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Biological denitrification using poly(butylene succinate) as carbon source and biofilm carrier for recirculating aquaculture system effluent treatment

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HIGHLIGHTS

- PBS polymer showed well performance for real RAS wastewater denitrification.
- High nitrate loading was favor to inhibit sulfate reduction and DNRA activity.
- Variation in microbial population was responsible for changed reactor performance.
- Precise carbon release was crucial for RAS denitrification process in practice.

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ABSTRACT

Nitrate removal is essential for the sustainable operation of recirculating aquaculture system (RAS). This study evaluated the heterotrophic denitrification using poly(butylene succinate) as carbon source and biofilm carrier for RAS wastewater treatment. The effect of varied operational conditions (influent type, salinity and nitrate loading) on reactor performance and microbial community was investigated. The high denitrification rates of 0.53 ± 0.19 kg NO_3^--N m^{-3} d^{-1} (salinity, 0‰) and 0.66 ± 0.12 kg NO_3^--N m^{-3} d^{-1} (salinity, 25‰) were achieved, and nitrite concentration was maintained below 1 mg/L. In addition, the existence of salinity exhibited more stable nitrate removal efficiency, but caused adverse effects such as excessive effluent dissolved organic carbon (DOC) and dissimilation nitrate reduce to ammonia (DNRA) activity. The degradation of PBS was further confirmed by SEM and FTIR analysis. Illumina sequencing revealed the abundance and species changes of functional denitrification and degradation microflora which might be the primary cause of varied reactor performance.

1. Introduction

The indoor recirculating aquaculture system (RAS) is a potential sustainable alternative to traditional aquaculture systems (Midilli et al., 2012). It provides opportunities to reduce water consumption and to improve waste treatment and nutrient recycling, which makes intensive fish production compatible with environmental sustainability (Martins et al., 2010). During the process, RASs employ biological filters to oxidize ammonia to nitrate with nitrite as intermediate product, since ammonia and nitrite are toxic to cultured species (Gutierrez-Wing and Malone, 2006). Thus, high concentration of nitrate can be accumulated in intensive RASs due to the continuous nitrification and less effluent discharge (van Rijn et al., 2006). Compared with ammonia and nitrite, though nitrate has relatively low stress effect to aquatic animals temporarily, long-time threat was detected to cultured species (van Bussel et al., 2012). Meanwhile, nitrate was considered as one of the main reasons for terrestrial ecosystems eutrophication (McIsaac et al., 2001). Therefore, nitrate removal has become an inevitable potential problem to be solved in RAS practice, considering the aquatic animal welfare, environment pollution and production sustainability (van Rijn et al., 2006).

Biological heterotrophic denitrification was proved to be an efficient way for nitrate removal in wastewater treatment (Pan et al., 2015). In this process, heterotrophic microorganisms use organic carbon as electron donor, while nitrate as electron acceptor, and ultimately change nitrite or nitrate into nitrogen (N_2). Therefore, sufficient organic carbon concentration or suitable C/N ratio was demonstrated as a crucial factor to ensure the desired nitrate

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removal efficiency (Chen et al., 2015). For RAS, daily feed contained relative high protein and low carbohydrate, which resulted in low C/N ratio of water quality characteristics. Thus, added liquid organic carbons such as methanol or ethanol were needed for RAS effluent denitrification (Müller-Belecke et al., 2013). However, the supplied organic carbon might cause water quality fluctuation as the continuous water recirculation. Because pulses addition organic carbon would result in organic carbon loss and water dissolved oxygen (DO) drop, which was a stress to fish and might cause increase mortality. Meanwhile, less amount of liquid organic cannot achieve acceptable denitrification performance while more liquid organic would add organic loading to the bio-filter, which would have a negative effect on the system stability. On the other hand, the fluctuation of nitrate concentration in RAS caused by different species and cultured objectives also increase the difficulty for precise dosage of liquid carbon. Though bypass system can solve the above concerns, it lead to more footprint, tanks and equipment, which increases the complexity and cost of control process.

An interesting alternative is to use insoluble solid carbon for denitrification, which is mainly divided into two methods. One is using natural materials (e.g., straw, and wood) which were demonstrated to have acceptable nitrate removal efficiency (Saliling et al., 2007), but the excessive effluent dissolved organic carbon (DOC) concentration and color problems limit its universality (Boley et al., 2000). The other one is using biodegradable polymers as solid carbon source and biofilm carrier for denitrification. It can release organic carbon slowly and automatically, which means it can be used directly in the system, and provides an alternative to sustainable RAS. For instance, polycaprolactone (PCL) (Chu and Wang, 2013) or starch/polycaprolactone (SPCL) (Shen et al., 2015), polyhydroxybutyrate (PHB) (Gutierrez-Wing et al., 2012) and poly(butylene succinate) (PBS) (Luo et al., 2014; Wu et al., 2013) were all proved to have high nitrate removal efficiency. Because of the specific material component and reaction product, denitrification based on biodegradable polymers seemed to be more convenient and competitive when the treatment objects have high water quality requirement (e.g., drinking water, groundwater, and RAS water) (Lucas et al., 2008). However, previous researches of solid-phase denitrification mostly focused on synthetic wastewater (Luo et al., 2014; Shen and Wang, 2011; Shen et al., 2013, 2015; Wu et al., 2013), whose composition is relatively unitary, while real RAS wastewater were seldom studied. Though these target wastewaters share the common water quality characteristic of low C/N ratio, RAS effluent contained more complex substrate component due to the interaction of cultured animals and feeding (van Rijn, 2013). Moreover, the increase in production of saltwater species appeal to more suitable denitrification systems, because salinity was demonstrated to be detrimental as its effect to bacteria osmotic pressure and enzyme inhibition (Lefebvre and Moletta, 2006). Therefore, further researches of real wastewater treatment were necessary for the sustainable and practical application of RAS.

