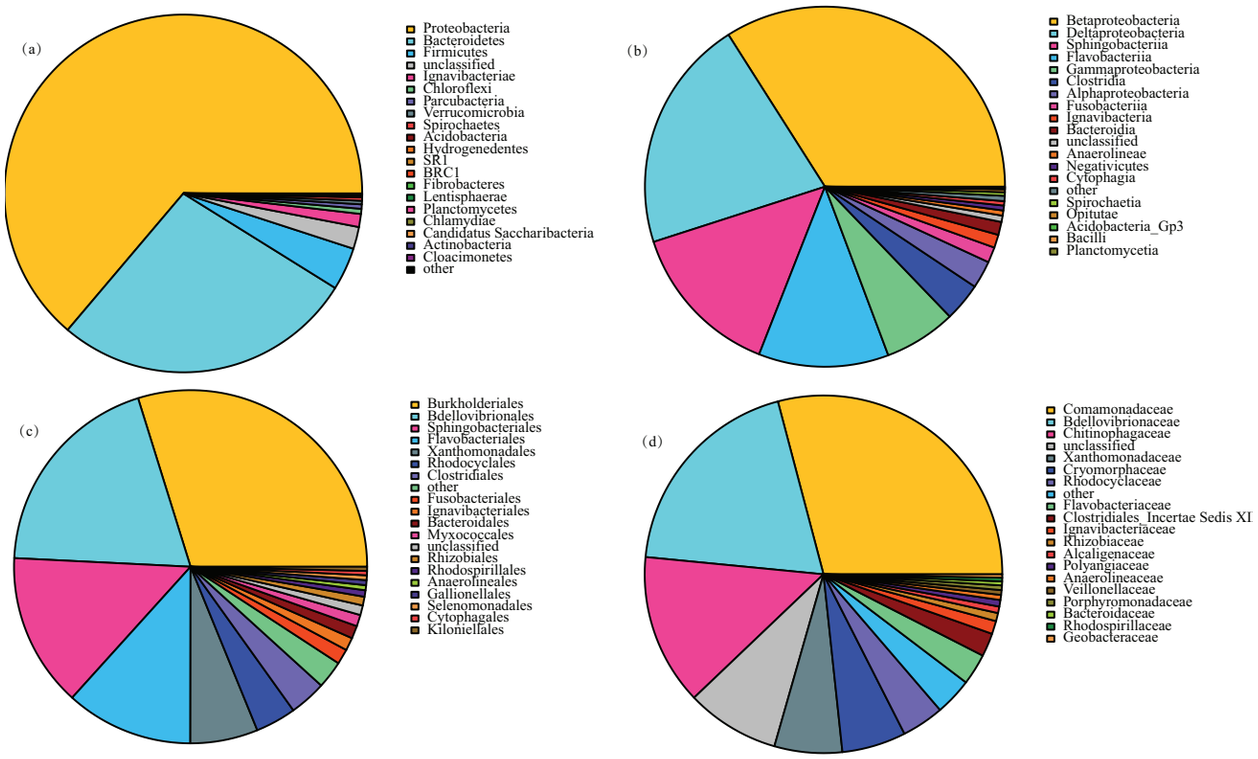

Fig. 5. Taxonomic affiliation and relative abundance of bacterial groups at phylum (a), class (b), order (c), and family (d) levels.

Table 1
Taxonomic affiliation and relative abundances of major bacteria groups at the genus level (abundance > 1%)

Genus	Bands	Abundance (%)	Phylum	Degradation of organic matters	Denitrifying	Carbon source	Reference
Bdellovibrio	3175	19.35	Proteobacteria	√		PCL	[19]
Comamonas	2810	17.13	Proteobacteria	√	√	PBS, Woodchips	[40,41]
Sediminibacterium	1764	10.75	Proteobacteria		√		
Fluviicola	934	5.69	Bacteroidetes	√			[42]
Xenophilus	625	3.81	Proteobacteria				[42]
Acidovorax	544	3.32	Proteobacteria	√	√	PHBV, PHA, PCL	[43-45]
Cecembia	481	2.93	Bacteroidetes		√		[46]
Ornithobacterium	443	2.70	Actinobacillus				
Azospira	407	2.48	Proteobacteria	√	√	PCL	[47]
Terrimonas	404	2.46	Bacteroidetes				
Frateuria	400	2.44	Proteobacteria	√	√	Polycyclic aromatic hydrocarbons	[48]
Dokdonella	366	2.23	Proteobacteria				
Acidaminobacter	343	2.09	Firmicutes	√			
Psychrilyobacter	223	1.36	Fusobacteria		√		
Thermomonas	216	1.32	Proteobacteria g		√	PCL	[49]
Ignavibacterium	194	1.18	Bacteroidetes	√	√		[50]
Diaphorobacter	182	1.11	Proteobacteria	√	√	PCL, PHB, PHBV	[32,38,47]

