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Simultaneous nitrogen and phosphorus removal in the sulfur cycle-associated Enhanced Biological Phosphorus Removal (EBPR) process

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ABSTRACT

Hong Kong has practiced seawater toilet flushing since 1958, saving 750,000 m^3 of freshwater every day. A high sulfate-to-COD ratio (>1.25 mg SO_4^{2-}/mg COD) in the saline sewage resulting from this practice has enabled us to develop the Sulfate reduction, Autotrophic denitrification and Nitrification Integrated (SANI®) process with minimal sludge production and oxygen demand. Recently, the SANI® process has been expanded to include Enhanced Biological Phosphorus Removal (EBPR) in an alternating anaerobic/limited-oxygen (LOS-EBPR) aerobic sequencing batch reactor (SBR). This paper presents further development – an anaerobic/anoxic denitrifying sulfur cycle-associated EBPR, named as DS-EBPR, bioprocess in an alternating anaerobic/anoxic SBR for simultaneous removal of organics, nitrogen and phosphorus. The 211 day SBR operation confirmed the sulfur cycle-associated biological phosphorus uptake utilizing nitrate as electron acceptor. This new bioprocess cannot only reduce operation time but also enhance volumetric loading of SBR compared with the LOS-EBPR. The DS-EBPR process performed well at high temperatures of 30 °C and a high salinity of 20% seawater. A synergistic relationship may exist between sulfur cycle and biological phosphorus removal as the optimal ratio of P-release to SO_4^{2-}-reduction is close to 1.0 mg P/mg S. There were no conventional PAOs in the sludge.

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1. Introduction

Today, water stress, in terms of both water shortage and declining water quality, has become a global issue due to its impacts on agricultural yields, energy conservation, and environmental protection (UNDP, 2006). The situation is exacerbated along with climate change and increasing population growth (Bates et al., 2008). To alleviate water stress, exploitation of seawater is considered as an economic and low-carbon solution for coastal cities (van Loosdrecht et al., 2012).

The exploitation of seawater for toilet flushing has been practiced in Hong Kong since 1950s (Tang et al., 2007), currently serving 80% of its 7 million inhabitants. This application has helped to relieve its serious water stress problem by saving 750,000 m^3/day, or 22% of freshwater demand, primarily imported from Dong-jiang River of Guangdong Province of China (Leung et al., 2012). Seawater toilet flushing also reduced the energy consumption for water supply by 37%. This is mainly attributed by the minimum treatment required, i.e. screening and electro chlorination, for seawater supply resulting in the difference in unit electricity consumption rate between 0.629 kWh/m^3 of freshwater and 0.396 kWh/m^3 of seawater (HKWSD, 2012).

Furthermore, a novel sulfur cycle-based biological nitrogen removal process, namely Sulfate reduction, Autotrophic denitrification and Nitrification Integrated (SANI®) process, has been developed for the treatment of Hong Kong's saline sewage (Wang et al., 2009). The SANI® process makes use of sulfate, resulting from the toilet flushing seawater, as an electron carrier for the sulfur cycle biotransformation to remove organic carbon and nitrogen pollutants (Lu et al., 2009). Three groups of slow-growing microorganisms namely, heterotrophic sulfate-reducing bacteria, autotrophic sulfur-oxidizing denitrifiers and nitrifiers, are dominant in the SANI® process, which greatly reduce the biological sludge production (Lu et al., 2009, 2012a). As compared to conventional biological wastewater treatment processes with sludge incineration, the SANI® process can save about 35% of the energy consumption and reduce 36% of the greenhouse gas emissions (Lu et al., 2012b). As a result, the combined seawater toilet flushing and SANI® process serves as a new horizon for urban water management in coastal cities.

To avoid harmful algal bloom and eutrophication in coastal areas (WRI, 2012), nitrogen (N) and phosphorus (P) removal from sewage is necessary (Howarth and Paerl, 2008). In conventional Biological Nutrient Removal (BNR) process, this is achieved by combining EBPR with nitrification and denitrification through subjecting a single sludge to alternating anaerobic/anoxic/aerobic conditions (Wentzel et al., 1992). The microorganisms responsible for EBPR, known as polyphosphate accumulating organisms (PAOs) (Wentzel et al., 1988; Van Loosdrecht et al., 1997; Nielsen et al., 2012). However, it appears that PAOs cannot compete against glycogen accumulating organisms (GAOs) in warm climate, rendering its limited applications in tropical and sub-tropical regions (Cao, 2011).

In addition to the well-studied conventional BNR process, researches have been conducted to extend the SANI® process for biological P removal (Wu et al., 2012, 2013) through

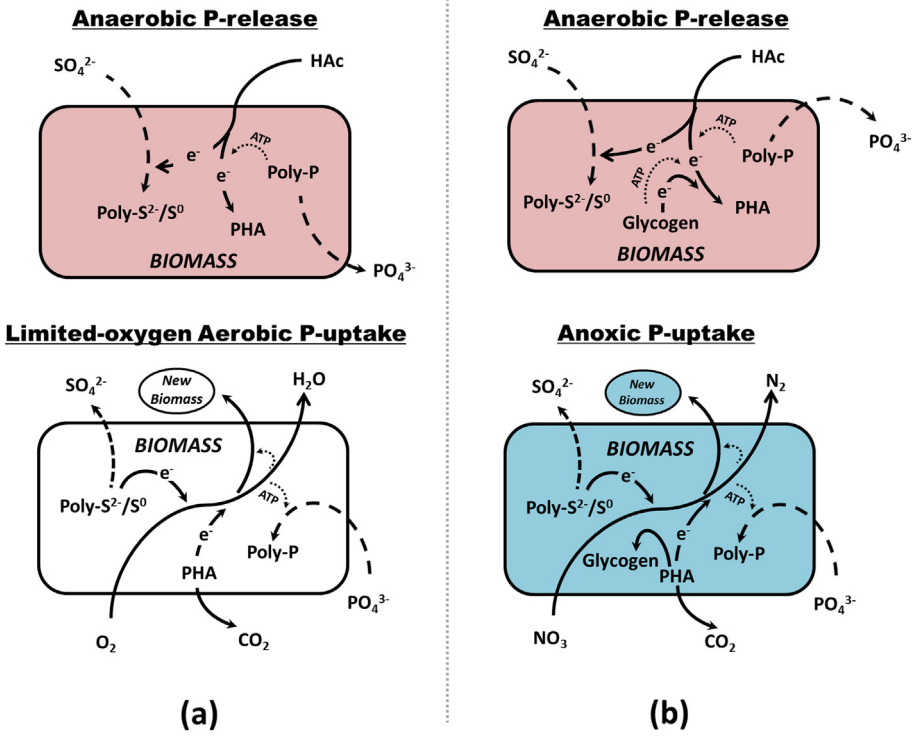

Fig. 1 – Possible reactions of sulfur cycle associated EBPR: (a) Limited-Oxygen Sulfur cycle-associated EBPR, (b) Denitrifying Sulfur cycle-associated EBPR.

exploring a sulfur cycle-associated EBPR. In this innovative addition to the SANI® process, which consists of three separate reactors, the cultivation of PAOs was not possible without sludge recirculation between these reactors. Therefore, the possibility of a sulfur cycle-associated EBPR has been explored and confirmed in an alternating anaerobic/limited-oxygen aerobic sequencing batch reactor (SBR), with acetate fed as the sole electron donor and sulfate as the sulfur source, at a low influent total organic carbon (TOC) to sulfur ratio (i.e. $C_{in.}/S_{in.}$ = 1.1–3.1 mg C/mg S) (Wu et al., 2013). Although the exact reason for this EBPR process is unknown, a synergy between the sulfur cycle and poly-phosphate (poly-P) accumulating metabolism is envisioned (Wu et al., 2013); the hypothetical diagram of this Limited-Oxygen Sulfur cycle-associated EBPR (LOS-EBPR) are shown in Fig. 1a. This new EBPR process has all the aforementioned advantages of the SANI® process within the future water sensitive urban water cycle scenario.

