Environmental Engineering and Management Journal

January 2018, Vol.17, No. 1, 1-9
http://www.eemj.icpm.tuiasi.ro/; http://www.eemj.eu

"Gheorghe Asachi" Technical University of Iasi, Romania

EFFECT OF INFLUENT C/N AND C/P RATIOS ON NUTRIENTS REMOVAL INVESTIGATED VIA CHEMICAL AND MICROBIAL ANALYSES IN A NOVEL BNR-IC PROCESS

Haiming Zou[1,2], Xiwu Lu[2*], Abualhail Saad[2,3]

[1]Department of Resource and Environment, Anhui Science and Technology University, Donghualu Road, Fengyang 233100, PR China
[2]School of Energy and Environment, Southeast University, Sipailou Road, Nanjing 210096, PR China
[3]Department of Civil Engineering, College of Engineering, University of Basrah, Basra, Iraq

Abstract

This work describes the variations in nitrogen (N) and phosphorus (P) removal performances and bacterial population dynamics in response to different influent C/N and C/P ratios in a novel BNR-IC process combining biological nutrients removal (BNR) with induced crystallization (IC). A good nutrients removal performance was achieved at 7.8 of C/N ratio and 23.3 of C/P ratio, where P and TN removal efficiencies were 94.0±2.4% and 90.5±4.3%, respectively. Moreover, significant changes of microbial community structure were found with the variations of influent C/N and C/P ratios. Increasing influent C/N and C/P ratios favored the development of DPAO, thus enhancing the TN and P removal efficiencies and causing a decrease in microbial community biodiversity. However, excessive P load in the influent strongly influenced the P removal performance when C/N ratio was maintained at appropriate levels. PCR-DGGE showed that microbial populations in sampled sludge were classified into five different phylum or class (*Alpha*-, *Beta*-, and *Gammaproteobacteria*, as well as *Firmicutes*, *Actinobacteria*).

Keywords: denitrifying polyphosphate accumulating organisms, enhanced biological phosphorus removal, nutrients removal, polymerase chain reaction-denaturing gradient gel electrophoresis, wastewater treatment

Received: July, 2013; Revised final: April, 2014; Accepted: April, 2014; Published in final edited form: January 2018

1. Introduction

Although nitrogen (N) and phosphorus (P) are essential nutrients for the growth of biomass, excess discharges of N and P into water bodies could lead to a threat to aquatic ecosystems, especially potential contributions to eutrophication, occurring often in three lakes of China (i.e. Taihu Lake, Chaohu Lake and Dianchi Lake). According to the Liebig's law, P is the key limitation factor for prevention eutrophication compared with N. A concentration of 0.1 mg P/L in surface waters has been specified as an indicator of possible current or future algal blooms (Young et al., 1999). An important process for P removal from domestic wastewater is generally regarded as an effective strategy to prevent eutrophication (Bashiri et al., 2017; Young et al., 1999). Therefore, legislations on P concentration in the effluent from wastewater treatment plants (WWTP) are incrementally becoming stricter worldwide. For example, permit limit of P concentration in the effluent from WWTP was 0.1 mg/L or lower in Europe and North American (Boltz et al., 2012), less than 0.2 mg P/L in South Korea (Choi et al., 2012), and likewise, not more than 0.5 mg P/L required by Chinese government.

To meet the requirements mentioned above, enhanced biological phosphorus removal (EBPR) has

* Author to whom all correspondence should be addressed: e-mail: hmzou@126.com; xiwulu@seu.edu.cn; Phone: +86 25 83794171; Fax: +86 25 83792614

been adopted in lab- or full- scale reactors to remove P from wastewater (Coma et al., 2012; Saba et al., 2017; Slater et al., 2010; Tobajas et al., 2014; Shi et al., 2012; Zhou et al., 2010). During this system, P removal is achieved mainly by polyphosphate accumulating organisms (PAO), presenting anaerobic P release and aerobic excessive P uptake. However, a competition for carbon sources between denitrification and anaerobic P release conducted by their respective microorganisms (such as denitrifying bacteria and PAO) exists in the conventional EBPR system (Wang et al., 2012). Moreover, available carbon source in the real domestic wastewater (so-called low C/N ratio) are often insufficient for N and P removal, which has become a challenge for nutrients removal effectively in WWTP (Ryu et al., 2008). To supplement the lack of carbon source, injection of additional external carbon source such as sodium acetate, methanol and glucose into influent was generally performed (Podedworna and Żubrowska-Sudoł, 2012), thus probably leading to a significant increase in the operational costs.

As an alternative to conventional EBPR processes running in the anaerobic/aerobic modes for P removal, recently denitrifying simultaneous N and P removal process has been proposed (Acevedo et al., 2012; Günther et al., 2011; Pijuan et al., 2008; Shi et al., 2012; Tsuneda et al., 2006). During this process, denitrifying polyphosphate accumulating organisms (DPAO) are capable of using nitrate rather than oxygen as an electron acceptor for denitrifying simultaneous N and P removal in anoxic conditions, compared with PAO, which can save aeration (30%), minimize sludge production (50%) and reduce the demand for carbon sources (50%) (Jiang et al., 2010). More important, DPAO offers an appropriate measure to treat domestic wastewater with a low C/N ratio. Numerous operational factors (such as pH, carbon source types, temperature and influent nutrients composition) affecting the DPAO performance have been researched (Kim et al., 2006; Panswad et al., 2003; Wang et al., 2012). In contrast, the nutrients ratios in the influent, i.e. ratios of C/N and C/P, have a strongly influence on the nutrient removal efficiencies in EBPR system (Wang et al., 2009). Based on the DPAO theory (Guisasola et al., 2009), the ratios of C/N and C/P in the influent have a profound impact on the amounts of synthesized PHB anaerobically, in turn, which determines greatly the N and P removal capacities in sequential anoxic condition. In fact, nutrients ratios of influent affect directly the abundance of particular functional microorganisms (DPAO) during the operational process. However, there is no information available on the shift of the microbial community structure in respond to different influent C/N and C/P ratios in EBPR system.

