Environ Sci Pollut Res (2015) 22:5887–5894
DOI 10.1007/s11356-014-3755-1

RESEARCH ARTICLE

Enhancement of post-anoxic denitrification for biological nutrient removal: effect of different carbon sources

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Received: 21 August 2014 / Accepted: 20 October 2014 / Published online: 30 October 2014

Abstract Previous research has demonstrated that post-anoxic denitrification and biological nutrient removal could be achieved in the oxic/anoxic/extended-idle wastewater treatment regime. This study further investigated the effect of different carbon sources on post-anoxic denitrification and biological nutrient removal. Acetate, propionate (volatile fatty acids (VFAs)), glucose (carbohydrate), methanol, and ethanol (alcohol) were used as the sole carbon source, respectively. The experimental results showed that VFA substrates led to an improvement in nitrogen and phosphorus removal. The total nitrogen and phosphorus removal efficiency values driven by acetate achieved 93 and 99 %, respectively. In contrast, glucose present in mixed liquor deteriorated total nitrogen and phosphorus removal efficiency values to 72 and 54 %. In the reactors cultured with methanol and ethanol, 66 and 63 % of the total nitrogen were removed, and phosphorus removal efficiency values were 78 and 71 %, respectively. The mechanism studies revealed that different carbon sources affected the transformations of intracellular polyhydroxyalkanoates (PHAs) and glycogen. PHAs are the dominant storages for microorganisms cultured with VFA substrates. Though glycogen is not the favorable energy and carbon source for polyphosphate-accumulating organisms, it can be consumed by microorganisms related to biological nitrogen removal and is able to serve as the electron donor for post-anoxic denitrification.

Keywords Biological nutrient removal · Carbon source · Oxic/anoxic/extended-idle process · Sequencing batch reactor · Polyhydroxybutyrates

Responsible editor: Gerald Thouand

Electronic supplementary material The online version of this article (doi:10.1007/s11356-014-3755-1) contains supplementary material, which is available to authorized users.

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Introduction

Biological nutrient removal (BNR) processes have been extensively used for removing nitrogen and phosphorus from wastewaters (Kuba et al. 1996). In BNR processes, microbes take up volatile fatty acids (VFAs) presented in wastewaters and store them as polyhydroxybutyrates (polyhydroxyalkanoates (PHAs)) under anaerobic conditions, with glycogen providing reducing equivalents for PHA accumulation (Arun et al. 1988). During the subsequent aerobic period, PHAs are consumed to replenish glycogen and to serve as energy and carbon sources for microbial growth and phosphorus uptake (Pereira et al. 1996).

The performance of BNR processes is directly related to the available carbon sources present in wastewaters (Osaka et al. 2008). The most suitable carbon source for BNR is generally considered to be short-chain VFAs (Abu-ghararah and Randall 1991; Osaka et al. 2008). In enhanced biological phosphorus removal (EBPR) systems, polyphosphate-accumulating organisms (PAOs) accumulate P and store it as intracellular polyphosphate (poly-P) under alternating anaerobic/aerobic conditions (Chen et al. 2004; Mullan et al. 2006) while another group of microorganisms known as glycogen-accumulating organisms (GAOs) can compete with PAOs for organic substrates without contributing to P removal,

which commonly caused process upsets and deterioration of EBPR systems (Thomas et al. 2003). Different carbon sources have been proven to have an impact on PAO-GAO competition and intracellular polymer synthesis (Oehmen et al. 2007). Acetate was proved to be the most prevalent VFA in EBPR plants and is used for poly-3-hydroxybutyrate (PHB) synthesis (Hesselmann et al. 1999; McMahon et al. 2002). Recent studies have suggested that propionate may be a more favorable substrate than acetate for successful EBPR performance (Thomas et al. 2003; Chen et al. 2004). Although propionate has been hypothesized to be effective in selecting against *Competibacter*, another group of GAOs named *Alphaproteobacteria* has the capacity to compete with PAOs for propionate, leading to the deterioration of P removal performance (Oehmen et al. 2006; Meyer et al. 2006).