However, the LOS-EBPR process needs further development. Due to the low oxygen transfer rate, the hydraulic retention time of the oxygen-limiting P-uptake phase is unacceptably long for full-scale applications. On the contrary, supplying more oxygen will inactivate biological sulfate reduction occurring in the anaerobic P-release phase (Kjeldsen et al., 2004). In addition, insufficient chemical oxygen demand (COD) in the influent will limit both biological P removal and denitrification simultaneously (Ekama and Wentzel, 1999).

To resolve these problems, an improved Denitrifying Sulfur cycle-associated EBPR (DS-EBPR) process, with nitrate as the electron acceptor, was proposed and examined in this study. As illustrated in Fig. 1b, this process makes use of denitrifying poly-phosphate accumulating organisms (DPAOs) for simultaneous anoxic P-uptake and denitrification (Kuba et al., 1993; Zeng et al., 2003a,b). The objectives of this study were to investigate the possibility of utilizing nitrate as electron acceptor for anoxic P uptake, to evaluate the performance of simultaneous N and P removal in association with the sulfur cycle, and to assess the effects of temperature and salinity on the operation of DS-EBPR process.

2. Methods and materials

2.1. Reactor design and operating conditions

A lab-scale SBR was made of transparent PVC, having a total reactor volume of 5 L (L) (4 L reaction volume and 1 L headspace), as shown in Supplementary Figure S1a. The reactor was tightly sealed and continuously operated in darkness for 211 days, with mixing by a mechanical mixer, at 250 rpm. The reactor pH was controlled between 6.9 and 7.9 by adding 0.5 N HCl/NaOH, and the temperature was maintained at 20 ± 1 °C and 30 ± 1 °C, respectively, during different experimental stages. The reactor was seeded with 4 L of sludge used in a previous study of sulfur cycle associated EBPR process, which was developed under alternating anaerobic/limited-oxygen aerobic conditions (Wu et al., 2013). Following the method of Kuba et al. (1993), this SBR was operated under alternating anaerobic/anoxic conditions for the purpose of developing sulfur cycle associated EBPR (DS-EBPR) in the reactor.

The composition of synthetic saline sewage as described in Wu et al. (2012), contains about 60 mg NH_4^+–N/L, 20 mg PO_4^{3-}–P/L, 400 mg COD/L. In this study, the only carbon source was sodium acetate, and the sulfur source was provided in two ways to meet the low influent C/S ratio: (i) adding only sodium sulfate solution to the synthetic sewage (from day 0 to day 145), and (ii) using 20% of real toilet flushing seawater instead of distilled water in the synthetic sewage (from day 146–211). The averaged initial concentrations of acetate, phosphate, sulfate and salinity during different stages (to be described as below) are shown in Table 1. The concentrated sodium nitrate solution was dosed into the SBR as an electron acceptor (Table 1). The cyclic operation of this anaerobic/anoxic-SBR, comprised (i) feeding of 2 L synthetic sewage (in 10 min), (ii) reaction phase R, i.e. P-release phase (phase time as shown in Table 1), (iii) addition of nitrate (200 mL of the sodium nitrate solution pumped into the reactor in 10 min), (iv) reaction phase U, i.e. P-uptake phase (phase time as shown in Table 1), (v) settling (110 min) and (iv)

Table 1 – Experimental stages and initial *in-situ* concentrations of acetate, sulfate, phosphate and nitrate in SBR (in the reactor after feeding or dosing).

Experimental stages		SBR operational condition			SBR reaction phase R					SBR reaction phase U	
Stages	Days	T (°C)	Sulfur-source (salinity %)	Cycle time (h/cycle)	Time (h)	In. HAc[a] (mg C/L)	In. SO_4^{2-}[a] (mg S/L)	In. PO_4^{3-}[a] (mg P/L)	$C_{in.}/S_{in.}$[b] (mg C/mg S)	Time (h)	In. NO_3^-[c] (mg N/L)
Stage 1	40	20 ± 1	$NaSO_4$ (0.05%)	24	7.83	–	–	–	–	13.83	–
Stage 2	46	20 ± 1	$NaSO_4$ (0.05%)	24	7.83	58 ± 3.7	114 ± 5.8	9.7 ± 1.5	0.51 ± 0.04	13.83	45
Stage 3	60	30 ± 1	$NaSO_4$ (0.05%)	12	5.83	52 ± 5.1	118 ± 2.4	8.7 ± 1.0	0.44 ± 0.04	3.83	45
Stage 4	65	30 ± 1	Seawater (0.7%)	12	5.83	57 ± 2.5	129 ± 9.3	12.8 ± 1.6	0.45 ± 0.03	3.83	45

[a] The initial concentrations of acetate, sulfate and phosphate were measured at the beginning of reaction phase R, specified as mg carbon and sulfur per liter of reactor. The data shown in this table are "Measured Average Value ± Standard Deviation".

[b] The influent ratio of TOC to sulfur ($C_{in.}/S_{in.}$) was calculated from the initial concentration of acetate-carbon divided by the initial concentration of sulfate-sulfur. The EBPR function can be successfully developed in the conventional sewage with a TOC to SO_4^{2-}–S ratio of 16 (mg C/mg S) (Tchobanoglous et al., 2004). In addition, the EBPR under the low $C_{in.}/S_{in.}$ ratio of 1.1–3.1 was investigated in Wu et al. (2013). A value of $C_{in.}/S_{in.}$ close to 0.5 mg C/mg S was investigated in the present study.

[c] The initial concentration of nitrate at the beginning of reaction phase U was calculated theoretically and it specified in terms of the entire reactor liquid volume (4 L).

Table 2 – Overall reactor performance (average value ± standard deviation).

	Volumetric HAc uptake rate[a] (mg C/L/d)	Volumetric nitrate consumption rate[b] (mg N/L/d)	Volumetric sulfate reduction rate[a] (mg S/L/d)	Volumetric P removal rate[a] (mg P/L/d)	VSS in reactor (g VSS/L)	TSS in reactor (g SS/L)	SRT (d)	ORP (mV, SHE)
Stage 2	58 ± 3.7	45	27 ± 3.6	7 ± 1.4	2.4 ± 0.2	3.1 ± 0.4	87 ± 11	−250 to −130
Stage 3	104 ± 10.2	90	46 ± 6.8	12 ± 1.7	3.1 ± 0.4	4.0 ± 0.4	68 ± 9	
Stage 4	115 ± 4.8	90	48 ± 6.9	7 ± 3.2	3.3 ± 0.3	4.3 ± 0.4	79 ± 11	

[a] The volumetric loading of acetate uptake, sulfate reduction and phosphate removal were calculated based on {[[Measured initial concentration of the reaction phase R] − [Measured end concentration of decant phase]] × [2L influent/cycle] × [Cyclic run numbers/day}/[4$L_{reactor}$]] basis to describe the overall performance of the reactor, not for evaluating the maximum reaction rate.
[b] The nitrate was completely consumed during the reaction phase U, the specific nitrate removal rate depended on the theoretical concentration of the additional nitrate solution.

decantation (2 L of supernatant being decanted over 10 min after settling), as illustrated in Supplementary Figure S1b.

Since this study was aimed to investigate the possibility of simultaneous N and P removal in association with sulfur cycle and the effects of some key factors (such as temperature, loading and salinity) on this new DS-EBPR process, the reactor operation was divided into four stages (Table 1) viz. Stage 1 start-up and Stage 2 both at 20 °C and 24 h cycle time; then 30 °C and 12 h cycle time in Stages 3 and 4. In Stage 4, real toilet flushing seawater was used in the synthetic sewage to study the salinity effect on this process. In stages 2–4, the SBR was tested 2 to 3 times per week by taking samples at the beginning and end of the reaction phases R and U.