In this context, a novel BNR-IC process proposed in our previous research was operated over 180 days with feeding different nutrient loads in this study to investigate how ratios of C/N and C/P in the influent could affect denitrifying simultaneous N and P removal performance coupled with microbial community structure. This result obtained here, combining chemical analysis with microbial analysis, may serve as a new suggestion for application of DPAO to treatment real domestic wastewater, especially for low C/N ratio sewage.

2. Material and methods

2.1. BNR-IC process and operation

The BNR-IC process designed in our previous research (Zou et al., 2013) consisted of biological nutrients removal (BNR) system (adopting denitrifying simultaneous N and P removal) and induced crystallization column (IC) for recovery P, which has been applied for a parent (No. 201110431802.4) in China. The main advantages of the BNR-IC process are lower oxygen and carbon sources requirement, minimal production of excess sludge and recovery of P in wastewater. In our previous research, DPAO was successfully enriched in the BNR-IC system during the 55 days cultivation process, where the seed sludge was collected from an aerobic basin of the Chengdong Municipal Wastewater Treatment Plant, Nanjing, China with AA–aerobic (A^2O) configuration and it presented, to some extent, a good aerobic phosphorus uptake capacity.

2.2. Experimental design

The tests with different C/N and C/P ratios in the influent (basic composition shown in Table 1), given as Runs 1-6, were divided into two operational stages (Table 2). During stage one, the concentrations of P and N in the influent were maintained constant, but the carbon source dosage was increased gradually to enhance the C/N and C/P ratios.

During the other stage, influent COD as well as N loading did not changed while P concentration in the influent was gradually decreased to change the C/P ratio. Variations of P and N concentrations in the effluent with time were monitored during the experimental period. Sludge samples were collected as well at the end of each Run to investigate the shift in microbial community structure.

2.3. Chemical analysis

Liquid samples from effluent were filtered through a 0.45 μm microporous membrane filter firstly. Chemical oxygen demand (COD) and mixed liquor suspended solid (MLSS) were determined according to the standard methods for the examination of water and wastewater (APHA, 2005). PO_4^{3-}-P and total nitrogen (TN) were analyzed by segmented flow analysis (AutoAnalyzer3, SEAL, UK). Dissolved oxygen (DO), pH and Oxidation-reduction potential (ORP) were measured by a DO meter analyzer (YSI DO200, USA) and a pH and ORP meter analyzer (YSI pH100, USA).

Table 1. Composition of wastewater used for experiment

Composition of feeds	***Concentration, g/L***	***Composition of nutrient solution***	***Concentration, g/L***
CH_3COONa	0.322	$FeCl_3 \cdot 6H_2O$	1.50
KH_2PO_4	0.044	H_3BO_3	0.15
$(NH_4)_2SO_4$	0.047	$CuSO_4 \cdot 5H_2O$	0.03
$CaCl_2$	0.005	KI	0.18
$MgSO_4 \cdot 7H_2O$	0.050	$MnCl_2 \cdot 4H_2O$	0.12
Nutrient solution	0.30 mL/L	$Na_2MoO_4 \cdot 2H_2O$	0.06
		$ZnSO_4 \cdot 7H_2O$	0.12
		$CoCl_2 \cdot 6H_2O$	0.15
		EDTA	10.00

Table 2. Variations of C/N and C/P ratios in the influent

Run	***COD mg/L***	***TN mg/L***	***PO_4^{3-}-P mg/L***	***COD/N***	***COD/P***	***Removal efficiencies %***	
						P	***TN***
1(1-30days)	150.0	56.0	15.0	2.7	10.0	37.4±16.3	50.4±9.9
2(31-60days)	250.0	56.0	15.0	4.5	16.7	76.1±4.8	65.6±6.8
3(61-90days)	350.0	56.0	15.0	6.3	23.3	94.0±2.4	90.5±4.3
4(91-120days)	350.0	56.0	20.0	6.3	17.5	76.5±3.5	84.7±1.2
5(121days-150days)	350.0	56.0	10.0	6.3	35.0	92.7±3.8	84.1±1.3
6(151-180days)	350.0	56.0	5.0	6.3	70.0	93.7±3.7	83.1±0.4

2.4. Microbial analysis

At the end of each Run (as described above), 100 mL activated sludge was sampled to analyze the bacterial community by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) according to the following rules.

2.4.1. Samples pretreatment

Activated sludge samples were homogenized with 3 mm diameter glass beads for 30 min, and then centrifuged at 5000 g for 5 min. Treated samples were stored at -20°C until further analysis after washed three times with 0.1 M PB (phosphate buffer), pH 7.2.