In this study, we evaluated the performance of two up-flow fixed bed reactors (salinities, 0‰ and 25‰) packed with PBS granules under different operational conditions. The feasibility and efficiency of nitrate removal were studied in terms of denitrification efficiency and effluent characteristics using synthetic and real RAS wastewater as influent, respectively. To have a better understanding of the PBS biodegradation process, the granules before and after experiment were characterized by FTIR and SEM observation. Furthermore, the microbial diversities during different operational conditions were also analyzed to elucidate the variation in microbial population and reactor performance.

2. Methods

2.1. Reactors and biodegradable PBS materials

The schematic representation of the two up flow fixed-bed PBS denitrification reactors is shown in Fig. 1. The two identical reactors were 60 mm inner diameter and 1000 mm height, with the PBS granules packed the column up to the height of 900 mm. The varied types of influents were fed into the two reactors by peristaltic pumps (BT100-2J, Baoding Longer Precision Pump Co., Ltd., China) and the effluents were collected for further analysis. The overall apparatus was placed in a dark artificial climate room to keep the temperature at 19 ± 1 °C and 24 ± 1 °C (Table 1), which were thought to be the suitable temperatures for the cold-water fish and warm-water fish, respectively.

The biodegradable injection level PBS granules were purchased from a commercial company, and the main physical characteristics are as follows according to the manufacturer description: density (25 °C), 1.26 kg/L; melt flow index, 21 g/10 min; tensile strength, 39%; and flexural strength, 27 MPa. The PBS granule has cylindrical size of 3 mm × 5 mm (diameter × height), and the porosity is 34.6%.

2.2. Experimental procedure

The different operational conditions during whole stage were shown in Table 1. For inoculation, 20 mL deep deposit-mixtures from local fresh lake and intertidal zone (Luchao port, Shanghai, China) were used to seed Reactor I and Reactor II, respectively. During stage I, synthetic RAS wastewater used as influent for both reactors was according to (mg/L): 360 KNO_3 (around 50 mg/L NO_3^--N), 78 K_2HPO_4, 31 KH_2PO_4, 95 $MgSO_4{\cdot}7H_2O$, 37 KCl, and 0.2% (v/v) trace element (mg/L): 640 EDTA, 550 $FeSO_4{\cdot}7H_2O$, 230 $ZnSO_4{\cdot}7H_2O$, 340 $MnSO_4{\cdot}H_2O$, 75 $CuSO_4{\cdot}5H_2O$, 47 $Co(NO_3)_2{\cdot}6H_2O$, 25 $(NH_4)_5Mo_7O_{24}{\cdot}4H_2O$ (Ruan et al., 2011). For other stages (II–VI), the real RAS wastewater was used to feed the reactors with only moderate added KNO_3 (around 50–150 mg/L NO_3^--N). The tilapia RAS contained with four tanks (4 m^3) and one MBBR bio-filter (0.5 m^3) was relative stable (survival rate, 95.8%) operated in our laboratory for nearly 1 year. Besides, the artificial sea salt (Blue starfish salt product Co., Ltd., Zhejiang, China) was used to keep the salinity at 25‰ for Reactor II in the whole stage, while the Reactor I was continuously operated at 0‰. In addition, DNA samples were also collected from the two reactors in different stages to evaluate the bacterial community variation.

The nitrate removal efficiency and denitrification rate of the reactors were calculated based on the following equation:

$$R_e = (C_{in} - C_{ef})/C_{in} \times 100\%$$

$$DEN_r = 0.024Q(C_{in} - C_{ef})/(\eta V)$$

where R_e (%) and DEN_r (kg NO_3^--N m^{-3} d^{-1}) were the nitrate removal efficiency and denitrification rate, respectively. C_{inf} (mg/L) and C_{eff} (mg/L) were the concentrations of NO_3^--N in the influent and effluent, respectively. Q (L/h) was the flow rate, V (L) was the bulk volume of the PBS, and η was the porosity of the PBS.

2.3. Analytical methods

The water samples were filtered through 0.45 μm filter membrane before analysis. Total ammonia nitrogen (TAN), NO_2^--N and NO_3^--N concentration were analyzed according to standard methods (SEPA, 2002). DOC was measured using a TOC analyzer (Multi N/C 2100, Analytik Jena, Germany). The morphology of PBS granule and attached biofilm was observed by scanning electron microscope (SEM) (TM-1000 and SU8010, Hitachi High-Technologies Corporation, Japan) on days 0, 25 and 205.

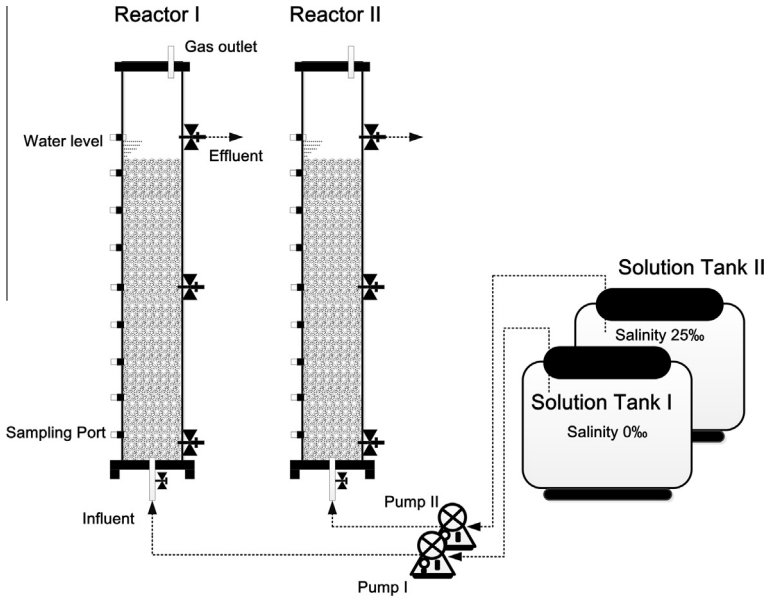

Fig. 1. Schematic representation of the two up flow fixed-bed reactors.