No sludge was purposely wasted during the entire operation. The sludge loss was self-regulated, as a result of sludge used for samples and batch tests or sludge escaping via the effluent. From the measurement of these sludge losses the sludge retention time (SRT) was calculated to be 87, 68 and 79 days in Stages 2, 3 and 4 respectively (see Table 2). In order to remove accumulated P from the biomass, the chemical P-harvesting was carried out periodically (about every 20 days), according to the method described in Wu et al. (2013).

2.2. Additional experiments

2.2.1. Determination of SBR cyclic performance

The DS-EBPR cyclic behavior was tested in the SBR reactor over three SBR-cycle tests C1 to C3. Tests C1 and C2 were carried out at the end of Stages 2 and 3 respectively to investigate the performance of SBR under 20 °C and 30 °C. Test C3 was carried out at the end of Stage 4 to investigate the effect of seawater toilet flushing on the SBR-cycle performance. Microbial inclusions (i.e. PHB, PHV, Glycogen and Poly-S) and bulk liquid components (i.e. acetate (HAc), ortho-phosphate (OP), sulfate, and nitrate) were measured during these tests.

2.2.2. Batch tests

In addition to the SBR-cycle tests, three types of batch test were conducted when the reactor was operating in Stage 3 to investigate: 1) the effect of temperature (10, 20 and 30 °C) on the physiology (in term of stoichiometry and kinetics) of the DS-EBPR (Type T); 2) the relationship between biological P removal and sulfur cycle (Type S); and 3) the effect on the anoxic P uptake performance of the sludge in phase U with and without acetate addition in the preceding anaerobic reaction phase R (Type U). All these batch tests were carried out in darkness in a 450-mL batch reactor, comprising 400 mL effective volume and 50 mL headspace. The batch reactor was sealed and equipped with a magnetic stirrer and a pH controller (6.9–7.9) (Supplementary Figure S1c). For all these batch tests, 400 mL of sludge was taken from the SBR at the end of reaction phase U, and nitrate solution was dosed at the end of anaerobic reaction phase R to provide the electron acceptor for anoxic reaction phase U. After each batch test, the residual sludge was put back to the SBR reactor. The batch test conditions are summarized in Table 3. Details of the batch

Table 3 – Batch test conditions.

		Type-T			Type-S					Type-U
		T10	T20	T30	S1	S2	S3	S4	S5	U
Day		127	129	131	100	109	114	118	120	116
Temperature (°C)		10	20	30	20	20	20	20	20	20
Number of batch tests		3	3	3	3	3	3	3	3	1
Feed synthetic sewage	HAc	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
	PO_4^{3-}	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	SO_4^{2-}	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes
Loading rate (mg COD/g VSS Cycle)		66.7	66.7	66.7	66.7	66.7	66.7	66.7	66.7	0
P/VSS (mg P/g VSS)		57	57	56	36	48	54	60	61	58
Reaction Phase R (h)		8	6	6	6	6	6	6	6	6
Reaction Phase U (h)		8	4	4	4	4	4	4	4	4
Bulk liquid compounds[a]		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Microbial inclusions[b]		Yes	Yes	Yes	No	No	No	No	No	Yes

[a] Bulk liquid compounds include SO_4^{2-}, PO_4^{3-}, HAc and NO_3^-.
[b] Microbial inclusions include PHA, Glycogen, Poly-phosphate and Poly-S^{2-}/S^0.

tests type-T, type-S and type-U are described in Supplementary Information 1.2.

2.3. Analytical methods

Mixed liquor suspended solids (SS), volatile suspended solids (VSS) and sulfide were determined according to the Standard Methods (APHA, 1998). Key anions in the bulk liquid, including acetate, chlorate, nitrate, phosphate, sulfate and thiosulfate were determined by ion chromatography (Shimadzu Prominence Liquid Chromatograph). Poly-β-hydroxyalkanoates (PHA), including poly-β-hydroxybutyrate (PHB) and poly-β-hydroxyvalerate (PHV), were determined by gas chromatography (Oehmen et al., 2005). Glycogen was analyzed using the anthrone method according to Jenkins et al. (2004). Polysulfide and elemental sulfur (Poly-S^{2-}/S^0) were determined by means of the sulfite method (Jiang et al., 2009). Details of the analytical methods of PHA, glycogen, and poly-S^{2-}/S^0 are described in Wu et al. (2013). The concentrations of degraded and synthesized poly-P (in mg P/L) and poly-S^{2-}/S^0 (in mg S/L) were calculated from the changes in the bulk liquid phosphate and sulfate concentration in the P-release and P-uptake phases (1 mol poly-P equals to 1 mol phosphate) (Smolders et al., 1994), for instance, when the stored poly-S was not measured, it was calculated based on the difference between bulk liquid sulfate concentrations.

The performance data of SBR operation during different stages and batch tests (Type-T and Type-S) were statistically compared through the variance analysis, with 95% confidence (Sheskin, 2000).

2.4. Microbial community analysis

The sludge sample was taken from the SBR at day 130, representing the cultivated sludge. The diversity of the microbial community was revealed by 454-pyrosequencing of 16S rRNA gene, according to Lee et al. (2011) and Zhang et al. (2012). Details of the methods of DNA extraction, PCR amplification, pyrosequencing and data analysis is provided in Supplementary Information 2.

3. Results and discussion

3.1. SBR operation and performance

Initially (Stage 1), the inoculate sludge was unable to use nitrate as electron acceptor, but over 40 days of acclimation, the sludge developed denitrification ability. At the end of Stage 1, the objectives of utilizing nitrate as electron acceptor and removing P under anoxic conditions were achieved. Thereafter, the SBR was continuously operated for 171 days (from day 41–211) which was sub-divided into Stages 2, 3 and 4. The general performance of the reactor during Stages 2–4 is summarized in Table 2; the key processes of P-release, P-uptake and P-removal, and the associated sulfur cycle conversions (bulk liquid sulfate changes) are shown in Fig. 2a–c.

Compared with LOS-EBPR, the DS-EBPR SBR cycle time was significantly reduced because nitrate was available as an electron acceptor for the majority of the anoxic period. The cycle time required for LOS-EBPR SBR was over 42 h/cycle due to the very slow supply of oxygen (Wu et al., 2013), whereas that for the DS-EBPR anaerobic/anoxic SBR could be achieved in 24 h/cycle during Stage 2, and further reduced to 12 h/cycle during Stages 3 and 4 at 30 °C.

Decreasing the cycle time increases the volumetric loading rates of the reactor. The volumetric acetate uptake rate increased from 34 ± 7 mg C/($L_{reactor}$ d) in LOS-EBPR to 58 ± 3.7 mg C/($L_{reactor}$ d) in Stage 2 and 115 ± 5.5 mg C/($L_{reactor}$ d) in Stages 3 and 4 of this study; with a concomitant increase in volumetric sulfate reduction rate from 17.8 ± 5.2 mg S/($L_{reactor}$ d) in LOS-EBPR to 27 ± 3.6 mg S/($L_{reactor}$ d) in Stage 2 and 48 ± 6.9 mg S/($L_{reactor}$ d) in Stages 3 and 4 for DS-EBPR (acetate removal rate in COD = mg C/(L d) × 2.67 mg COD/mg C). This was confirmed to be a statistically significant improvement by multiple student's *t*-test with $\alpha = 0.05$. Moreover, the volumetric anoxic nitrate consumption rate was zero in LOS-EBPR, but it was 45 mg N/($L_{reactor}$ d) in Stage 2, and 90 mg N/($L_{reactor}$ d) in Stages 3 and 4 in DS-EBPR. These nitrate consumption rates in the DS-EBPR would have been higher, had more nitrate been dosed at the start of the anoxic phase U.. Curiously, the volumetric P removal rate from the bulk liquid of LOS-EBPR and DS-EBPR remained similar, i.e. 6.3 ± 1.4 mg P/($L_{reactor}$ d) in the LOS-EBPR and 7.0 ± 1.4 mg P/($L_{reactor}$ d) and 12.0 ± 1.7 mg P/($L_{reactor}$ d) and 7.2 ± 3.2 mg P/($L_{reactor}$ d) in Stages 2, 3, and 4 of the DS-EBPR (Student's *t*-test, $\alpha = 0.05$).