2.4.2. DNA extraction and PCR amplification

Total community DNA in 300 mg of sludge sample was extracted using a commercial FastDNA SPIN Kit for Soil (MP-Bio, USA) according to the manufacture's instruction, and stored at -20°C. About 10 ng of DNA was used as a template for PCR amplification with universal primers (341F, 5'-CCTACGGGAGGCAGCAG-3' and 534R, 5'-ATTACCGCGGCTGCTGG-3') (Liu et al., 2012), targeting the conserved regions of 16S rRNA genes in the variable V3 region of 16S rDNA. The PCR amplification was performed in a PCR thermal cycler dice (Takara, Japan), as previously reported (Li et al., 2012).

2.4.3. DGGE analysis and sequencing of PCR fragments

Dcode TM system (Bio-Rad, USA) was used for DGGE according to the previously described method (Li et al., 2012). The main DGGE bands in different lanes were excised for cloning and then sequenced, which were performed by a Biotech Co., Ltd., Shanghai, China. These sequencing results obtained here were compared with the closest known relatives using the BLAST program from the National Center for Biotechnology Information (NCBI) database. Phylogenetic tree was carried out using the neighbour-joining method (Tortoli, 2012) with MEGA version 5.0 software, employing 1000 bootstrap resampling and similarities in DGGE profiles were also calculated via Dice coefficient with MEGA.

3. Results and discussion

3.1. Effects of influent C/N and C/P ratios on the nutrient removals

With the different operational strategies (Table 2), the BNR-IC was continuously operated over 180 days, divided into two stages (i.e. Run 1-Run 3 and Run 4-Run 6) and 30 days in each Run, where the side-stream ratio was maintained with a constant value of 35% based on the results of previous research, in which the removal efficiencies of N and P were not influenced by the IC column. The concentrations of PO_4^{3-}-P and TN in the effluent fluctuated, and were averaged and are shown in Table 2 and Fig. 1. During the stage one, with an increase in the influent organic loading (consistently, the C/N and C/P ratios increased as well), the removal efficiencies of P and TN increased significantly, ranging from 37.4±16.3% to 94.0±2.4% and 50.4±9.9% to 90.5±4.3%, respectively. During

the second stage, the influent P concentration ranged from 20 mg/L to 5 mg/L, leading to C/P ratio increased gradually while C/N ratio was constant. There was a significant difference in removal efficiency between P and TN in Run 4-Run 6. The P removal efficiency increased gradually, varying from 76.5±3.5% to 93.7±3.7% while that of TN was rather stable with values constantly in the range 84.7±1.2-83.1±0.4%. Despite the competition for carbon source existing in nutrient removal, both P and TN removal efficiencies reached a maximum, as in Run 3, where the C/N and C/P ratios were 6.3 and 23.3, respectively.

Clearly, the relatively lower amount of available organic loading, i.e. low C/P and C/N ratios, negatively affected the removal performances of phosphorus and nitrogen, as displayed in Run 1 and Run 2. For EBPR system, insufficient carbon source in the influent can deteriorate the function of anaerobic P release and anoxic P uptake (Carvalho et al., 2007), leading to a decrease in the amount of PHB responsible for denitrifying N and P removal simultaneously. Wang et al. (2012) also clearly demonstrated that the influent C/N and C/P ratios affected the PHB synthesized in the anaerobic period. In Run 3, good P and N removal performances, reaching maximum values throughout the whole experimental processes arranged in this study, were both achieved compared with Run 1 and Run 2 when the influent COD concentration was 350 mg/L. This suggests that relatively high C/N and C/P ratios can effectively promote DPAO activity in EBPR process. Moreover, the removal efficiencies of P and TN in the present study were higher than that obtained in other studies, even when a higher influent C/P ratio of 54.2-56.2 was used in anaerobic/anoxic/aerobic (A/A/O), Oxidation ditch and sequencing batch reactor (SBR) processes (Pei et al., 2008; Peng et al., 2008; Zeng et al., 2003). One reason of this advantage may be that DPAO is capable of utilizing intracellular carbon source of PHB to reduce nitrate and accumulating P stored as Poly-p in the cell simultaneously. During this process, 50 % carbon source for conventional denitrification used can be saved (Jiang et al., 2010). As a result, EBPR system running in the mode of anaerobic-anoxic may be more suitable for treatment of low C/N ratio wastewater compared with conventional wastewater treatment processes.

When the influent's COD and TN loads were stable at 350 mg/L and 56 mg/L respectively and load of P in the influent was decreased from 20 mg/L to 5 mg/L, the P removal efficiency greatly varied from 76.5±3.5% to 93.7±3.7% while that of TN slightly changed, as Run 4-Run 6, indicating that a relative stable TN removal performance can be obtained when maintaining an appropriate C/N ratio in the influent (for example, C/N ratio of 6.3 was used in this study), regardless of influent C/P ratio. This may have occurred because low C/P ratio deteriorates DPAO activity but promotes denitrifying microorganism functioning, which can maintain stable nitrogen removal efficiency in EBPR system. However, DPAO seemed to thrive better than denitrifying bacteria as the C/P ratio increased quickly with the decreasing in P loading in the influent, as given in Run 5 and Run 6. These findings indicate that the dominant microorganism, such as DPAO or denitrifying bacteria, in the EBPR system may have changed along with decreasing the P load, as described in section 3.2.