Though acetate, propionate, and other VFAs have been successfully applied to improve BNR performance (Oehmen et al. 2007), the addition of VFA substrates considerably increases the overall treatment costs. Several recent studies have shown that glucose, ethanol, and other non-VFA substrates could drive high BNR performance (Oehmen et al. 2007; Yang et al. 2012). Many non-VFA substrates have been shown to be fermented to VFAs such as acetate and propionate prior to substrate uptake by PAOs or GAOs (Oehmen et al. 2007). The most widely tested carbon substrate other than VFAs is glucose, which is required for energy generation to be used in PHAs and poly-P synthesis (Wang et al. 2002). However, other substrates such as alcohol have been few reported.

In our previous studies, introducing an anoxic period after the aeration (i.e., oxic/anoxic/extended-idle (O/A/EI) process) to achieve post-anoxic denitrification driven by PHB was proven to be feasible in a feast-famine wastewater treatment condition (Chen et al. 2013). Further investigations showed that excellent wastewater BNR was also obtained in the modified O/A/EI process (Li et al. 2014). Though it is known that the accumulation of PHAs and glycogen is mainly dependent on the type of carbon sources, the effect of different carbon sources on BNR performance induced by post-anoxic denitrification is unclear.

The purpose of this work was to investigate the effect of different carbon sources on post-anoxic denitrification and BNR performance. First, N and P removal performances in the process fed with different carbon sources were compared. Then, cyclic variations of N, P, and intracellular polymers in the reactors were analyzed. Finally, the mechanisms for different substrates affecting post-anoxic denitrification and BNR performance were explored by investigating microbial population abundance, key enzyme activities, and intracellular PHA and glycogen synthesis.

Materials and methods

SBR operation

Experiments were performed in five lab-scale sequencing batch reactors (SBRs) each with a working volume of 12 L. Activated sludge collected from a wastewater treatment plant in Changsha, China, was inoculated into the five SBRs. The initial concentration of mixed liquor suspended solids (MLSSs) was about 4,000 mg L^{-1}. Aeration was supplied through an air diffuser placed in the bottom of the SBRs at a flow rate of 4 L min^{-1}, and mixing was accomplished using magnetic stirrers. Temperature inside the SBRs was maintained at 23±2 °C with a thermostatic heater, and the pH value was controlled at 7.0±0.2 by dosing 1 M HCl or 1 M NaOH. All SBRs were operated sequentially in 6-h cycle. The cycling profile comprised 120-min aeration, 90-min anoxic mix, followed by 25-min settling, 5-min decanting, and 120-min idle periods. Supernatant (10 L) was discharged from the SBRs at the end of settling phase and was replaced with 10-L synthetic medium. Sludge mixtures (1.5 L) were discharged once per day at the end of aerobic zone but before settling, resulting in a sludge retention time and hydraulic retention time of 8 h.

Wastewater

The synthetic feeding medium used as influent contained 40 mg L^{-1} NH_4^+, 15 mg L^{-1} PO_4^{3-}, and 300 mg L^{-1} COD. Acetate, propionate (VFAs), glucose (carbohydrate), methanol, and ethanol (alcohol) were used as carbon sources, respectively. The concentrations of the other nutrients in the synthetic medium were presented as below: 0.01 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.005 g L^{-1} $CaCl_2$, and 0.5 mL L^{-1} trace element solution described by Zheng et al. (2011).

Analytical methods

NH_4^+, NO_2^-, NO_3^-, SOP, COD, MLSS, and mixed liquor volatile suspended solids (MLVSSs) were measured according to the standard methods for the examination of wastewater (APHA 1998). Sludge glycogen, PHB, poly-3-hydroxyvalerate (PHV), and poly-3-hydroxy-2-methylvalerate (PH2MV) were measured according to the methodology described by Wang et al. (2009). The total PHAs were calculated as the sum of PHB, PHV, and PH2MV. Analysis of exopolyphosphatase (PPX), polyphosphate kinase (PPK), ammonia monooxygenase (AMO), nitrite oxidoreductase (NOR), nitrate reductase (NR), and nitrite reductase

(NIR) activities was performed according to the reference (Zheng et al. 2011).