The COD loading rate of Stages 3 and 4 doubled that of Stage 2 and increased the number of cycle/d (n) from 1 to 2 per day, i.e. COD loading rate = [(400 mg COD/$L_{influent}$) × (2L influent/cycle) × (n cycles/d)]/(4$L_{reactor}$) = 200 n mg COD/($L_{reactor}$ d). As compared with LOS-EBPR (where $n = 0.5$), the COD loading rate at Stages 3 and 4 were 4 times higher. Moreover, the reactor VSS concentration was also higher, increasing from 1.2 g VSS/L in LOS-EBPR to 2.4 g VSS/L in Stage 2 and 3 g VSS/L in Stages 3 and 4. These results indicate that the sulfur cycle-associated EBPR process can be significantly intensified volumetrically by making use of nitrate as an electron acceptor for potential full-scale application. The specific rates of C, N, S and P relating to the biomass activities are described in Section 3.3 below.

The P accumulation in the biomass was calculated from the difference between the influent and effluent P flux and the P removed from the SBR via sampling, batch tests and P-harvesting. The P/VSS ratio over time in Stages 2, 3 and 4 is shown in Fig. 2d, which shows that the P content increased from about 26 to 70 mg P/g VSS over about 100 days in Stages 2 and 3, but decreased sharply to 30 mg P/g VSS after chemical harvesting and changing to seawater during the transition of Stage 3 and 4. However, it gradually increased again in Stage 4–60 mg P/g VSS. This P content is quite low compared with the LOS-EBPR (180 mg P/g VSS, Wu et al., 2013) and also very low compared with conventional aerobic EBPR (380 mg P/g PAO-VSS, Wentzel et al., 1989).

Temperature is a key factor affecting the EBPR process (Brdjanovic et al., 1997, 1998; Lopez-Vazquez et al., 2009a,b). Hence, the temperature effect was investigated by comparing the performances of the reactor operated under different temperatures. In this study, the reactor was operated at 20 °C (46 days in Stage 2) and 30 °C (60 days in Stage 3), respectively,

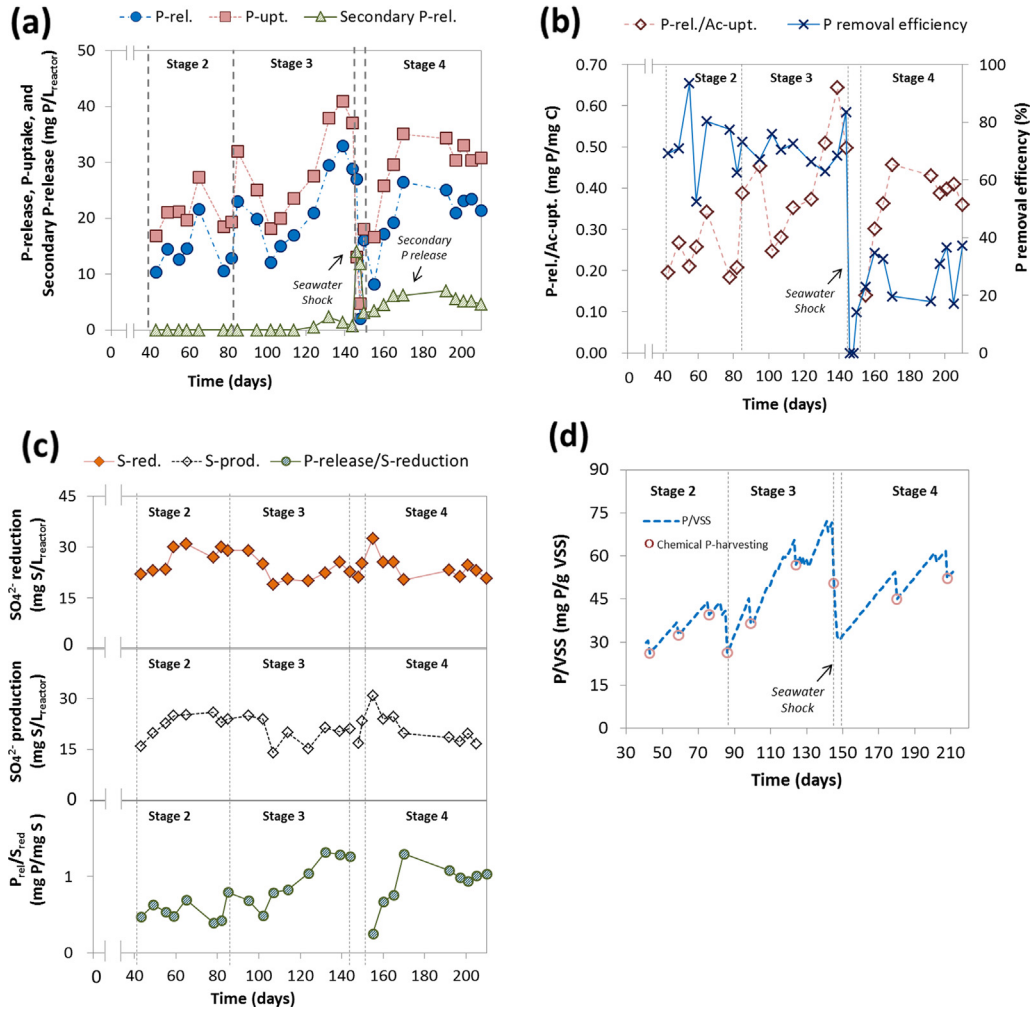

Fig. 2 – The biological phosphorus release (difference between end and initial P concentrations of anaerobic period R) and uptake (difference between initial and end P concentrations of anoxic period U) performance and associated sulfate reduction (difference between and initial and end SO_4^{2-}–S concentrations of anaerobic period R) and generation (difference between initial and end SO_4^{2-}–S concentrations of anoxic period U) over the long period SBR operation of Stage 2, 3 and 4: (a) P-release (●), P-uptake (■) and "Secondary" P-release (▲); (b) P-release to HAc-uptake ratio (◇) and P removal efficiency (×); (c) Sulfate-reduction (▯), Sulfate-production (◇) and P-release to S-reduction ratio (●); (d) the phosphorus content in sludge mass (mg P/g VSS) and the times of P-harvesting treatment (○).

to simulate the average temperature in winter and summer of the Hong Kong sewage (Yu et al., 2002). The P-release, P-uptake and P-removal efficiency are shown in Fig. 2a and b. The P-release and P-uptake at 20 °C was 15 ± 4.8 and 22 ± 5.1 mg P/$L_{reactor}$, respectively, while at 30 °C they were 22 ± 7.6 and 29 ± 8.7 mg P/$L_{reactor}$, respectively. The P removal efficiency, $P_{eff.} = (P_{in.} - P_{end.})/P_{in.} \times 100\%$, where $P_{in.}$ = OP concentration at the start of reaction phase R while $P_{end.}$ = OP concentration at the end of reaction phase U, remained on the average of 72 ± 12.2 and 71 ± 6.5% at 20 °C and 30 °C, respectively. The results support the idea that the sulfur cycle EBPR is capable of tolerating high temperatures. As compared to conventional EBPR processes of which the P removal efficiencies had been reported to be negatively affected by warm climates or even during the summer seasons in temperate climates (Cao, 2011), this new EBPR process appears to be able to withstand the negative impact of temperature changes and maintain good P removal. A possible reason of the warm temperature tolerance may be due to the less potential for the populations shift from PAOs to GAOs during the 60 days period of the long SRT DS-EBPR system (about 1 SRT) than that of the conventional EBPR (about 3–6 SRT). A further study will be carried out to test this view by operating a DS-EBPR system under 30 °C in a longer period (equivalent to three times the SRT or longer).