Table 1
The different operated conditions of the two PBS reactors.

Stage	Influent type	Influent NO_3^--N (mg/L)		Temperature (°C)	Hydraulic retention time (h)	Operated time (d)	DNA sampling	
		Reactor I salinity: 0‰	Reactor II salinity: 25‰				Sampled time (d)	Number
I	Synthetic wastewater	52.34 ± 4.57	52.33 ± 10.85	19 ± 1	8	1–61	13	1
							50	2
II	Real RAS wastewater	59.14 ± 15.29	59.24 ± 17.78		8	62–106		
III		155.50 ± 25.46	164.84 ± 25.42		8	107–116	110	3
IV		149.56 ± 10.82	148.67 ± 10.25	24 ± 1	8	117–147	138	4[a]
								5[b]
V		118.51 ± 19.48	131.12 ± 19.33		4	148–156		
VI		145.88 ± 19.87	147.01 ± 27.37		2	157–230		

[a] Number 4 was sampled from the middle of the two reactors;
[b] Number 5 was sampled form the bottom the two reactors.

Changes of major functional groups of PBS were characterized by a fourier transform infrared spectroscopy (FTIR) spectrometer (Avatar 370, Thermo Nicolet, USA) on days of 0, 50 and 230. The samples were analyzed through the scanning wave number from 4000 cm^{-1} to 550 cm^{-1} with 32 scanning times and the resolution of the spectrum was 4 cm^{-1}.

2.4. Microbial community analysis

2.4.1. DNA extraction and PCR amplification

The attached biofilms of different operation stages were sampled from the PBS granules through ultrasonic treatment (SK3200HP, Kedao Ultrasonic Instrument Co., Ltd., China) for 1 min at a frequency of 53 kHz, and then shaking for 5 min using a vortex shaker (WH-861, Hualida laboratory instrument Co., Ltd., China). After filtered through 0.22 μm sterile membrane, the biofilm samples were used for genomic DNA extraction by FastDNA® Spin Kit for Soil (MP Biomedicals, CA, USA). PCR amplification of 16S rRNA (V3–V4 hypervariable regions) genes from extracted DNA was performed using the primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) as previously described (Herlemann et al., 2011). The PCR mixture contained 10 ng DNA template, 1.0 μl primer mix (50 μM), 0.5 μl dNTP mixture (10 mM each), 5.0 μl 10× PCR buffer (2.5 mM), 0.5 μl Plantium Taq (5 U/μl) and other RNase-free water for the mixture up to 50 μl volume. PCR amplification program included an initial denaturation step of 3 min at 94 °C, followed by 5 cycles (94 °C for 30 s, 45 °C for 20 s, 65 °C for 30 s) and 20 cycles (94 °C for 20 s, 55 °C for 20 s, 72 °C for 30 s), with an final extension step of 5 min at 72 °C.

2.4.2. Illumina MiSeq sequencing

The PCR amplifications were extracted from 2% agarose gels and purified using the Agencourt® AMPure XP Beads (A63881, Beckman, USA). Then, the quality of gene library was detected using Qubit® 2.0 Fluorometer (Q32866, Invitrogen, USA) and Agilent 2100 Bioanalyzer (G2939AA, Agilent, USA). After that, the qualified amplifications were paired-end sequenced (2 × 250/300 nt multiplex) on an Illumina MiSeq platform at Zhejiang Institute of Microbiology (Hangzhou, Zhejiang, China) according to the standard protocols and software (Data collection software, Illumina).

3. Results and discussion

3.1. Long-term denitrification performance of the two reactors

The nitrate removal efficiency and volumetric removal rate of the two reactors during the whole experimental period are shown in Fig. 2. Both Reactor I and Reactor II achieved appreciable nitrate removal efficiency with an exception of stage I during which

synthetic RAS wastewater was used (Fig. 2A and B). In stage I, the average denitrification rates of both reactors were approximate 0.02 kg NO_3^--N m^{-3} d^{-1} (Fig. 2C and D). This result was relative lower when compared with other report that achieved approximate 0.3 kg NO_3^--N m^{-3} d^{-1} denitrification rate after 70 days experiment at 25 °C (Wu et al., 2013). In this study, low temperature (19 ± 1 °C) during the start-up period might be the main reason, since it strongly affected the hydrolysis efficiency of carbon source and the activity of the denitrifying bacteria (Chu and Wang, 2013).