Note: polyhydroxyalkanoate, PHA; polycaprolactone, PCL; polybutylene succinate, PBS; polylactic acid, PLA; polyhydroxybutyrate-hydroxyvalerate (PHBV), polyhydroxybutyrate (PHB).

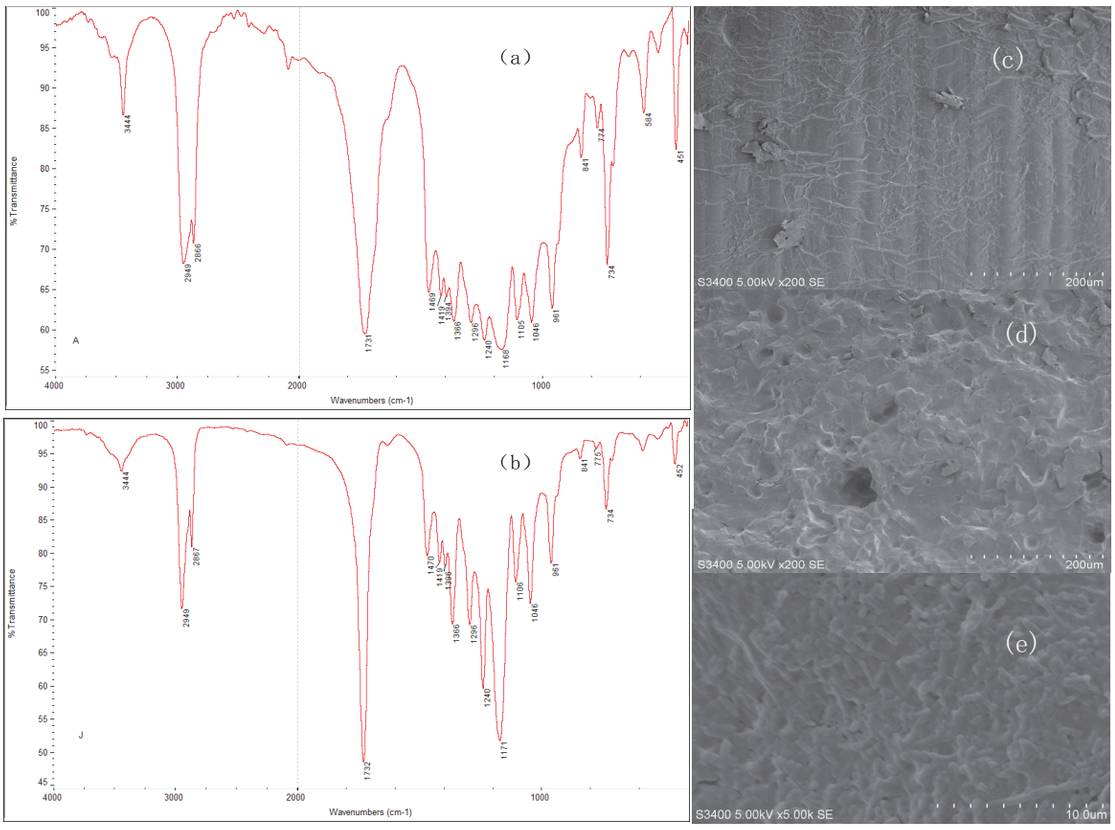

Fig. 6. FT-IR spectra of fresh PCL (a), used PCL (b), SEM observations of the surface of fresh PCL (c), PCL after biofilm detachment (d), and the biofilm attached to PCL (e) at the end of the experiment.

microorganisms. This indicated that biological utilisation in the reactors did not significantly change the chemical structure of the PCL [17,58]. As shown in Fig. 6e, the biofilm on the surface of PCL was mainly composed of *Bacillus*, which can take full advantage of carbon sources for denitrification [17].

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