In Hong Kong, the saline sewage is rich in salts (e.g. 4000 mg Cl^-/L, Yu et al., 2002). The salt inhibition on EBPR in nutrient removal systems has been reported in the literature (Uygur and Kargi, 2004; Bassin et al., 2011). Thus, there is a need to evaluate the salinity effect on the new EBPR process, with a view to examine the possibility of developing the sulfur

cycle-associated EBPR for saline sewage. In Stage 4, the SBR was continuously operated at 30 °C, with introduction of the seawater as the sulfur source.

When the feed was switched to seawater, the P uptake behavior was seriously affected. In the initial days of Stage 4, the effluent P was higher than the influent with the result that the P/VSS ratio decreased (Fig. 2d). After 4 days, almost all the poly-P accumulated in Stage 3 was released, and the P content dropped to that of the beginning of Stage 3. Thereafter, the P uptake behavior recovered and the P/VSS ratio gradually increased. After 58 days, i.e. near the end of Stage 4 (day 207), the P/VSS ratio reached a maximum of 62 mg P/g VSS, which is similar to the maximum reached at the end of stage 3 (day 144), i.e. 72 mg P/g VSS. However, the P accumulation rate (when no P losses from P-harvesting and batch test) in Stage 4 was slower (0.77 mg P/(g VSS d), day 148–178) than in Stage 3 (1.6 mg P/(g VSS d), day 87–98).

Despite the P uptake behavior recovering during Stage 4, there was a major difference in the behaviors between Stages 3 and 4. In Stage 3, the maximum P uptake (i.e. minimum P concentration in the anoxic phase) occurred at the end of the anoxic period even though the nitrate concentration might have reached zero before the end of the anoxic period. In Stage 4, the maximum P uptake took place at the time the bulk liquid nitrate concentration reached zero; and if this took place before the end of the anoxic period, P release ("secondary") took place. This can be seen in Fig. 3b (stage 3, day 142) and Fig. 3c (stage 4, day 202). Simultaneously with this different P uptake behavior was a shift in biomass glycogen content-in stage 3 the glycogen concentration (with respect to bulk liquid) decreased by only 14 mg C/L between the start and the end of the anaerobic period (Fig. 3b) whereas in Stage 4 it decreased by 32 mg C/L (Fig. 3c).

The deteriorating P removal efficiency in Stage 4 caused by the "secondary P-release", was on average of about 4 ± 2.2 mg $P/L_{reactor}$ (as shown in Fig. 2a, between days 160 and 211). The phenomenon of the secondary P-release has already been reported in the literature on denitrifying EBPR (Kuba et al., 1993), which is possibly the reason for the decrease in efficiency in the EBPR process (Cao, 2011). This secondary P-release can be interpreted as the process of degrading intracellular poly-P in order to provide ATP for anaerobic maintenance when the nitrate reaches zero (Kuba et al., 1993; Smolders et al., 1994; Brdjanovic et al., 1997). Following the method of Smolders et al. (1994), the average anaerobic ATP maintenance coefficient (m_{atp}^{an}) of the DS-EBPR (when fed with seawater) was calculated to be 0.45 ± 0.2 mmol ATP/(g VSS h). This value is within the range of the (m_{atp}^{an}) values (0.3–3.6 mmol ATP/(C mol active.biomass h) obtained in the conventional EBPR study (Brdjanovic et al., 1997). Also, Uygur and Kargi (2004) suggested that the low efficiency of P removal from the salty wastewater could result from the secondary P-release. This secondary P-release was possibly due to insufficient nitrate dosed in the anoxic phase U. An insufficient nitrate dosing might cause the reactor eventually to collapse, had it been operated for a longer time. With regard to real reactor operation, this secondary P-release could be avoided if nitrate is adequately available for the entire anoxic phase U. However, no significant impacts of seawater shock and secondary P-release were observed in different DS-EBPR reactors (a parallel study), in which the seawater was fed from the beginning of the reactor start-up period.

The sludge production rate in the reactor was estimated based on the calculation of the sludge age by dividing the total reactor biomass by the average daily biomass wastage via effluent and sampling. The sludge production rates were 28 ± 5, 45 ± 5, and 40 ± 5 mg VSS/(L d) in Stages 2, 3 and 4, respectively. In addition, the sludge yield was calculated to be 0.13–0.17 g VSS/g COD. Little biological sludge was produced in this DS-EBRP process, as little as the biological sludge production in the SANI® process (Lu et al., 2012a), which is possibly due to the involvement of the sulfur cycle.

3.2. Microbial community

The diversity of microbial community of the cultivated sludge (day 130) was examined by 454-pyrosequencing analysis. After quality filtering, 7904 sequences was acquired. The microbial community in the sludge sample was well represented by these sequences as shown in the rarefaction curves (Figure S2a) and Good's coverage (≥93%, Table S2). Supplementary Figures S2b show the relative bacterial community abundances of the bacterial genus level.

Nearly all living organisms have poly-P compounds (Kornberg, 1995), but only some bacteria can accumulate the poly-P in large amounts, e.g. *Accumulibacteria* (Hesselmann et al., 1999) and *Tetrasphaera* (Nguyen et al., 2011). These bacteria were found to be the major poly-P accumulating organisms (PAO) in the wastewater treatment plant or lab-scale EBPR system (Nielsen et al., 2012). However, in this study, no 16S rRNA gene sequences related to known PAOs were detected in the cultivated sludge sample (Figure S2b). Instead, fermentative bacteria *Lactococcus*-like species (13.8%), *sphingomonas*-like species (14.3%), sulfate reducing bacteria (*Desulfobulus*-like species (10.6%) and *Desulfomicrobium*-like species (10.5%)) and unclassified genera (17.1%) were identified to be the predominant groups in the cultivated sludge. The *sphingomonas*-like species was reported as a potential glycogen-accumulating organisms (GAOs) species which were reported to be competing carbon source with PAOs (Beer et al., 2004). Therefore, the bacteria performing poly-P accumulation in our reactor should belong to genera other than *sphingomonas*. Although the exact species are not known, the results demonstrated the possible presence of unknown PAOs in the sulfur cycle associated P removal process. Further studies are required for the identification as well as phylogenetic and physiology analysis of the major functional populations.

3.3. SBR cyclic performance

Fig. 3a–c show the cyclic performance of typical SBR-cycles in Stages 2, 3 and 4, as measured in the "in-situ" cyclic tests C1 (day 83), C2 (day 142) and C3 (day 202), respectively. According to these observed cyclic tests, the microbial conversions that occurred can be described as: carbon storage (i.e. PHA formation), glycogen degradation and sulfate reduction occurring simultaneously with P-release, which implied electrons transferring to sulfate through the sulfur cycle forming poly-S^{2-}/S^{0} in reaction phase R. Almost no thiosulfate and sulfide