The nitrate removal efficiencies of both reactors reached to approximately 90% in stage II when real RAS wastewater was used. It might be because of the abundant substrate composition, such as DO and DOC in the real aquatic water, since liquid organic carbon can also be utilized for denitrification. Meanwhile, when influent nitrate concentration changed from approximately 50 mg/L to 150 mg/L (stage III) and HRT decreased to 2 h (stage VI), the nitrate volumetric removal rates increased as well (Fig. 2C and D), which showed that the biofilm got mature gradually after acclimation under high nitrate loading. In stage VI, the average denitrification rates of the two reactors were 0.53 ± 0.19 kg NO_3^--N m^{-3} d^{-1} and 0.66 ± 0.12 kg NO_3^--N m^{-3} d^{-1}, which were consistent with SPCL denitrification process (0.64 ± 0.06 kg NO_3^--N m^{-3} d^{-1}) (Shen et al., 2013), and higher than PCL denitrification process (0.192 ± 0.031 kg NO_3^--N m^{-3} d^{-1}) (Chu and Wang, 2013), but lower than PHB denitrification process (1 kg NO_3^--N m^{-3} d^{-1}) (Gutierrez-Wing et al., 2012). This result might be caused by the different degradability and structural characteristics of varied materials and feeding types. From the perspective of stability, obvious fluctuations of effluent nitrate and were found in reactor I when operational conditions changed (Fig. 2, Table 1). The nitrate removal rates at the first 10 days of stage IV and VI in Reactor I were 71.77 ± 20.98% and 69.09 ± 27.86%, respectively, while 96.73 ± 2.90% and 98.98 ± 0.67% were achieved in Reactor II under similar influent nitrate concentration. This result indicated that the microflora in reactor II had relative higher capability to tolerate and adapt the environmental impacts, such as varied temperature and increased nitrate loading shock. Otherwise, the denitrification efficiencies of the two reactors decreased after approximate 200 days operation, which was also found in previous study (Chu and Wang, 2013). This might be because of the increased thickness of biofilm that limited the mass transfer and might cause turbulence in partial microenvironment. Therefore, regular backwash should be considered when using PBS denitrification reactor in RAS practice, especially under long breeding time. Nevertheless, backwash would result in biomass loss in the reactor which leads to the fluctuation of effluent. But it should not be lethal since part biofilm was existed in the core of PBS granules and further study of the in-depth impact was needed.

The increased DOC concentration in effluents of the two reactors indicated obvious biodegradation of the PBS polymer (Fig. 3). During stage I, the effluent DOC concentrations of Reactor I and Reactor II were 14.37 ± 8.44 mg/L and 23.83 ± 14.58 mg/L, respectively. The relative high DOC concentration at the first 10 days was caused by the PBS dissolution, and then decreased by the utilization of denitrifiers. Similar result of approximate 30 mg/L effluent DOC concentration was also found in previous study (Shen and