Additionally, the difference of TN removal efficiency between Run 3 and Run 4-6 may result from the change of microbial community structure, as described in PCR-DGGE analysis. In Run 3, the maximum N and P removal efficiencies were obtained, suggesting that the optimum C/N and C/P ratios were presented in the influent for DPAO activities, i.e., denitrifying simultaneous N and P removal. Compared with Run 3, although the C/N ratio was constant in Run 4-6, the TN removal efficiency slightly decreased, probably due to the fact that change of C/P ratio didn't support the growth of DPAO but may support the denitrifying microorganisms and the carbon resources was inadequate for denitrifying nitrogen removal, thus probably leading to a decrease in TN removal efficiency.

3.2. Effect of influent C/N and C/P ratios on microbial community structure

The different denitrifying N and P removal performance observed in Run 1-Run 6 (Table 2) could be due to the variations in the dominant microorganism when given with different influent C/N and C/P ratios, as discussed in section 3.1. Alternatively, it could be explained by the development of different microbe in the BNR-IC system. For this, The shift of microbial community structure during the experimental period was investigated by using PCR-DGGE analysis of 16Sr RNA genes (Fig. 2 (left)); lanes comparison and Dice coefficients on the DGGE patterns are shown in Fig.3 (right) and Table 3, respectively; the sequencing and BLAST searching results of the DGGE bands are present in Table 4 and phylogenetic affiliation of the 16S rDNA of DEEG bands was conducted by using software of MEGA version 5.1 as well, as illustrated in Fig. 3. In total, 18 DGGE bands of highest species richness (band 1-band18), as shown in Fig. 3 (left), were visually detected at the different influent C/N and C/P ratios operational period for 180 days, showing the diversity of microbial ecologies in the sampled sludge. Increasing the organic load in the influent (stage one) from 150 mg/L to 350 mg/L caused noticeable shift in the bacterial community structure, where a sharply decreasing number of bands was observed in the lanes of DGGE, indicating a decreasing microbial diversity.

Conversely, in stage two, an increase in the number of bands was displayed with the decreasing influent P load from 20 mg/L to 5 mg/L, suggesting the increasing microbial diversity.

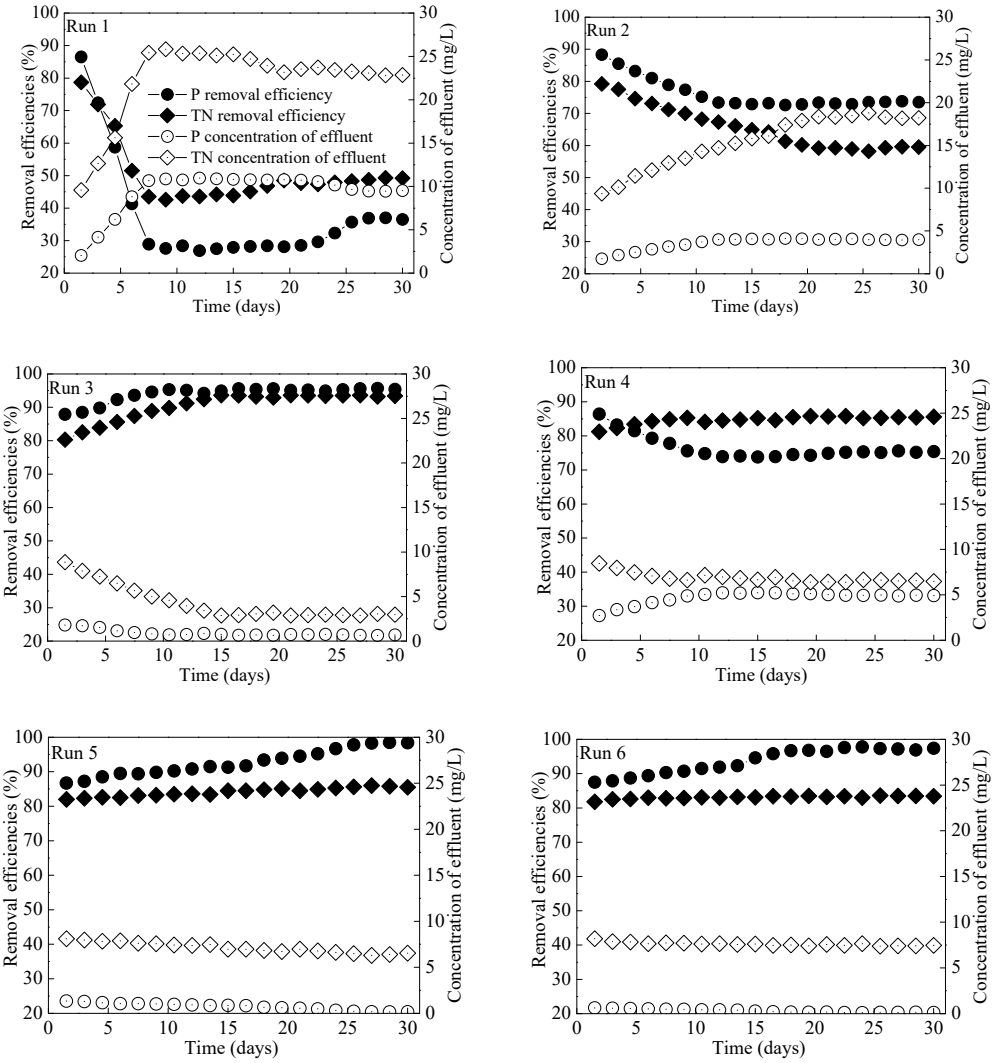

Fig. 1. Phosphorus and total nitrogen concentrations in the effluent and removal efficiency during tests