4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) staining was carried out to analyze the presence of intracellular poly-P granules (Mullan et al. 2006). Sludge samples taken at the end of the aerobic period were used for staining. The analysis of fluorescence in situ hybridization (FISH) technique was the same as described in the works of literature (Carvalho et al. 2007; Wong et al. 2005). Sludge samples taken from the aerobic period at various times were analyzed by FISH for PAOs, GAOs, nitrite-oxidizing bacteria (NOB), and ammonia-oxidizing bacteria (AOB). The probes used for FISH are listed in Table S1 of Supporting Material (SM). FISH quantification was performed by Image-pro plus 7.0 Software; the relative abundance of bacteria of interest was determined as the mean percentage of all bacteria.

Statistical analysis

All measurements were performed in triplicate. An analysis of variance was used to evaluate the significance of results, and $p<0.05$ was considered to be statistically significant.

Results and discussion

Variations of MLSSs and MLVSSs in the SBRs

It is well known that activated sludge biomass concentration was important to the system performance in activated sludge processes. From Table 1, no enormous difference was observed among the MLVSSs/MLSSs in the five SBRs, displaying the fact that MLSS and MLVSS concentrations were differed in these SBRs. This suggested that different carbon sources resulted in different levels of biomass. Moreover, MLSSs and MLVSSs in VFA-cultured SBRs were higher than those in non-VFA SBRs, which indicated that VFA substrates are conducive to the growth of biomass.

Table 1 Variations of MLSS and MLVSS in the SBRs during steady-state operation

Carbon source	MLVSS	MLSS	MLVSS/MLSS
Acetate	2603±141	3679±226	0.71±0.03
Propionate	2578±137	3736±243	0.69±0.02
Glucose	2461±124	3418±231	0.72±0.04
Methanol	2418±118	3312±224	0.73±0.04
Ethanol	2397±126	3329±227	0.72±0.03

Results are averages and their standard deviations, and data are obtained during day 106 and day 136

Biological nutrient removal in the SBRs

COD removal

The SBRs fed with different carbon sources were operated steadily for 150 days. The data of the effluent NH_4^+, NO_2^-, NO_3^-, SOP, and COD concentrations and the NH_4^+, total N (TN), SOP, and COD removal efficiency values of five SBRs during steady-state operation are summarized in Table 2.

From Table 2, no enormous difference of COD removal was observed among the five reactors because the COD removal efficiency values in the five SBRs were all above 90 % and $p>0.05$ (Fig. S2). This is expected since all the feeding substrates are readily biodegradable organic compounds.

SOP removal

Table 2 depicts that 98 and 99 % of the influent SOP were removed, respectively, when acetate and propionate were respectively used as the sole carbon source. However, in the SBR fed with methanol and ethanol, obvious decreases of SOP removal were detected. Effluent SOP concentration in methanol and ethanol-cultured SBRs was 3.32 and 4.35 mg L^{-1}, respectively, far higher than the 0.13 mg L^{-1} in SBR fed with propionate ($p<0.05$). In glucose-cultured SBR, SOP removal efficiency was decreased to 54 %, and effluent SOP was above 6.87 mg L^{-1}, which was far higher than the 3.32 mg L^{-1} in methanol-cultured SBR ($p<0.05$). Different carbon sources have been shown to have different impacts on the PAO-GAO competition (Oehmen et al. 2007). The data of SOP removal in the SBRs suggested that SOP removal efficiency values depended strongly on the type of carbon source. It can be concluded from Table 2 that SOP removal efficiency driven by VFA substrates was found to be higher than that driven by non-VFA substrates.

To express the SOP removal performance in the SBRs more accurately, SOP release and uptake were also transformed relative to the mean VSS during the steady-state operation (Fig. 1). It can be found that 5.2-mg SOP release and 10.3-mg SOP uptake per gram of VSS were detected in propionate-cultured SBR, reflecting the good SOP removal performance of the reactor. In contrast, SOP release and uptake in the SBR fed with glucose were just 1.2 and 4.3 mg P g^{-1} VSS, respectively. Furthermore, the cyclic variations of SOP in the SBRs are shown in Fig. 2. The high SOP release and uptake in VFA-cultured SBRs suggest that VFA substrates can drive higher SOP removal than non-VFA substrates (Fig. 3). From Fig. 2, it can be concluded that denitrifying P removal occurred in the SBRs, and a part of the BNR should be contributed by nitrite or nitrate-driven denitrifying P removal.