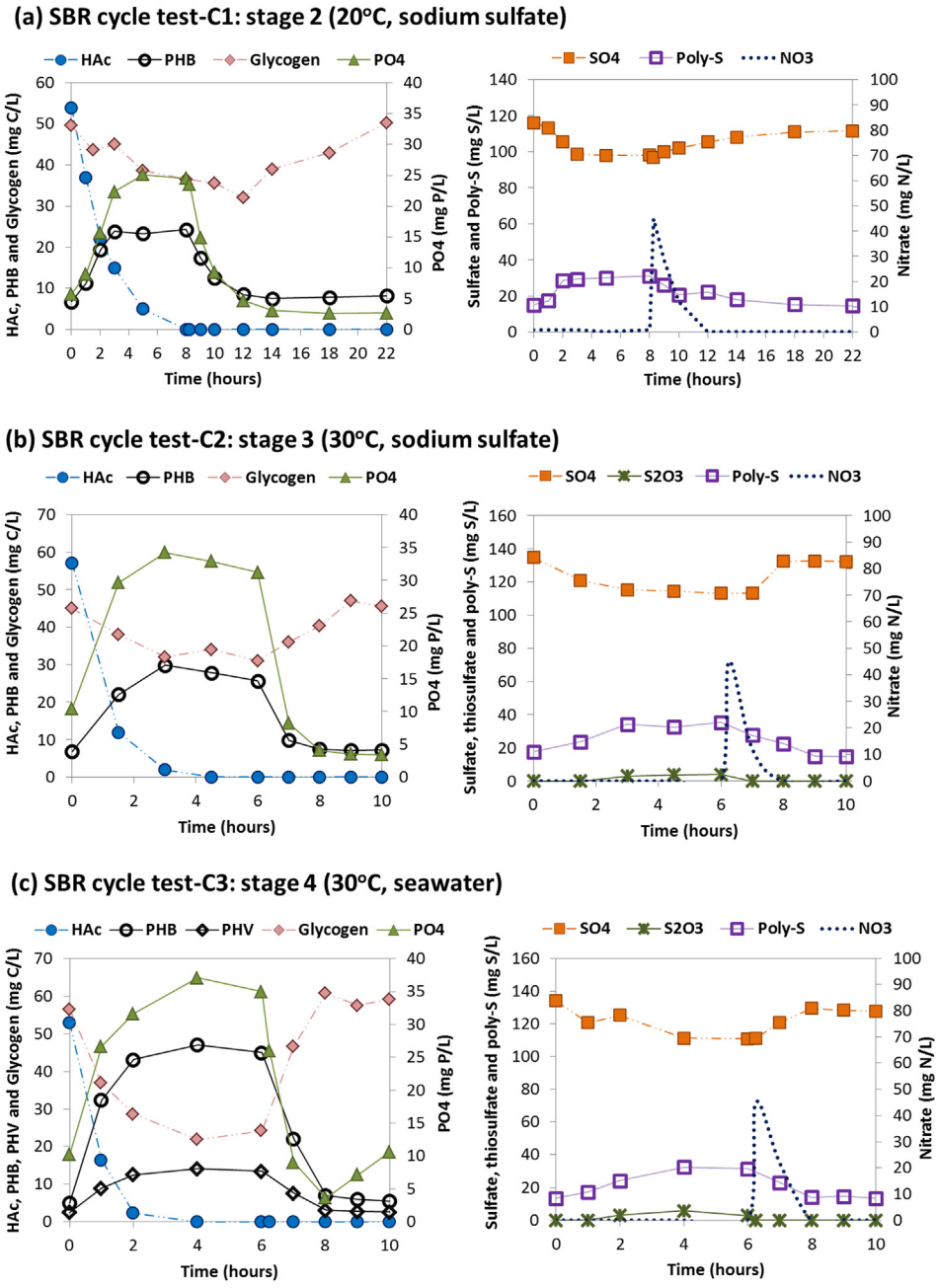

Fig. 3 – Cyclic changes of the key EBPR and associated compounds in the SBR system measured in the cyclic tests C1 to C3: (a) – (c) shows the SBR cycle tests C1 (day 83), C2 (day 142) and C3 (day 202), respectively. (HAc (●), PHB (○), PHV (◇), Glycogen (), Phosphate (▲), sulfate (■), thiosulfate (×), poly-S^{2-}/S^0 (□) and nitrate (··).

were detected as the end products of sulfate reduction. Subsequently in reaction phase U, the stored PHA and poly-S^{2-}/S^0 were oxidized using nitrate as an electron acceptor, P-uptake from and SO_4^{2-} release to the bulk liquid was observed together with an increase in intracellular poly-P and glycogen.

The important stoichiometric ratios and kinetic rates of the key EBPR process and associated sulfur cycle are summarized in Table 4. From these ratios and rates, some observations of the DS-EBPR reactor performance are outlined below. Firstly, comparing cycle tests C1 and C2 at 20 °C and 30 °C shows that the stoichiometric ratios are similar but the kinetic rates are significantly higher at 30 °C, especially those under the anoxic conditions — oxidizing the stored PHA and Poly-S with nitrate (Table 4). These results, also confirmed by batch tests T10, T20 and T30 below, indicated that the biological mechanisms of the sulfur cycle EBPR behaved stably under different operating temperatures. So it is speculated that the dominating microbial populations could persist without a significant shift while the process is operated under different temperatures. In addition to this tolerance to temperature change, the

Table 4 – Stoichiometric ratios and kinetic rates (average value ± standard deviation).

Experiments	Stoichiometric ratios					Kinetic rates[a]							
	Key EBPR ratios				S Cycle	Anaerobic condition				Anoxic condition			
	$P_{rel.}$/ $Hac_{upt.}$ (mg P/ mg C)	$PHA_{form.}$/ $Hac_{upt.}$ (mg C/ mg C)	$P_{upt.}$/ $No_{3cons.}$ (mg P/ mg N)	$P_{upt.}$/ $Pha_{deg.}$ (mg P/ mg C)	$P_{rel.}/S_{red.}$ (mg P/ mg S)	$HAc_{upt.}$ (mg C/g VSS/h)	$P_{rel.}$ (mg P/g VSS/h)	$PHA_{form.}$ (mg C/g VSS/h)	$S_{red.}$ (mg S/g VSS/h)	$PHA_{deg.}$ (mg C/g VSS/h)	$P_{upt.}$ (mg P/g VSS/h)	$NO_{3cons.}$ (mg N/g VSS/h)	$S_{oxid.}$ (mg S/g VSS/h)
SBR cycle test-C1 (20 °C)	0.36	0.32	0.5	1.3	1.1	7.1	2.3	2.6	2.4	4.9	6.4	7.0	1.4
SBR cycle test-C2 (30 °C)	0.42	0.4	0.68	1.4	1.1	9.7	4.1	3.2	3.0	6.5	7.4	11	3.1
SBR cycle test-C3 (30 °C)	0.51	1.0	0.74	0.67	1.4	11	4.9	10.4	2.7	9.6	7.8	7.0	3.0
Batch test T10 (10 °C)	0.36 ± 0.02	0.70 ± 0.04	0.94 ± 0.06	0.69 ± 0.04	2.5 ± 0.35	4.4 ± 0.9	0.8 ± 0.05	2.2 ± 0.3	0.7 ± 0.3	2.4 ± 0.1	2.7 ± 0.2	1.8 ± 0.1	0.8 ± 0.04
Batch test T20 (20 °C)	0.58 ± 0.02	0.67 ± 0.02	0.84 ± 0.03	0.62 ± 0.02	2.2 ± 0.19	7.4 ± 0.7	2.9 ± 0.2	4.3 ± 0.4	2.1 ± 0.3	6.9 ± 0.4	10 ± 0.7	7.1 ± 0.3	2 ± 0.1
Batch test T30 (30 °C)	0.61 ± 0.04	0.58 ± 0.04	0.87 ± 0.04	0.75 ± 0.02	1.4 ± 0.09	8.6 ± 0.7	4 ± 0.7	4.5 ± 0.3	3.8 ± 0.7	8.3 ± 0.9	11 ± 1.2	9.8 ± 0.5	3.7 ± 0.2
LOS-EBPR[b] (20 °C)	0.8 ± 0.15	0.32 ± 0.03	NA	3.6 ± 0.6	1.5 ± 0.3	7.2 ± 1.5	5.6 ± 1.6	NA	3.4 ± 1.3	NA	1.2 ± 0.1	NA	0.6 ± 0.1

[a] The kinetics rates were calculated from the first 2 h of the obtained data to represent the maximum kinetics rates.
[b] The stoichiometric ratios and kinetic rates in Stage 4 in Wu et al. (2013) were used as the representative values for LOS-EBPR.

persistence of stable dominant populations could be a benefit from the slow growth and long SRT of the sulfur cycle system. However, a disadvantage of these slow growth bioprocesses is that, if the bioprocesses of this new EBPR system are disrupted and deteriorate, the system will take long time to recover. Also, the inert solids accumulation in the sludge within a long SRT system will decrease the specific activity of the VSS in the full-scale application.