Similarly, comparison of microbial community structure among Run 1-Run 6, performed by quantity one software of version 4.6.2 (Bio-Rad, USA), also demonstrated the change of microbial diversity, as illustrated in Fig. 2 (right). Differences of bands distribution and intensities in six lanes may result from the different influent C/N and C/P ratios provided in this study, since different carbon, nitrogen and phosphorus loads in the influent may be contribution to the development of different dominant microorganism to be extent in EBPR system (Chuang et al., 2011). Moreover, microbial community similarities in six lanes were analyzed via Dice coefficients (all less than 70%), as shown in Table 3, suggesting that a large bacterial community changes occurred when given different influent C/N and C/P ratios as well, where the Dice coefficient of 42.0% between Lane 1 and Lane 6 was minimum, resulting from the change in the influent conditions, which is agreement with the discussion mentioned above. The total 18 bands, as depicted in Fig. 2 (left), were excised from the DGGE gel and conducted for cloning and sequencing.

The affiliations of the 16Sr DNA sequences (Table 4) were investigated by comparison against the International GenBank database (www.ncbi.nlm.nih.gov) and phylogenetic tree (Fig. 3) was performed to obtain a better understanding of variations in the microbial community structure dependent of operational conditions.

At a generally glance, the microbial populations correspond to 18 bands were classified into five different phylum or class, including *Alpha-*, *Beta-*, and *Gammaproteobacteria,* as well as *Firmicutes*, *Actinobacteria*, as shown in Table 4 and Fig. 3. Six brightness bands (band 2, 6, 9, 11, 14 and 16) were commonly present in all lanes, which related to *Azonexus.spp*, *Thiothrix.spp*, *Acidovorax.spp*, *Acinetobacter.spp*, *Rhodocyclus.spp*, *Agrobacterium.spp*, respectively; four bands (band 1, 3, 5 and 10, related to *Variovorax.spp*, *Microlunatus.spp*, *Hydrogenophaga.spp* and *Tetrasphaera.spp* respectively) were present in all lanes expect for land 1; and the rest were only found in land 1.

Table 3. Dice coefficients comparing the similarities of DGGE fingerprints

	Lane 1	***Lane 2***	***Lane 3***	***Lane 4***	***Lane 5***	***Lane 6***
Lane 1	100.0	47.8	54.9	63.0	57.0	42.0
Lane 2	47.8	100.0	51.9	57.6	67.1	58.0
Lane 3	54.9	51.9	100.0	65.8	63.0	44.0
Lane 4	63.0	57.6	65.8	100.0	65.4	49.6
Lane 5	57.0	67.1	63.0	65.4	100.0	65.3
Lane 6	42.0	58.0	44.0	49.6	65.3	100.0

Table 4. NCBI BLAST search results of sequences from DGGE bands

Band	***Closest match***	***Similarity %***	***Phylum/Class***
1	*Variovorax.spp*	100	*Betaproteobacteria*
2	*Azonexus.spp*	95	*Betaproteobacteria*
3	*Microlunatus.spp*	100	*Actinobacteria*
4	*Agrobacterium.spp*	98	*Alphaproteobacteria*
5	*Hydrogenophaga.spp*	99	*Betaproteobacteria*
6	*Thiothrix.spp*	89	*Gammaproteobacteria*
7	*Methylocaldum.spp*	100	*Gammaproteobacteria*
8	*Desulfitibacter.spp*	87	*Firmicutes*
9	*Acidovorax.spp*	92	*Betaproteobacteria*
10	*Tetrasphaera.spp*	100	*Actinobacteria*
11	*Acinetobacter.spp*	98	*Gammaproteobacteria*
12	*Methylococcus.spp*	99	*Gammaproteobacteria*
13	*Rhabdochromatium.spp*	100	*Gammaproteobacteria*
14	*Rhodocyclus.spp*	94	*Betaproteobacteria*
15	*Trichococcus.spp*	99	*Firmicutes*
16	*Agrobacterium.spp*	96	*Alphaproteobacteria*
17	*Rhabdochromatium.spp*	100	*Gammaproteobacteria*
18	*Halochromatium.spp*	99	*Gammaproteobacteria*

3.3. Relationship between nutrients removal and shift in microbial community structure when varying influent C/N or C/P ratio

Analyses of both chemical and PCR-DGGE, as described in section 3.1 and 3.2, showed that influent C/N and C/P ratios influenced not only the N and P removal performance but also microbial community structure in the BNR-IC system. This seems to have a correlation between nutrient removal efficiencies and change of microbial populations with the operational period (Fig. 2).