Table 2 Summary of biological nutrient removal performance in the SBRs during steady-state operation

Carbon source	N						SOP		COD	
	Influent NH_4^+-N (mg L^{-1})	Effluent NH_4^+-N (mg L^{-1})	Effluent NO_2^--N (mg L^{-1})	Effluent NO_3^--N (mg L^{-1})	TN removal efficiency (%)	Influent SOP (mg L^{-1})	Effluent SOP (mg L^{-1})	SOP removal efficiency (%)	Effluent COD (mg L^{-1})	COD removal efficiency (%)
Acetate	40	0.16±0.04	0.83±0.01	1.81±0.01	93±2.3	15	0.31±0.02	99±1.3	18±0.52	94±2.3
Propionate	40	0.13±0.03	2.56±0.01	3.31±0.64	85±2.7	15	0.15±0.06	98±0.7	21±0.52	93±1.6
Glucose	40	0.21±0.12	6.32±0.13	4.67±0.14	72±1.8	15	6.87±0.12	54±1.6	23±0.52	92±1.8
Methanol	40	0.24±0.13	6.87±0.12	6.49±0.16	66±2.1	15	3.32±0.18	78±2.3	22±0.52	93±2.1
Ethanol	40	0.29±0.13	8.94±0.17	5.57±0.13	63±2.4	15	4.35±0.24	71±1.8	26±0.52	91±2.7

Results are averages and their standard deviations

Figure 4 shows the DAPI staining results of the sludge samples taken from the SBRs. More blue areas in Fig. 4a, b were found than in other pictures, suggesting that more poly-P granules were accumulated in VFA-cultured SBRs than in non-VFAs-cultured SBRs, which was in accordance with the data of SOP release and uptake in Fig. 2. In addition, it has been reported that a low MLVSS/MLSS ratio usually implied that a high level of poly-P was stored in the sludge (Oehmen et al. 2005; Wang et al. 2012). In Table 1, the MLVSS/MLSS ratio in the SBR cultured with propionate was lower than those in other SBRs, which was consistent with the DAPI staining results and supported the data of the SOP removal presented in Table 2.

Nitrogen removal

As shown in Table 2, most of the NH_4^+ was oxidated to NO_2^- and NO_3^- in the SBRs. It can be seen that 13.6 and 14.8 mg L^{-1} of TN were monitored in the effluent of methanol and ethanol-cultured SBRs, respectively, which suggested that only 66 and 63 % of the influent TN were removed. However, a high level of TN removal was achieved in the two SBRs fed with VFA substrates ($p<0.05$). Effluent TN in acetate and propionate-cultured SBRs was kept about 2.8 and 6.1 mg L^{-1}, and the TN removal efficiency values driven by acetate and propionate were above 93 and 85 %, respectively. It was clearly observed that TN removal driven by VFA substrates was higher than that driven by non-VFAs ($p<0.05$), and glucose drove a higher TN removal than methanol and ethanol ($p<0.05$). These results were consistent with the works of literature (Osaka et al. 2008; Yang et al. 2012).

Figure 2 shows the cyclic variations of NH_4^+, NO_2^-, and NO_3^- in the SBRs feed with different substrates. Of note, nearly complete nitrification was observed in the SBRs, but NO_2^- and NO_3^- accumulation and denitrification driven by VFA substrates were higher than those driven by non-VFA substrates. Especially, in the SBR fed with acetate, NO_2^- and NO_3^- accumulation was achieved at 12.4 and 23.5 mg L^{-1}, respectively, which was higher than those in other SBRs. In Fig. 1, it can be found that though no enormous difference was observed among the specific nitrification rates in the SBRs, the specific denitrification rate in acetate-cultured SBR was higher than that in other SBRs.