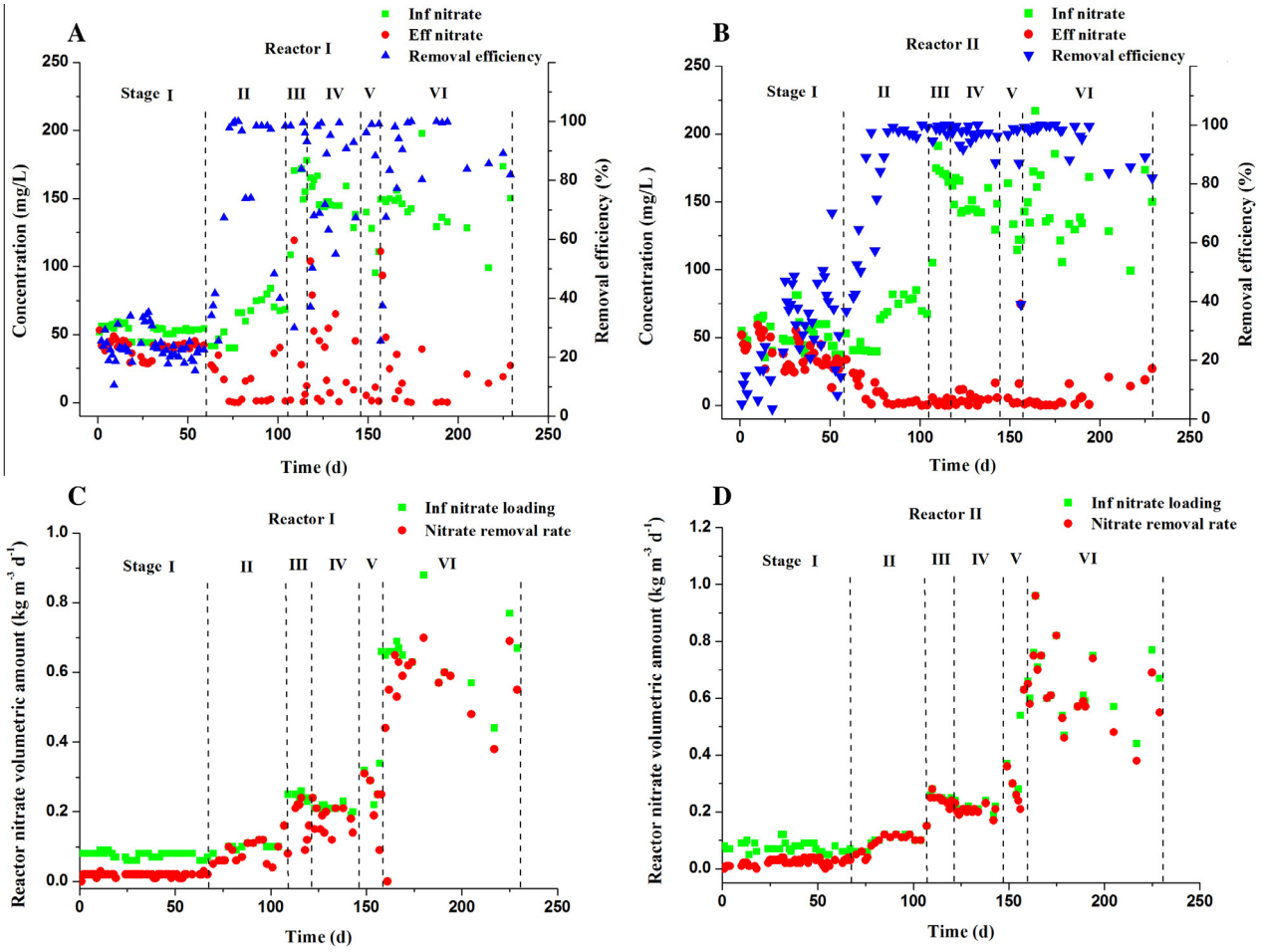

Fig. 2. The nitrate removal efficiency (Panel A and B) and volumetric removal rate (Panel C and D) of the two reactors.

Wang, 2011). However, it should be noted that the effluent DOC concentration increased obviously when real RAS wastewater was used. The increase of DOC was directly caused by the high influent DOC concentration of the real RAS wastewater, which could act as an alternative liquid carbon source. Besides, the complex influent component could also stimulate the microbial degradation activity. For example, DO was thought to accelerate the biodegradation of PBS (Luo et al., 2014). In our study, the DO was approximate 6.5 mg/L in the real RAS wastewater, while in synthetic wastewater the DO was approximate 1.5 mg/L. Moreover, as the degradation of PBS, more cores were formed and provided more specific surface area for degradation bacteria attachment. Overall, this phenomenon indicated that the released dissolved organic carbon exceeded the need of microbes for both growth and denitrification. Meanwhile, Reactor II had higher average effluent DOC concentration (202.51 ± 118.90 mg/L) than that of reactor I (136.11 ± 49.52 mg/L) under similar nitrate loading during stage II–VI, which can be concluded that the microbial biodegradation were more active with the existence of salinity. This phenomenon also existed in previous studies that used aliphatic polyester materials, such as PHB (Gutierrez-Wing et al., 2012) and PCL (Rutkowska et al., 1998) for denitrification, and the salinity was thought favor to hydrolytic efficiency and denitrification performance. Since synthetic polymers can generally be attacked either biochemically (enzymes and organisms) or mechanically by environmental factors (Lucas et al., 2008), the exceeded DOC release in this study might due to salinity accelerated surface erosion, and real RAS wastewater caused transformation of functional degradation bacteria (Table 2).

Though sufficient carbon source is indispensable to denitrification, excessive DOC not only waste PBS consumption but also causes effluent contamination. Moreover, as the bacteria depleted nitrate along with the up-flow reactor column, higher C/N ratio was favor to the activity of sulfate-reducing bacteria (SRB) that uses sulfate as electron donor, which result in the formation of toxic sulfides as the redox potential decreased (Gutierrez-Wing et al., 2012). However, at the bottom the relative high DO and nitrate concentrations are superior to sulfate for electron competition, due to its greater energy yield during the respiratory processes (Gutierrez-Wing et al., 2012). This hypothesis was confirmed by the existence of SRB species (*Desulfopila*) detected at the middle instead of bottom of reactor II during stage IV (Table 2). Theoretically, conventional strategy to control residual DOC is to increase influent nitrate loading, since the degradation process and denitrification process were considered mutually independent (Chu and Wang, 2013). However, sharply decrease of DOC concentration was not detected when both reactors achieved stable denitrification performance after the sudden increase of influent nitrate loading in stage III, V and VI (Figs. 2 and 3). This might be not only because the two reactors did not suffer the maximum loading but also that abundant ecological niche of degradation bacteria had already been established for the continuous DOC release. Therefore, the reactors still have more potential for denitrification performance improvement. Otherwise, improving the control of PBS degradation instead of enhanced nitrate loading might be a potential alternative in practice. Such strategy as regulation of microorganism activity by manipulating its cell to cell communication was demonstrated feasible in wastewater treatment biofilms (Shrout and Nerenberg, 2012). Hence, to clarify the metabolic and regulatory relationships between PBS degradation and denitrification pathways by quorum sensing (QS) might be an important subject awaiting future studies.

No obvious accumulation of nitrite (below 1 mg/L) was observed during the whole stage in both two reactors apart from operational conditions changed (Fig. 4, Table 1). Sudden increase of influent nitrate loading would result in decline of nitrate removal efficiency (Fig. 2) and nitrite fluctuation, which was more obvious in Reactor I (Fig. 4). The acceptable effluents nitrite concentration in both reactors indicated that sufficient organic could supply enough electrons for denitrification, since the accumulation of nitrite intermediate is often a result of electron competition among N-reductases involved in the denitrification process (Ge et al., 2012).

Increases of effluent TAN concentration were detected in both reactors (Fig. 4), which indicated that dissimilatory nitrate reduction to ammonium (DNRA) was occurred. In nitrogen cycle process, DNRA was considered as main substance and electron competitor with denitrification, and the influence factors such as higher temperature, carbon loads and increasing sulfide may favor DNRA over denitrification (Giblin et al., 2013). Our results confirmed the previous reports, as effluent TAN concentration of 5.35 ± 2.67 mg/L in Reactor II was observed when temperature shifted from 19 to 24 °C in stage IV. Besides, high residual DOC and sulfide concentration produced by SRB in Reactor II can also be used as substrates for nitrate reduction through both fermentative-DNRA and autotrophic-DNRA pathways (Giblin et al., 2013). Reactor I also achieved its average effluent TAN concentration of 1.33 ± 0.75 mg/L, but less than Reactor II. It was worth noting that