Increasing influent C/N and C/P ratios (as Run 1-Run 3,) promoted the N and P removal in the BNR-IC system (Table 2 and Fig. 1), while, bacterial diversity of activated sludge decreased, as shown in Fig. 3, probably supporting a higher DPAO accumulation. Comparing six lanes, some group of bacterial belonging to GAO or heterotrophic bacteria instead of DPAO were found in land 1, but disappeared in other lanes, including *Agrobacterium.spp* (band 4), *Methylocaldum.spp* (band 7), *Desulfitibacter.spp* (band 8), *Methylococcus.spp* (band 12), *Rhabdochromatium.spp* (band 13), *Trichococcus.spp* (band 15), *Rhabdochromatium.spp* (band 17) and *Halochromatium.spp* (band 18). However, four group of bacterial responsible for N or P removal were present in all lanes expect for land 1. *Hydrogenophaga.spp* (band 5) and *Tetrasphaera.spp* (band 10) can reduce nitrate and utilize PHB simultaneously (Karkman et al., 2011), displaying the characterization of DPAO.

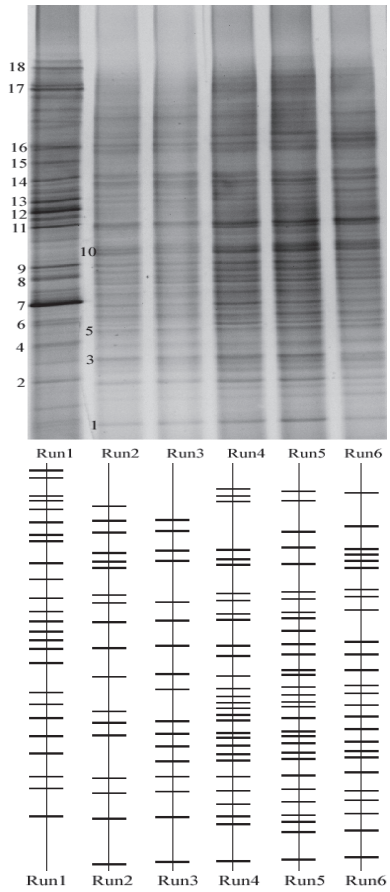

Fig. 2. DGGE fingerprint of 16Sr DNA fragments (left) and lanes comparison (right) generated from six batch tests (Run1-Run6) given different influent C/N and C/P ratios

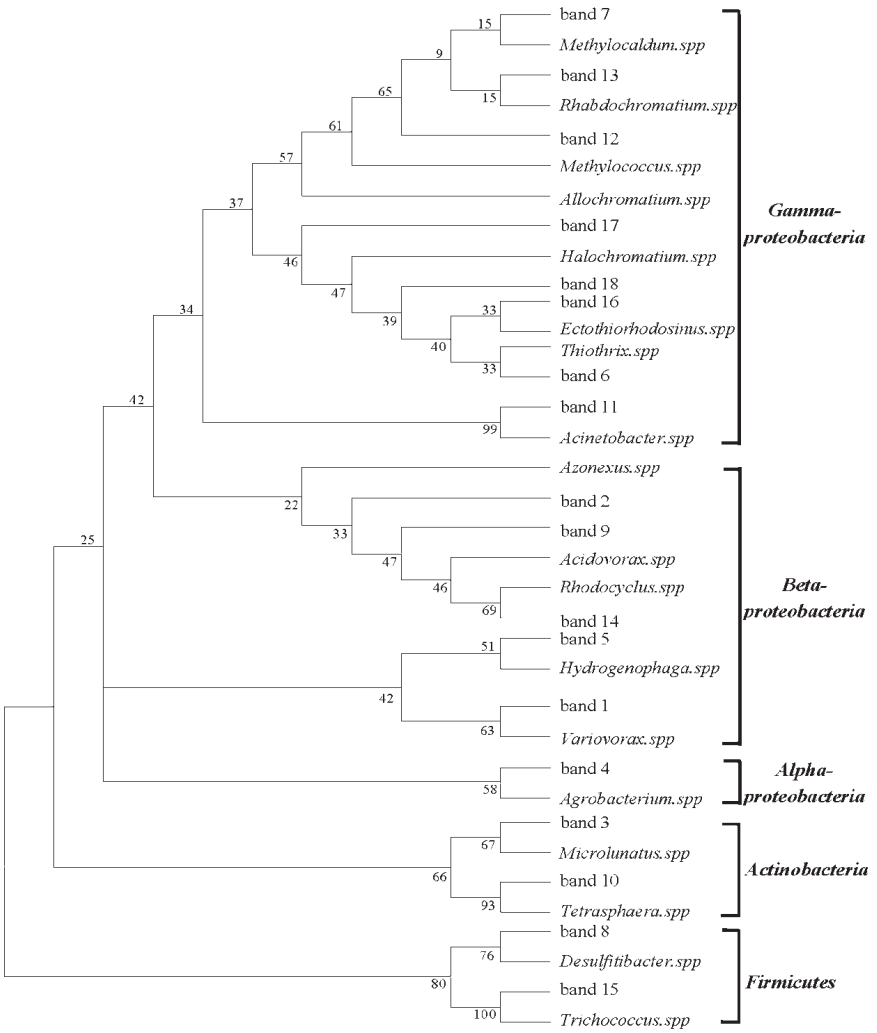

Fig. 3. Phylogenetic tree, carried out in the software of MEGA version 5.1, of 16SrDNA excised from DGGE showing the phylogenetic affiliations between strains obtained in activated sludge studied here and their closest relatives derived from GenBank

Similarly, *Variovorax.spp* (band 1) and *Microlunatus.spp* (band 3) are also capable for denitrifying phosphorus removal (Jackson et al., 2012. Moreover, 8 bands were present in all lanes, some of which have been demonstrated as DPAO, such as *Acinetobacter.spp* (band 11) and *Rhodocyclus.spp* (band 14), dominantly existing in various EBPR systems (Muszyński et al., 2013; Zengin et al., 2011). Overall, PCR-DGGE and chemical analysis showed increasing C/N and C/P ratios in the influent may be contribution to the higher DPAO accumulation, thus enhancing the N and P removal performances and decreasing the microbial diversity in EBPR system.