Microbial population with different carbon sources

First, the bacterial population in EBPR sludge was varied with different carbon sources (Lemaire et al. 2006; Oehmen et al. 2007). In addition, the microbial competition of NOB with AOB was considered to be responsible for partial nitrification to nitrite (Ma et al. 2009). Therefore, quantitative analysis of PAO, GAO, NOB, and AOB abundance in activated sludge was carried out via FISH technology (Table 3).

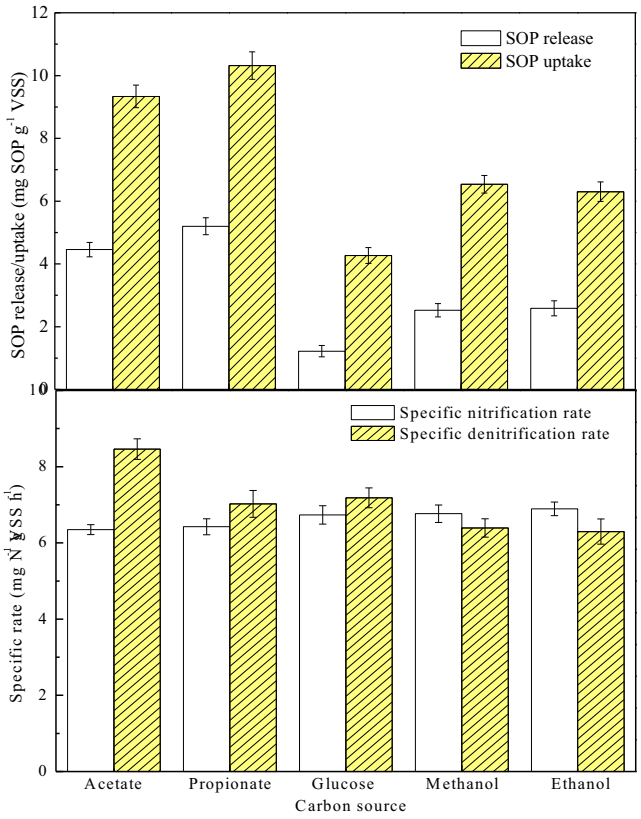

Fig. 1 SOP release and uptake and specific nitrification and denitrification in the SBRs during the steady-state operation. The data reported are the averages and their standard deviations in triplicate tests

The FISH analysis indicates that it can supply PAOs advantage in the competition with GAOs when fed with VFA substrates, but GAOs tend to become stronger competitors against PAOs in glucose-cultured SBR. These results are consistent with the data of SOP removal efficiency values and suggest that different carbon sources, VFAs, and non-VFA substrates have an impact on the PAO-GAO competition.

Moreover, the quantitative analysis showed that the abundance of NOB and AOB accounted for 21~24 % and 15~19 % of the total biomass in the SBRs, respectively, and the difference in NOB and AOB abundance in different carbon-source-cultured reactors was relatively small. This result was in accord with the similar specific nitrification rate in the SBRs and indicated that carbon sources had little influence on the abundance of NOB and AOB.

Key enzyme activities with different carbon sources

It is known that SOP release and uptake are directly related to PPX and PPK activities, respectively (Mino et al. 1998). In Table 4, the activities of PPX and PPK detected in the SBRs fed with VFA substrates were higher than those in non-VFA-cultured SBRs. Nevertheless, in consideration of the higher PAO abundance cultured by VFAs, no obvious variations in PPX and PPK activities per mean PAO abundance were observed in the SBRs. This suggested that VFA substrates improving SOP removal performance was ascribed to the