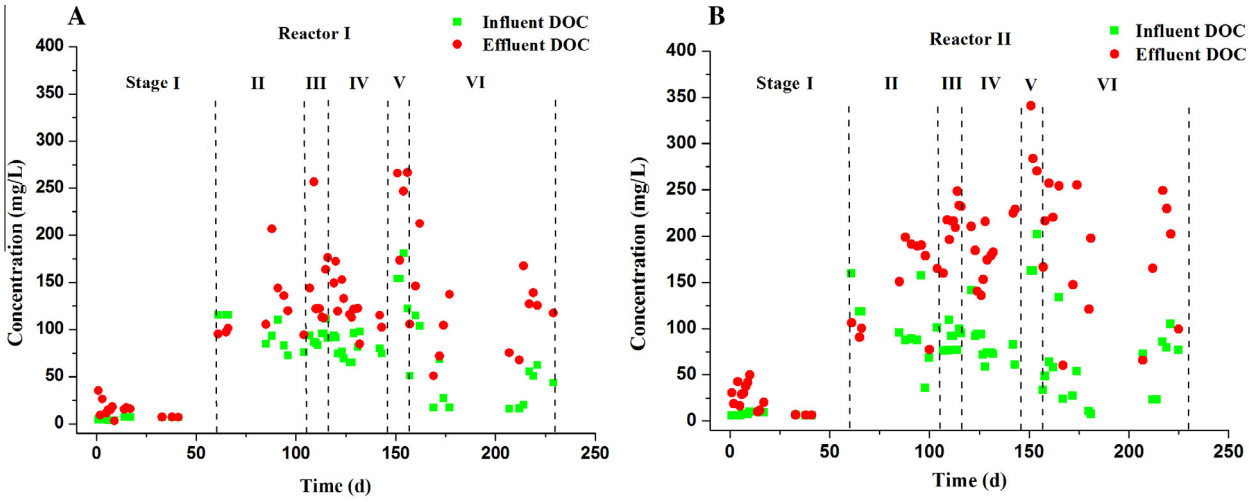

Fig. 3. The variation of DOC concentration in the influents and effluents of the two reactors (Panel A and B).

Table 2
Comparison of sequences percentage of the frequently identified genera in total number of sequences from the samples.

	Genus	Relative abundance (%)						
		Synthetic wastewater			Real RAS wastewater			
		1	2	Average	3	4[a]	5[b]	Average
Reactor I	*Acidovorax*[*]	49.5	8.7	29.1	3.4	1.5	1.6	2.2
	Afipia	0.0	2.3	1.2	0.3	24.7	9.3	11.4
	Azoarcus[*]	0.0	0.0	0.0	3.9	3.2	9.2	5.4
	Azospira	0.8	0.0	0.4	0.0	0.3	5.3	1.9
	Bdellovibrio	0.0	0.1	0.1	0.9	2.0	10.4	4.4
	Bradyrhizobium	0.3	16.8	8.5	16.9	12.2	5.6	11.6
	Brucella	0.3	0.3	0.3	0.3	0.0	0.3	0.2
	Comamonas	12.5	11.3	11.9	14.1	7.2	10.1	10.5
	Dechloromonas	1.5	4.8	3.2	4.0	3.3	4.8	4.0
	Ferruginibacter	0.0	1.1	0.6	0.6	1.8	1.0	1.1
	Oligotropha	0.1	5.7	2.9	5.1	6.8	0.0	4.0
	Pelomonas	0.2	2.8	1.5	2.8	0.2	0.0	1.0
	Rhizobium	6.0	8.8	7.4	1.3	4.3	2.1	2.6
	Sediminibacterium	0.0	5.6	2.8	1.2	0.2	0.6	0.7
	Simplicispira[*]	4.7	4.5	4.6	24.1	7.6	15.2	15.6
	Thermomonas	8.6	14.6	11.6	7.3	3.1	1.0	3.8
Reactor II	*Acidovorax*[*]	2.3	0.5	1.4	6.2	8.4	0.2	4.9
	Aequorivita	0.4	0.4	0.4	0.3	0.2	0.7	0.4
	Azoarcus[*]	34.7	23.7	29.2	31.4	19.5	35.0	28.6
	Colwellia	1.1	0.0	0.5	1.8	1.5	0.5	1.3
	Desulfopila	0.0	0.0	0.0	0.7	22.4	3.5	8.9
	Devosia	3.8	2.8	3.3	1.7	1.1	0.5	1.1
	Gracilimonas	0.0	0.7	0.4	2.2	2.6	1.7	2.2
	Hyphomonas	4.5	2.0	3.3	0.6	0.3	0.0	0.3
	Labrenzia	30.6	3.3	16.9	0.3	0.6	2.7	1.2
	OD1	0.0	0.0	0.0	17.6	14.0	16.3	16.0
	Owenweeksia	0.1	3.4	1.8	1.8	0.8	0.9	1.2
	Paracoccus	3.2	1.5	2.3	0.3	0.2	0.0	0.1
	Pseudomonas	3.8	0.8	2.3	2.8	1.6	1.1	1.8
	Simplicispira[*]	0.2	53.6	26.9	17.2	10.3	19.1	15.5
	Stappia	1.9	0.9	1.4	0.3	0.2	0.0	0.2
	Thalassospira	1.4	0.1	0.7	0.5	0.4	4.5	1.8

[a] Number 4 was sampled from the middle of the two reactors;
[b] Number 5 was sampled form the bottom the two reactors;
[*] Were the species present in the both reactors.