From Fig. 1 and Fig. 2, ratio of C/P appears to be a more significant factor affecting the P removal when given an appropriate C/N ratio in the influent. It is particularly noted that TN removal efficiency was stable about 84%, even through P removal efficiency greatly fluctuated with the increase in C/P ratios (Run 4-Run 6), which was probably due to the unfavorable change in the development of DPAO and good promotion of the denitrifying bacteria growth. Moreover, the bacterial composition of sludge collected in Run 4 was more diverse than that in Run 3, also indicating that excessive P may have a negative effect on the DPAO activity in EBPR system.

3. Conclusions

Influent C/N and C/P ratios greatly influenced N and P removal and microbial community structure due to considerable variations in the dominant microorganisms in the BNR-IC system. Increasing C/N and C/P ratios favored N and P removal and decreased microbial community biodiversity. However, excessive phosphorus load in the influent may deteriorate phosphorus removal when maintaining an appropriate C/N ratio.

Microbial populations in whole experimental period were classified into five different phylum or class and the determination of phylogenetic tree indicated that microorganisms related to DPAO mostly belonging to the phylum of *Betaproteobacteria*.

Acknowledgements

We wish to thank W.B.BAO for assistance with activated sludge treatment. This research is supported by grant 2012ZX07101-005 from National Key Technology in Water Pollution Control and Treatment in 12th Five-year Plan of China and grant 51078074 from National Natural Science Foundation of China.

References

Acevedo B., Oehmen A., Carvalho G., Seco A., Borras L., Barat R., (2012), Metabolic shift of polyphosphate-accumulating organisms with different levels of polyphosphate storage, *Water Research*, **46**, 1889-1900.

Bashiri S., Akbarzadeh A., Zarrabi M., Yetilmezsoy K., Fingas M., Moosakhaani M., (2017), Using PCA combined SVM in the classification of eutrophication in DEZ reservoir (Iran), *Environmental Engineering and Management Journal*, **16**, 2139-2146.

Boltz J.P., Morgenroth E., Daigger G.T., Debarbadillo C., Murthy S., Sørensen K.H., Stinson B., (2012), Method to identify potential phosphorus rate-limiting conditions in post-denitrification biofilm reactors within systems designed for simultaneous low-level effluent nitrogen and phosphorus concentrations, *Water Research*, **46**, 6228-6238.

Carvalho G., Lemos P.C., Oehmen A., Reis M.A.M., (2007), Denitrifying phosphorus removal: linking the process performance with the microbial community structure, *Water Research*, **41**, 4383-4396.

Choi Y., Kwon K., Kim S., Lee S., Min K., (2012), Optimization of phosphorus reduction in BNR process for urban watershed management, *Desalination and Water Treatment*, **38**, 216-221.

Chuang S., Chang W., Huang Y., Tseng C., Tai C., (2011), Effects of different carbon supplements on phosphorus removal in low C/P ratio industrial wastewater, *Bioresource Technology*, **102**, 5461-5465.

Coma M., Verawaty M., Pijuan M., Yuan Z., Bond P.L., (2012), Enhancing aerobic granulation for biological nutrient removal from domestic wastewater, *Bioresource Technology*, **103**, 101-108.

Guisasola A., Qurie M., Vargas M.D.M., Casas C., Baeza J.A., (2009), Failure of an enriched nitrite-DPAO population to use nitrate as an electron acceptor, *Process Biochemistry*, **44**, 689-695.

Günther S., Koch C., Hübschmann T., Röske I., Müller R.A., Bley T., Harms H., Müller S., (2011), Correlation of community dynamics and process parameters as a tool for the prediction of the stability of wastewater treatment, *Environmental Science & Technology*, **46**, 84-92.

Jackson V.A., Paulse A.N., Odendaal J.P., Khan S., Khan W., (2012), Identification of metal-tolerant organisms isolated from the Plankenburg River, Western Cape, South Africa, *Water SA*, **38**, 29-38.

Jiang X.X., Yang J.X., Ma F., Yang F.F., Wei L., Yi J., (2010), Denitrifying phosphorous removal in anaerobic/anoxic SBR system with different startup operation mode, *Journal of Harbin Institute of Technology*, **17**, 824-829.

Karkman A., Mattila K., Tamminen M., Virta M., (2011), Cold temperature decreases bacterial species richness in nitrogen-removing bioreactors treating inorganic mine waters, *Biotechnology and Bioengineering*, **108**, 2876-2883.

Kim H.T., Oh S.H., Lee Y.D., Kim G.S., (2006), The behavior of the DNPAOs at the anaerobic-anoxic process according to the change of the influent NO_3^--N loading, *KSCE Journal of Civil Engineering*, **10**, 399-403.