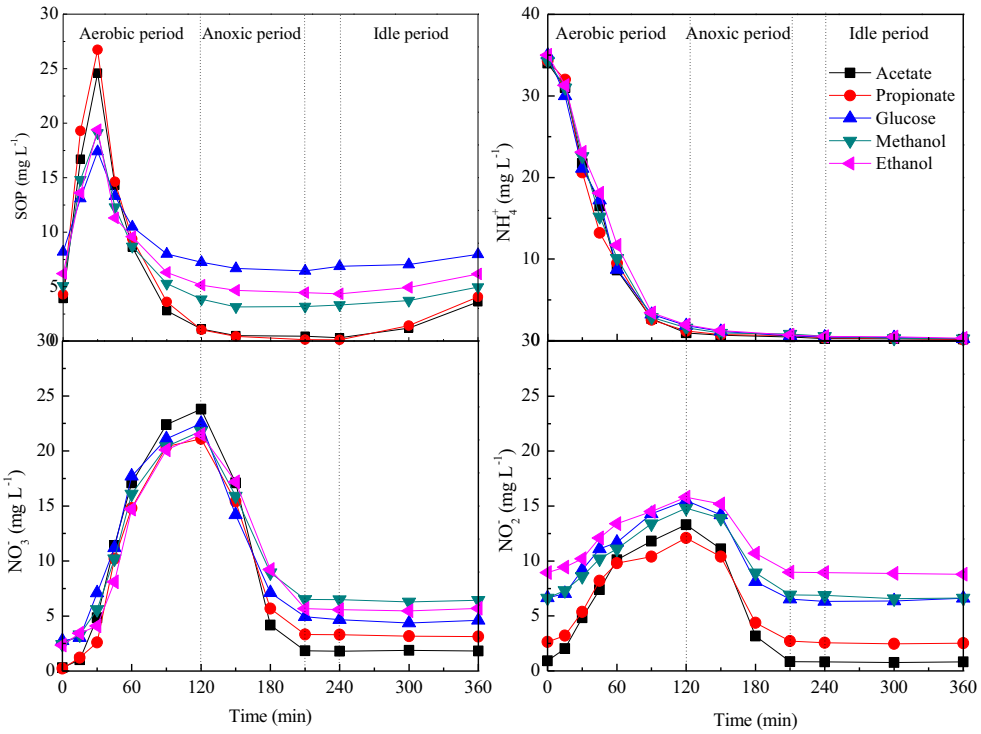

Fig. 2 Variations of SOP, NH_4^+, NO_2^-, and NO_3^- during one typical cycle of the SBRs. The data reported are the averages and their standard deviations in triplicate tests