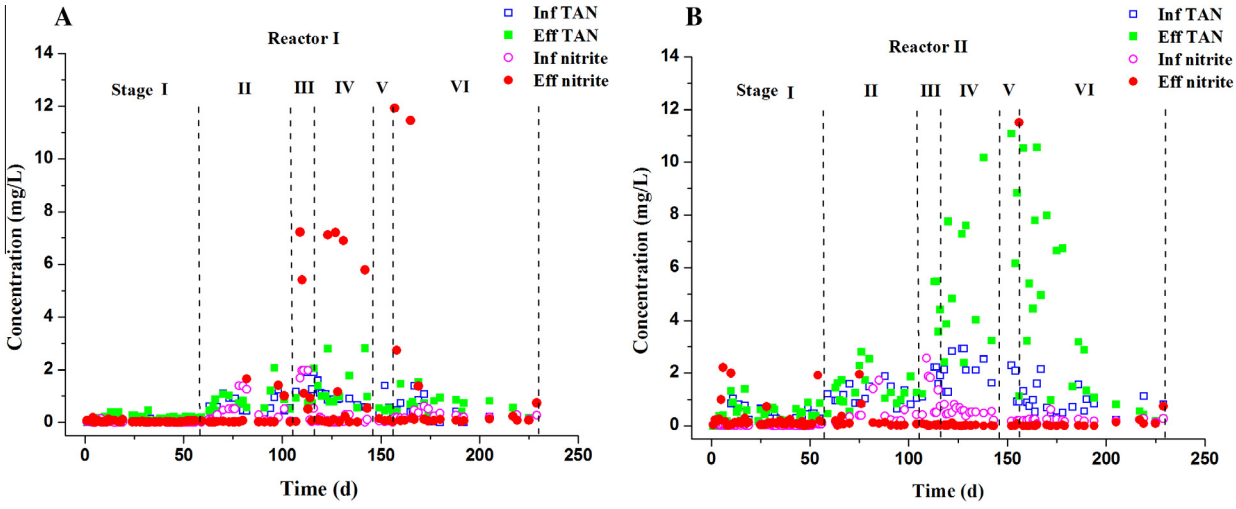

Fig. 4. The changes of inorganic-nitrogen concentration (TAN, NO_2^--N) in influents and effluents of the two reactors (Panel A and B).

when HRT sudden decreased to 2 h in stage IV, DNRA reduced significantly (Fig. 4B). This result indicated that high nitrate loading might favor denitrifiers re-domination in the reactor. In the stage V and VI, relative higher influent nitrate loading and more electron donors used by denitrifiers would also enhance denitrification performance, as it is more energy-gained pathway in such circumstance (Behrendt et al., 2014). Another reason might be that the high influent flow rate reduced the thickness of biofilm and brought in certain DO which suppressed DNRA activity (Wu et al., 2013). As the maximum TAN concentration allowed in the

aquatic water was 1.0 mg/L and 3.0 mg/L for cool-water fish and warm-water fish, respectively. Inhibition of the DNRA process in polymer denitrification system is needed in practice.

3.2. Degradative characterization of PBS by SEM and FTIR

Microscopic observation of the PBS polymer was shown in Fig. S1. The raw PBS granules exhibited a relative smooth and clear surface with attached massive crystalline precipitates. No obvious difference was shown between these two samples after 25 days of biological utilization, bacilli of similar feature were both observed sporadically in the surface of PBS. Further biodegrading was confirmed at day 205 of stage VI as obvious holes and hollows appeared in the surface of PBS. The biofilm became much richer and thicker especially in the holes which in return provided more anoxic microenvironment for bacterial attachment. Moreover, the sample in Reactor I appeared that the microbial groups were aggregated densely. Instead, the bacteria in reactor II seemed to be relatively incompact and more flagella were formed. Overall, observations from SEM micrographs provide clear evidence for PBS biodegradation and biofilm growth.

Changes in PBS functional groups before and after biodegradation were evaluated through FTIR spectroscopy (Fig. S2). The strong absorption peaks at 1722 and 1156 cm^{-1} were attributed to carbonyl (C=O) and —C—O—C— stretching in the ester group of fresh PBS. The absorption bands at 1329 cm^{-1} and 2945 cm^{-1} were caused by the symmetric and asymmetric deformational vibrations of —CH_2— groups in the main chains, respectively. The position of characteristic peaks had not changed a lot after biodegradation in both reactors. But, the peaks intensity at 1722 cm^{-1} and 1156 cm^{-1} increased in the samples of day 50 and 230, which indicated the hydroxylation of ester groups with more carboxylic end groups and ketone species were formed. Besides, the presence of vinyl groups was also detected in the unsaturated region between 800 and 1000 cm^{-1}, which further demonstrated the chain scission of polymer (Manzur et al., 2004). The relative lower density of peaks after 230 days biodegradation than that of 50 days might due to the degradation products leaching from the PBS surface and converting to CO_2 and water after being metabolized by microorganism (Phua et al., 2012).

3.3. Microbial community of biofilm during different stages

Illumina sequences of DNA extracted from each sample yielded more than 25,000 high quality reads, and the relative abundance of the main phylum was shown in Fig. 5. Based on the phylum results, the *Proteobacteria* (Reactor I, average 88.6 ± 2.6%; Reactor II, average 83.4 ± 10.5%) and *Bacteroidetes* (Reactor I, average 7.6 ± 2.1%; Reactor II, average 4.9 ± 0.9%) were most dominant over the samples, and then followed by *Actinobacteria* in Reactor I (average 0.6 ± 0.5%) and *Firmicutes* in Reactor II (average 1.4 ± 1.3%). This result was similar with previous solid-phase denitrification systems of PBS (Wu et al., 2013), PCL (Chu and Wang, 2013) or SPCL (Shen et al., 2013). Otherwise, to the best of our knowledge, the microbial communities of marine solid-phase denitrification system have not been studied yet. In our study, characteristic phylum of *OD1* was detected in reactor II (16.0 ± 1.8%) during stage II–VI, which has been widely detected in anaerobic environments sufficient with sulfur and DOC (Peura et al., 2012).