Li X., Xu F., Liu H., Liang B., Xu Y., Jin L., (2012), Application of polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis for analysis of microbiota on the tongue dorsa of subjects with halitosis, *African Journal of Microbiology Research*, **6**, 5789-5795.

Liu J., Lu Z., Yang J., Xing M., Yu F., Guo M., (2012), Effect of earthworms on the performance and microbial communities of excess sludge treatment process in vermifilter, *Bioresource Technology*, **117**, 214-221.

Muszyński A., Łebkowska M., Tabernacka A., Miłobędzka A., (2013), From macro to lab-scale: Changes in bacterial community led to deterioration of EBPR in lab reactor, *Central European Journal of Biology*, **8**, 130-142.

Panswad T., Doungchai A., Anotai J., (2003), Temperature effect on microbial community of enhanced biological phosphorus removal system, *Water Research*, **37**, 409-415.

Pei H., Hu W., Pan J., Zhang J., (2008), Simultaneous nitrogen and phosphorus removal using a double sludge switching sequencing batch reactor, *Journal of Environmental Engineering*, **134**, 923-927.

Peng Y., Hou H., Wang S., Cui Y., Zhiguo Y., (2008), Nitrogen and phosphorus removal in pilot-scale anaerobic-anoxic oxidation ditch system, *Journal of Environmental Sciences*, **20**, 398-403.

Pijuan M., Oehmen A., Baeza J.A., Casas C., Yuan Z., (2008), Characterizing the biochemical activity of full-scale enhanced biological phosphorus removal systems: A comparison with metabolic models, *Biotechnology and Bioengineering*, **99**, 170-179.

Podedworna J., Żubrowska-Sudoł M., (2012), Nitrogen and phosphorus removal in a denitrifying phosphorus removal process in a sequencing batch reactor with a forced anoxic phase, *Environmental Technology*, **33**, 237-245.

Ryu H., Kim D., Lim H., Lee S., (2008), Nitrogen removal from low carbon-to-nitrogen wastewater in four-stage biological aerated filter system, *Process Biochemistry*, **43**, 729-735.

Saba B., Zaman B., Mahmood T., Khan S.J., (2017), Treatment of wastewater with a high C/N ratio in sequencing batch bioreactor (SBBR) containing biocarrier, *Environmental Engineering and Management Journal*, **16**, 2485-2489.

Shi J., Lu X., Yu R., Zhu W., (2012), Nutrient removal and phosphorus recovery performances of a novel anaerobic-anoxic/nitrifying/induced crystallization process, *Bioresource Technology*, **121**, 183-189.

Slater F.R., Johnson C.R., Blackall L.L., Beiko R.G., Bond P.L., (2010), Monitoring associations between clade-level variation, overall community structure and ecosystem function in enhanced biological phosphorus removal (EBPR) systems using terminal-restriction fragment length polymorphism (T-RFLP), *Water Research*, **44**, 4908-4923.

Tobajas M., Polo A.M., Monsalvo V.M., Mohedano A.F., Rodriguez J.J., (2014), Analysis of the operating conditions in the treatment of cosmetic wastewater by sequencing batch reactors, *Environmental Engineering and Management Journal*, **13**, 2955-2962.

Tortoli E., (2012), Phylogeny of the genus Mycobacterium: many doubts, few certainties, *Infection, Genetics and Evolution*, **12**, 827-831.

Tsuneda S., Ohno T., Soejima K., Hirata A., (2006), Simultaneous nitrogen and phosphorus removal using denitrifying phosphate-accumulating organisms in a sequencing batch reactor, *Biochemical Engineering Journal*, **27**, 191-196.

Wang Y., Geng J., Ren Z., Guo G., Wang C., Wang H., (2012), Effect of COD/N and COD/P ratios on the PHA transformation and dynamics of microbial community structure in a denitrifying phosphorus removal process, *Journal of Chemical Technology and Biotechnology*, **88**, 1228-1236.

Wang Y., Peng Y., Stephenson T., (2009), Effect of influent nutrient ratios and hydraulic retention time (HRT) on simultaneous phosphorus and nitrogen removal in a two-sludge sequencing batch reactor process, *Bioresource Technology*, **100**, 3506-3512.

Young K., Morse G.K., Scrimshaw M.D., Kinniburgh J.H., MacLeod C.L., Lester J.N., (1999), The relation between phosphorus and eutrophication in the thames catchment, UK, *Science of the Total Environment*, **228**, 157-183.

Zeng R.J., Yuan Z., Keller J., (2003), Enrichment of denitrifying glycogen-accumulating organisms in anaerobic/anoxic activated sludge system, *Biotechnology and Bioengineering*, **81**, 397-404.

Zengin G.E., Artan N., Orhon D., Satoh H., Mino T., (2011), Effect of aspartate and glutamate on the fate of enhanced biological phosphorus removal process and microbial community structure, *Bioresource Technology*, **102**, 894-903.

Zhou S., Zhang X., Feng L., (2010), Effect of different types of electron acceptors on the anoxic phosphorus uptake activity of denitrifying phosphorus removing bacteria, *Bioresource Technology*, **101**, 1603-1610.

Zou H.M., Lu X.W., Shi J., (2013), Characteristics of nitrifier in sludge denitrifying phosphorus removal and recovery process, *Transactions of the Chinese Society of Agricultural Engineering*, **29**, 218-223.