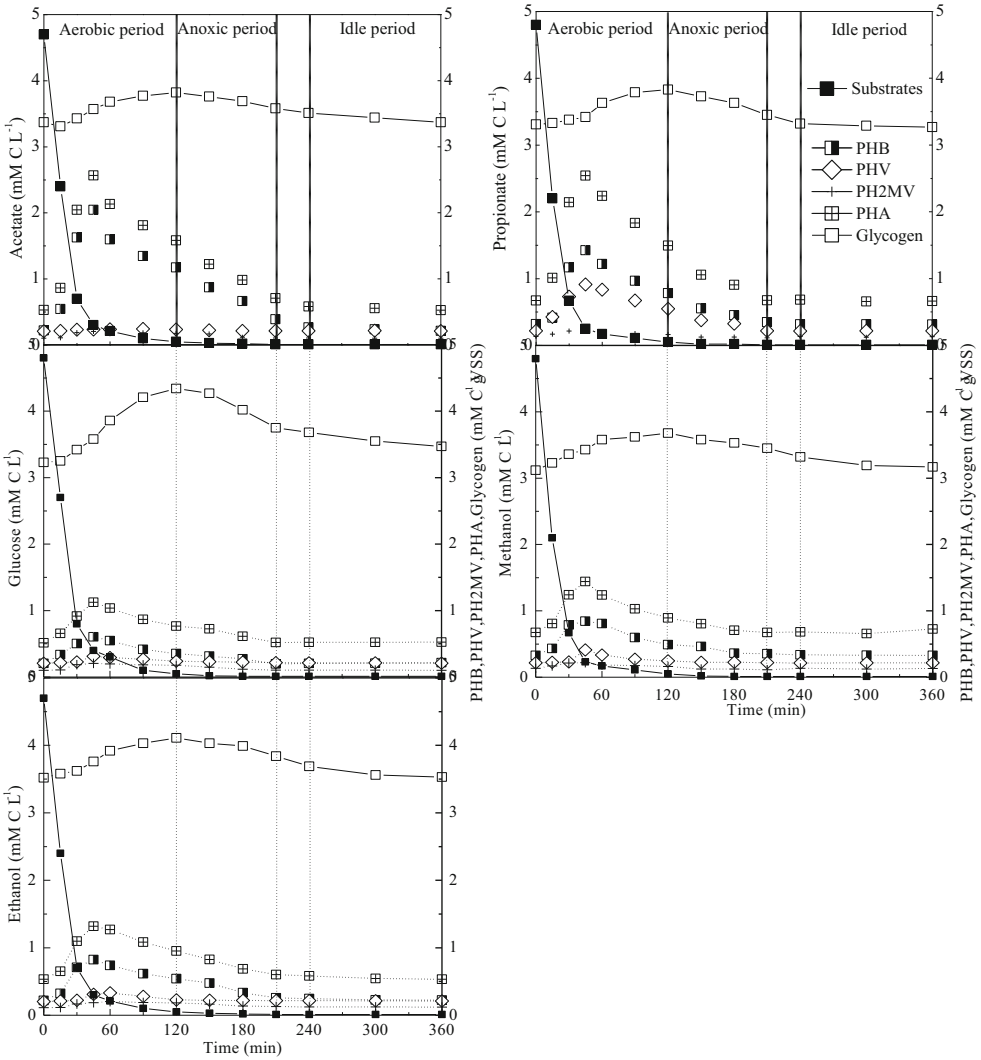

Fig. 3 Variations of substrates, PHB, PHV, PH2MV, total PHAs, and glycogen during one typical cycle of the SBRs. The data reported are the averages and their standard deviations in triplicate tests

increase of PAO abundance but not the enhancement of PPX and PPK activities.

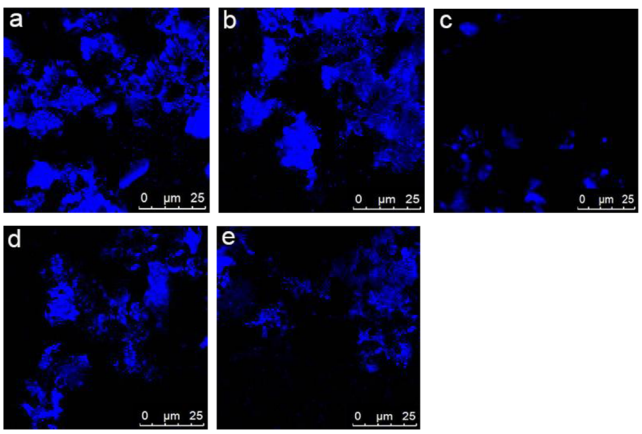

Fig. 4 Micrographs of DAPI staining of the activated sludge samples withdrawn at the end of aeration in the SBRs. *Scale bar*=5 μm

Further investigation showed that different carbon sources did not affect the activities of enzymes relevant to biological nitrogen removal. AMO and NOR are two key enzymes in nitrification, and the ratio of denitrification is correlated with NR and NIR activities (Kristjansson and Hollocher 1980). From Table 4, it can be found that no obvious variations of AMO, NOR, NR, and NIR activities were measured in the

Table 3 Bacterial population in the SBRs

Carbon source	PAOs (%)	GAOs (%)	NOB (%)	AOB (%)
Acetate	26±2.4	13±1.2	24±2.7	16±2.2
Propionate	28±2.7	16±1.8	23±3.2	15±1.6
Glucose	18±1.8	24±2.3	21±1.4	19±2.3
Methanol	23±2.4	17±1.4	23±2.1	16±1.7
Ethanol	21±1.6	19±2.1	22±1.6	18±2.1

Percentage to all bacteria (EUBmix probe). The values are averages and standard deviations

Table 4 Activities of the key enzymes related to biological nutrient removal in the SBRs

Carbon source	PPX[a]	PPK[b]	AMO[c]	NOR[c]	NR[c]	NIR[c]
Acetate	0.029±0.004	0.273±0.021	0.033±0.006	0.072±0.004	0.032±0.003	0.283±0.008
Propionate	0.026±0.003	0.265±0.018	0.028±0.004	0.073±0.005	0.041±0.004	0.268±0.005
Glucose	0.021±0.002	0.228±0.017	0.026±0.003	0.064±0.003	0.036±0.003	0.224±0.006
Methanol	0.023±0.004	0.237±0.014	0.024±0.005	0.075±0.008	0.043±0.007	0.257±0.004
Ethanol	0.025±0.002	0.261±0.017	0.023±0.003	0.067±0.004	0.039±0.003	0.254±0.003