The SBR-cycle performance fed seawater was investigated based on the results from the typical cyclic test (C3) in Stage 4. Some of the stoichiometric ratios and kinetic rates are now significantly different from those of cycle test-C2 performed at the same temperature (30 °C) without seawater in Stage 3. The ratios of $P_{rel.}/HAc_{upt.}$ and $P_{rel.}/S_{red.}$, and the specific rates of P-release ($P_{rel.}$) and sulfate reduction ($S_{red.}$) in cycle test-C3 are similar to those of cycle test-C2. This indicated that the activities of the responsible species for DS-EBPR had not failed when subjected to saline sewage but were in shock for a short period (4 days) by a sudden change to seawater. While the biomass lost much of its stored poly-P, P accumulation did recover after the seawater shock. However, it never recovered to the same rates as in Stage 3 (0.77 mg P/(g VSS d) vs. 1.6 mg P/(g VSS d)).

Furthermore, the ratio of $PHA_{form.}/HAc_{upt.}$ (1.0 mg C/mg C) in cycle test-C3 was significantly higher than the ratio in cycle test-C2 (0.4 mg C/mg C). Thereby, the amount of PHA formed anaerobically in cycle test-C3 could be twice as much as formed anaerobically in cycle test-C2. This higher PHA formation did not correspond with a higher P-release; on the contrary, the ratio of $P_{rel.}/PHA_{form.}$ decreased from 1.1 mg P/mg C in the cycle tests C1 and C2 to 0.5 mg P/mg C in cycle test-C3. Furthermore, the glycogen changes became significant in cycle test C3, and the ratio of $Gly_{deg.}/HAc_{upt.}$ increased from 0.24 in the cycle tests C1 and C2 to 0.6 in cycle test-C3. This illustrates that greater glycogen involvement emerged in the reactor after it was fed with seawater. Moreover, the PHA compounds contained not only PHB but also PHV; more PHV formation was due to more glycogen degradation (Zeng et al., 2003a,b). The phenomenon of the glycogen pathways gradually replacing the poly-P pathways for storing acetate occurs also in conventional EBPR, and can be explained as GAOs competing for carbon sources with PAOs in anaerobic conditions (Zeng et al., 2003a,b). This competition is intensified by environmental factors such as temperature (Lopez-Vazquez et al., 2007, 2009a,b) and salinity (Bassin et al., 2011; Welles et al., 2012). Based on the results in this study, it appears that the sulfur cycle EBPR is more tolerant to temperature change; however, the trend of glycogen accumulation under a salt stress condition (Bassin et al., 2011) could possibly result in EBPR deterioration. It is unclear that the salt stress caused the deterioration in P removal.

3.4. *Temperature effect on the stoichiometry and kinetics of the S cycle EBPR*

Temperature effect on the physiology of this new sulfur cycle EBPR sludge was carried out in the batch test type-T (see Supplementary Figures S3a–c). The anaerobic and aerobic stoichiometric ratios and kinetic rates observed at different operating temperatures are summarized in Table 4. In addition, the different specific (/g VSS) conversion rates of the biomass under different temperatures are shown in Supplementary Figures S4d–k and both simplified and extended Arrhenius equations were fitted to the data. The θ values for the various kinetic rates are shown in Figures S3d–k. These batch tests confirm the results of the C1 and C2 cycle tests, i.e. temperature does not affect the ratios of $P_{rel.}/HAc_{upt.}$, $PHA_{form.}/HAc_{upt.}$, $P_{upt.}/NO_{3cons.}$, $P_{upt.}/PHA_{deg.}$, and $P_{rel.}/S_{red.}$, but does increase the kinetic rates when temperature is raised from low (10 °C) to high (30 °C) in the batch tests. These were confirmed statistically by multiple student's *t*-test with $\alpha = 0.05$. This phenomenon on the temperature effect is also found in the conventional EBPR (Brdjanovic et al., 1997).

3.5. *Sulfur cycle effect on the phosphorus removal*

In this new DS-EBPR process, the sulfur transformations associated with the P-release and P-uptake was measured in batch tests S1 to S5 on days 100, 109, 114, 118 and 120 in Stage 3 in which the "P content" of the VSS was 36, 48, 54, 60, and

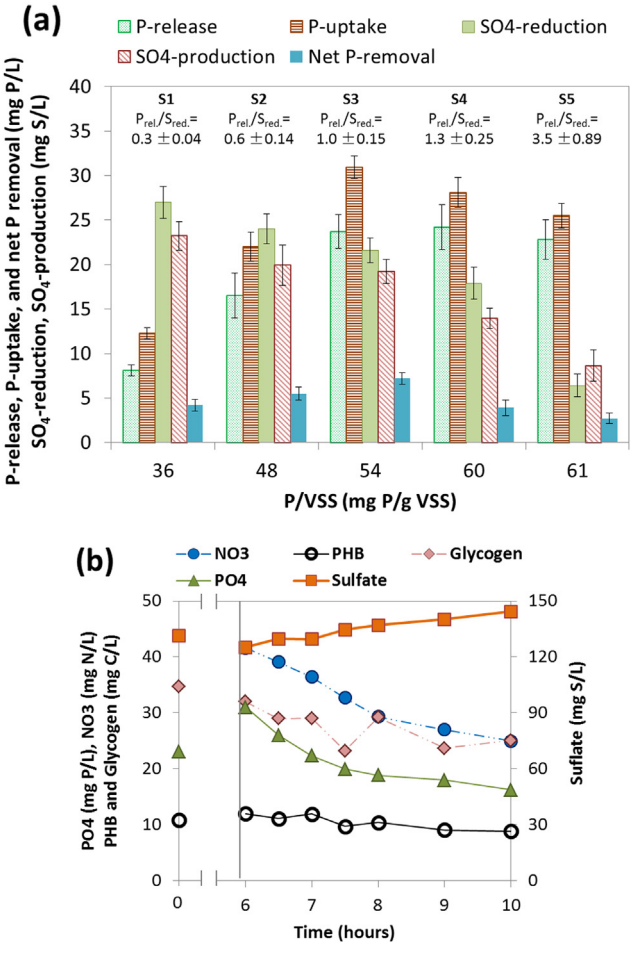

Fig. 4 – Sulfur cycle effect on phosphorus removal: (a) Results from Batch test Type-S (P-release (□), P-uptake (▤), Sulfate-reduction (▨), Sulfate-production (▧) and net P-removal (=$P_{upt.}$ – $P_{rel.}$, ■)) and (b) Results from Batch test Type-U (Nitrate (●), PHB (○), Glycogen (◇), Phosphate (▲), Sulfate (■)).

61 mg P/g VSS, respectively. Fig. 4a shows the results, the anaerobic P-release and SO_4^{2-}–S reduced, anoxic P-uptake and SO_4^{2-}–S produced, and the P removal ($P_{upt.} - P_{rel.}$) in these batch tests. The anaerobic P-release increased from 8 ± 0.6 mg P/$L_{reactor}$ to 24 ± 2.5 mg P/$L_{reactor}$ as the P/VSS ratio increased from 36 (S1) to 60 (S4) mg P/g VSS, while the sulfate reduction decreased from 27 ± 1.8 mg S/$L_{reactor}$ (S1) to 18 ± 1.8 mg S/$L_{reactor}$ (S4). From these results, the ratio $P_{rel.}/S_{red.}$ increased from 0.3 ± 0.04 to 1.3 ± 0.3 mg P/mg S in the batch tests S1 to S4. The P removal increased from 4.2 ± 0.64 to 7.2 ± 0.65 mg P/$L_{reactor}$ in the batch tests S1 to S3, but then decreased to 3.9 ± 0.85 mg P/$L_{reactor}$ in S4. All these results are statistically significant different (multiple student's *t*-test, $\alpha = 0.05$).

In batch test S5, no sulfate was added at the start of the anaerobic period so sulfate reduction associated with the P release could not take place. The P release was still as high as S5, but the SO_4^{2-} reduction was only 6 mg S/L (from residual sulfate in the bulk liquid of the sludge addition). However, the P removal now was only 2.7 ± 0.6 mg P/$L_{reactor}$ in S5.