On the genus level, 520 genera were assigned to all samples, of which 16 frequently detected genera of reactor I and II during four operational stages were shown in Table 2. As for reactor I, the dominate genera were assigned to genus *Acidovorax* (average, 29.1%), *Comamonas* (average, 11.9%) and *Thermomonas* (average, 11.6%) during synthetic RAS wastewater stages. Then, *Acidovorax* and *Rhizobium* were found decreased conspicuously with the increase

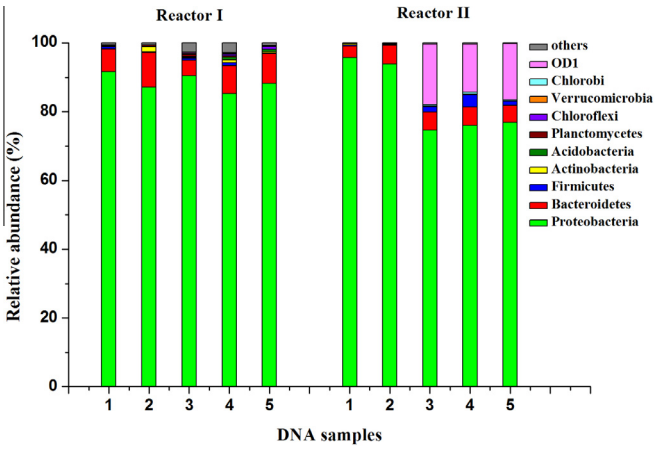

Fig. 5. Relative abundance of the main phylum identified in PBS biofilm samples.

of genera *Simplicispira* (average, 15.6%), *Bradyrhizobium* (average, 11.6%), *Afipia* (average, 11.4%) and *Azoarcus* (average, 5.4%) in the subsequent period when real RAS wastewater was used. Otherwise, *Comamonas* and *Dechloromonas* were relative stable during whole operational stage. Here, *Acidovorax*, *Azoarcu*, *Afipia* and *Comamonas* were considered to play partly polymer-degradation role (Chu and Wang, 2013; Horiba et al., 2005). Other denitrifying groups like *Thermomonas*, *Rhizobium*, *Simplicispira*, and *Bradyrhizobium* were also widely detected in wastewater denitrification systems (Heylen et al., 2006; Shen et al., 2013). In addition, *Bdellovibrio*, a predatory bacterium which attacks and consumes other bacterial strains, was detected with an extraordinary increase during stage V in reactor I. Thus, it can act as an potential antibiotic to control pathogenic bacteria in the aquatic system (Kandel et al., 2014). As for reactor II, *Azoarcus* was found dominant during the whole operational stage, which might be the main reason for high effluent DOC concentration and stable nitrate removal efficiency, as it has cooperative ability of denitrification and degradation (Horiba et al., 2005). Besides, denitrifying genera in activated sludge plant (Heylen et al., 2006), such as *Labrenzia* and *Paracoccus* showed high abundance during synthetic RAS wastewater period, and decreased when real RAS wastewater was used, while *Simplicispira* and *Acidovorax* showed adverse variation trend. Above all, the changes of abundance and species of functional denitrification and degradation microflora were the primary cause for the varied reactor performance.

3.4. Future research prospect

This study demonstrated that PBS could act as denitrification carbon source for RAS wastewater treatment. The existence of salinity (25‰) could improve the efficiency and stability of the system, which makes it possible for valuable marine species farming. However, how to control the DOC release and utilization and avoid sulfate reduction and DNRA electron competition with denitrification is crucial of this kind of polymers denitrification. Based on the organic carbon release regulation, potential solutions include cell to cell communication which could elucidate the mechanism of carbon metabolism pathways. Besides, blending PBS with cellulose materials such as bamboo powder is also feasible to slow the degradation of packing. Based on DOC utilization, avoiding nitrate exhaust in one circulation and increasing ORP by DO might be beneficial to reduce effluent DOC accumulation.

Considering RAS operation characteristic, the finding of this study suggested that nitrate should not be removed in only one

circulation since the continuous recirculation of water. Not like other studies that primary focus on high nitrate removal efficiency, the effluent nitrate residual is needed especially in marine system, which might simultaneously help to inhibit sulfate reduction and DNRA, as well as DOC residual. Moreover, since DO could promote PBS-denitrification process (Luo et al., 2014), potential implication of alternatively aerobic/anoxic operational conditions for simultaneous nitrification and denitrification in RAS wastewater treatment is an interesting research awaiting for future study.

4. Conclusion

High denitrification rates in PBS solid-phase packing reactors were achieved for real RAS wastewater treatment. The existence of salinity (25‰) exhibited more stable nitrate removal efficiency when suffering changed operational conditions, but caused adverse effects such as excessive effluent DOC and DNRA activity. The SEM and FTIR analysis demonstrated the degradation of PBS. Illumina sequencing revealed the changes of abundance and species of functional denitrification and degradation microflora which took the fundamental responsibility for the varied reactor performance. Overall, PBS showed great potential in denitrification but still needed further study on precise carbon release for RAS practice.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.06.021.

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