The data reported are the averages and their standard deviations in triplicate tests

[a] The unit is μmol *p*-nitrophenol/(min mg protein)

[b] The unit is μmol NADPH/(min mg protein)

[c] The unit is μmol nitrite/(min mg protein)

SBRs. These results indicate that different carbon sources have no obvious effect on the activities of enzymes relevant to biological nitrogen and phosphorus removal, and the reason for VFAs improving biological nitrogen and phosphorus removal is still unclear.

Intracellular polymer synthesis with different carbon sources

It was reported that post-anoxic denitrification is closely related to the transformations of intracellular PHAs and glycogen, which have been hypothesized to be important carbon sources driving post-anoxic denitrification (Vocks et al. 2005). Therefore, the high PHA accumulation detected in acetate-cultured SBR suggests high denitrification ability and results in high TN removal.

The cyclic variations of substrates, PHB, PHV, PH2MV, total PHAs, and glycogen in the SBRs are illustrated in Fig. 3. As shown in Fig. 3, substrates are fully depleted during the initial 60 min of the aeration, accompanied by the gradual accumulation of intracellular PHAs and glycogen. After substrates are consumed, PHA degradation and SOP uptake are observed concurrently. However, the SBRs cultured with VFA substrates show higher PHA variations.

Recently, some works of literature reported that post-anoxic denitrification could be driven by PHAs and glycogen (Coats et al. 2011). In this study, the SBRs have almost the same accumulation of PH2MV, but the PHB synthesis with acetate is higher than that in other SBRs (Fig. 3). Thus, the higher PHA synthesis cultured with acetate is due to the increase of PHB but not PHV and PH2MV production. Moreover, negligible glycogen is consumed during the anoxic period in VFA-cultured SBRs. Therefore, the carbon sources driving post-anoxic denitrification in the SBRs cultured with acetate and propionate are proven to be PHAs. And, high accumulation of PHAs (especially for PHB and PHV) in the nitrification makes the biomass cultured with acetate and propionate show high denitrification ability.

From Fig. 3, it can be inferred that external substrates are quickly depleted in the initial oxic period. Therefore, the whole process in this study can be divided into two periods: a feast period defined as the time when external substrates were consumed in the initial oxic period and a famine period when external substrates had been exhausted in the aerobic zone, the anoxic phase, as well as the extended-idle period. Since the bacteria is to encounter external substrate feast and famine process, it tends to store external substrates as internal storage compounds during the feast period, which hereby can be consumed to gain a more balanced growth (Carucci et al. 2001; Wang et al. 2009). The results in Fig. 3 indicate that PAOs could take up substrates quickly for PHA synthesis and exhibit a rapid SOP release in the initial oxic period, suggesting that the initial oxic phase could still facilitate the favorable proliferation of PAOs.

Figure 3 also shows that PHAs and glycogen were the dominant aerobic storages when acetate and glucose were fed to SBRs as the sole carbon source, respectively. In glucose-cultured SBR, PHA variation was low while glycogen consumption is high as compared with other SBRs. Though a low SOP removal was induced by glucose, TN removal efficiency in glucose-cultured SBR achieved 72 %. These results indicate that though glycogen was not the favorable energy source for PAOs, it could be used by microorganisms related to biological nitrogen removal and drove post-anoxic denitrification.

Conclusions

Post anoxic denitrification in feast-famine process depended strongly on the types of carbon sources, and high BNR removal was obtained when VFA substrates were used as carbon source. Different carbon sources affected the transformation of PHAs and glycogen. The biomass cultured with acetate accumulated more PHAs, which can drive post-anoxic denitrification and results in the high BNR performance.

Acknowledgments This research is based upon work supported by the project of the Hunan Provincial Innovation Foundation for Postgraduate

(CX2014B140), the Shanghai Tongji Gao Tingyao Environmental Science and Technology Development Foundation (STGEF), and the National Natural Science Foundation of China (NSFC) (Nos. 51278175 and 51378188).

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