In the conventional EBPR process, the carbon substrate uptake and PHA storage is linked with the poly-P degradation and P-release, the more poly-P degraded and P released, the more carbon substrate taken up and PHA stored in biomass; also, only if sufficient PHA is stored, can P-uptake and poly-P accumulation take place. In contrast, with DS-EBPR, the P-uptake and poly-P accumulation is not linearly related to the PHA stored in the DS-EBPR sludge. According to the results of above batch tests (Type-S), the maximal P removal (i.e. poly-P accumulated in the sludge) was achieved when the ratio P-release/S-reduced was around 1.0 mg P/mg S. The presence of this optional value of the $P_{rel.}/S_{red.}$ ratio is in agreement to the proposed idea (Wu et al., 2013) that a synergetic relationship exists between sulfate reduction and biological phosphorus removal. In conformity with this synergetic relationship, the electron flow of the intracellular storage for biological P removal was roughly estimated below (the calculation details are shown in Supplementary Information 4) for Stage 3 in this study (the ratio of $P_{rel.}/S_{red.} = 0.96 \pm 0.31$ mg P/mg S); the specific bioenergetics analysis for the S cycle EBPR will be addressed in a further study.

The storage compounds PHA and poly-S^{2-}/S^0 were formed from the acetate uptake (about 550 mg COD/cycle) in the reaction phase R. The average value of PHA produced via anaerobic organic-C storage in reaction phase R was 300 mg COD/cycle accounting for 54% of acetate uptake. The ratio of poly-P degraded to PHA synthesis was close to 1.0 mg P/mg C (0.4 mol P/mol C). The poly-S^{2-}/S^0 produced from SO_4^{2-} might account for 120–160 mg COD/cycle in the reaction phase R, i.e. 22–30% of the total acetate-COD. So a total of 76–84% of acetate taken up can be accounted for in PHA and poly-S formation. The synthesized PHA and poly-S^{2-}/S^0 in sludge could be utilized as the energy source for P removal in the reaction phase U (Schulz and Schulz, 2005; Wu et al., 2013). On the basis of the results and calculation, it appears that oxidizing 1 mg storage COD can remove 0.05 mg P from the influent in Stage 3 in this study. The remaining 16–24% acetate-COD was probably utilized in the reaction phase R with oxygen entrained into the reactor by mixing or stored as certain form of carbon compounds.

In addition, the role of stored poly-S (the end product of sulfate reduction) was investigated in batch test type-U, in which no acetate was dosed at the start of the anaerobic period but sulfate was (see Fig. 4b). In this test, the increase in PHA and bulk liquid OP and the decrease of glycogen and sulfate were very low during the 6 h anaerobic reaction phase R. This caused the behavior during the anoxic reaction phase U to be different from that of the normal SBR-cycle and batch tests S1 to S4. After adding the nitrate solution, the PHA started to decrease, but only to 3.1 mg C/L. However, there was 15 mg P/L of P-uptake, while the internal sulfur storage (poly-S) was oxidized resulting in 19 mg SO_4^{2-}–S/L released to the bulk-liquid. Also, the glycogen decreased, which was probably utilized by the biomass with nitrate. This result supports the speculation that energy from poly-S oxidation is utilized by the sulfur cycle P removing biomass for P uptake and poly-P synthesis.

4. Conclusion

In this investigation, a denitrifying sulfur cycle-associated enhanced biological phosphorus removal (DS-EBPR) bioprocess for simultaneous removal of organics, nitrogen and phosphorus was developed in this study. The main results are:

1) The 211 day stable SBR operation indicated that the S associated EBPR using nitrate as electron acceptor is possible and beneficial to increase the volumetric loading and shorten the cycle time of the SBR compared with the limited oxygen sulfur (LOS)-EBPR investigated earlier (Wu et al., 2013);
2) Operations of the SBR under different temperatures (20 and 30 °C), without and with salinity (20% seawater) showed this new EBPR consortium was capable of withstanding the temperature and salinity effects, and maintaining a good P removal capacity (highest P/VSS observed 70 mg P/g VSS).
3) 454-pyrosequencing analysis double confirmed that there were no conventional PAOs in the SBR sludge;
4) Relatively low phosphorus content of the biomass (30–70 mg P/g VSS) and sludge yield (0.13–0.17 g VSS/g COD) were found in this DS-EBPR system;
5) The stoichiometric ratios and kinetic rates were determined for this new EBPR process from *in-situ* SBR-cyclic tests and the batch tests under different temperatures (10, 20 and 30 °C). Increasing temperature did not significantly affect the stoichiometric ratios but kinetic rates increases with temperature.
6) A synergistic relationship was observed between sulfur cycle and biological phosphorus removal – an optimal ratio of P-release to SO_4^{2-}-reduction under anaerobic condition was observed at about 1.0 mg P/mg S.

The present DS-EBPR, operated as a granular UASB reactor, can be integrated with an attached-growth bioreactor for nitrification so that a BioP-SANI (as shown in Fig. 5) can be developed to simultaneously achieve effective removal of organics, nitrogen and phosphorus from saline sewage in warm

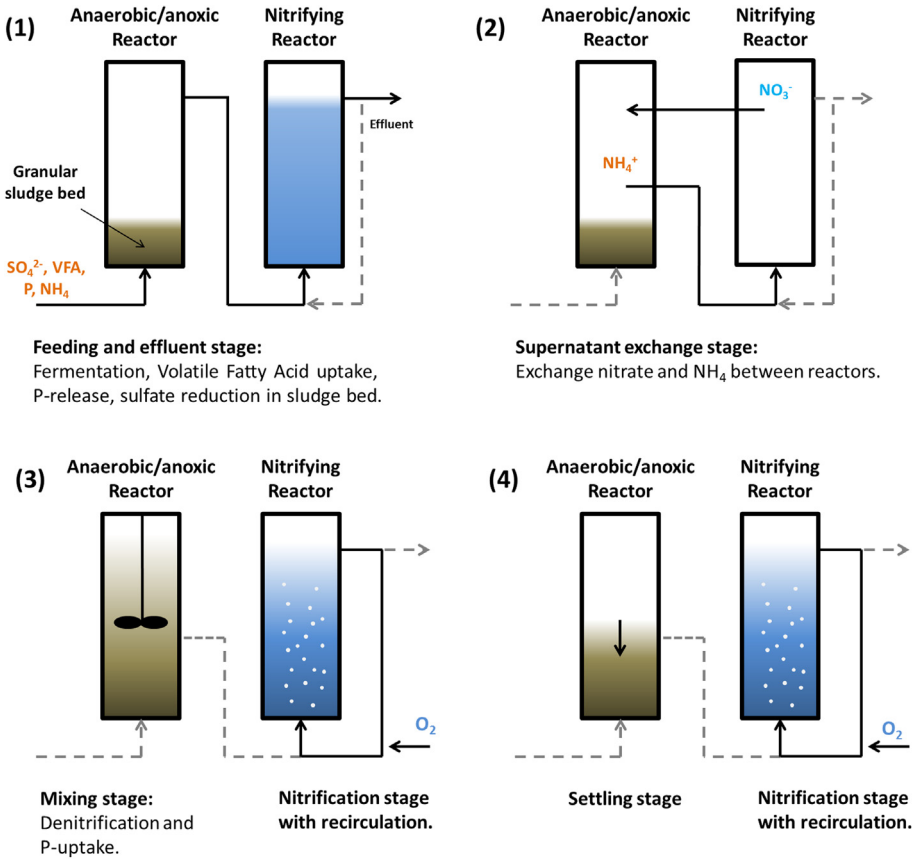

Fig. 5 – The conceptual BioP-SANI process.

climate regions with little biological sludge production and oxygen demand. To realize this BioP-SANI process, future studies will be focused on the microbial ecology, DS-EBPR mechanism and process optimization.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2013.11.029

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