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Gradual adaptation to salt and dissolved oxygen: Strategies to minimize adverse effect of salinity on aerobic granular sludge

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ABSTRACT

Salinity can affect the performance of biological wastewater treatment in terms of nutrient removal. The effect of salt on aerobic granular sludge (AGS) process in terms of granulation and nutrient removal was examined in this study. Experiments were conducted to evaluate the effect of salt (15 g/L NaCl) on granule formation and nutrient removal in AGS system started with flocculent sludge and operated at DO of 2.5 mg/L (phase I). In addition, experiments were conducted to evaluate the effect of gradually increasing the salt concentration (2.5 g/L to 15 g/L NaCl) or increasing the DO level (2.5 mg/L to 8 mg/L) on nutrient removal in AGS system started with granular sludge (phase II) taken from an AGS reactor performing well in terms of N and P removal. Although the addition of salt in phase I did not affect the granulation process, it significantly affected nutrient removal due to inhibition of ammonia oxidizing bacteria (AOB) and phosphate accumulating organisms (PAOs). Increasing the DO to 8 mg/L or adapting granules by gradually increasing the salt concentration minimized the adverse effect of salt on nitrification (phase II). However, these strategies were not successful for mitigating the effect of salt on biological phosphorus removal. No nitrite accumulation occurred in all the reactors suggesting that inhibition of biological phosphorus removal was not due to the accumulation of nitrite as previously reported. Also, glycogen accumulating organisms were shown to be more tolerant to salt than PAO II, which was the dominant PAO clade detected in this study. Future studies comparing the salinity tolerance of different PAO clades are needed to further elucidate the effect of salt on PAOs.

1. Introduction

Saline wastewaters are generated from a variety of industrial processes like seafood processing, vegetable pickling, leather tanning and petroleum refining (Lefebvre and Moletta, 2006). In some coastal cities, due to shortage of fresh water, seawater is used for toilet flushing, which also contributes to high salt concentration in municipal wastewater treatment plants (Wu et al., 2016). High salt can exert an adverse effect on biological wastewater treatment systems, since freshwater-based microorganisms are unable to survive at high osmotic pressures and their activities are inhibited by salt (Madigan et al., 2002). Under high salinity, a decrease of chemical oxygen demand (COD) and nutrient removal were often observed (Moon et al., 2003).

Aerobic granular sludge (AGS) process is a promising biotechnology for wastewater treatment (de Kreuk and van Loosdrecht, 2006). Compared with conventional activated sludge (CAS) process, AGS has a variety of merits including excellent settling properties, small footprint, ability to withstand shock and toxic loadings, and capability of simultaneous COD, nitrogen and phosphorus removal. (Liu and Tay, 2004; de Kreuk et al., 2005; Gao et al., 2011; Maszenan et al., 2011). Currently, there is an increasing interest in the use of AGS for saline wastewater treatment. A wide range of salt concentrations (2–80 g/L NaCl) has been tested in AGS (Table S1). In general, high salinity tends to stimulate extracellular polymeric substances (EPS) secretion (Wan et al., 2014; Wang et al., 2015; Corsino et al., 2016). Also, in most of the cases where reactors were seeded with flocculent sludge, granules were irregularly shaped and not smooth compared to reactors seeded with mature granules (Figueroa et al., 2008; Taheri et al., 2012; van den Akker et al., 2015). Organic carbon and ammonium removal efficiencies were kept high, but nitrite accumulation was observed in many

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studies (Figueroa et al., 2008; Bassin et al., 2011; Pronk et al., 2014; Wan et al., 2014; van den Akker et al., 2015; Wang et al., 2015; Corsino et al., 2016). The effect of salt on phosphorus removal was less studied. Bassin et al. (2011) showed that PAOs gradually disappeared from the sludge at high salt concentrations (22 and 33 NaCl g/L). Pronk et al. (2014) reported that in the absence of nitrite, bio-P removal was not much affected at salt concentrations up to 22 g/L NaCl, but was significantly inhibited at salt concentrations of 33 g/L NaCl.

All the aforementioned studies were conducted at high dissolved oxygen (DO) conditions (almost saturation level). However, exploring the effect of salt on AGS at moderate DO level is more realistic from a practical point of view. In addition, studies on the effect of salt on nutrient removal in AGS were often conducted in systems seeded with mature granules (Bassin et al., 2011; Pronk et al., 2014; Wan et al., 2014; Wang et al., 2015; Corsino et al., 2016). Therefore, it is tempting to see if comparable effect of salt on AGS in terms of aerobic granulation and nutrient removal can be achieved at moderate DO level and in systems seeded with flocculent sludge.

DO is an important operational parameter in AGS process. The oxygen concentration in the bulk liquid controls the oxygen penetration depth and the aerobic/anaerobic zone ratio, which are associated with the capacity for nitrification and denitrification (de Kreuk et al., 2005; Mosquera-Corral et al., 2005). DO also affects the granule stability (Mosquera-Corral et al., 2005) and the competition between PAOs and glycogen accumulating organisms (GAOs) (Carvalheira et al., 2014). Therefore, it is possible to minimize the adverse effect of salt on AGS systems by regulating the DO level.

Another important operational strategy that can be applied to minimize the effect of salt on nutrient removal in AGS is by allowing microorganisms to adapt to salt by gradually increasing the salt concentration. This strategy was successfully applied in a flocculent sludge system to minimize the adverse effect of salt on nitrification (Bassin et al., 2012).

Therefore, the objectives of this study were i) phase I: examine the effect of salt on aerobic granulation and nutrient removal in AGS operated at moderate DO concentration (2.5 mg/L) and seeded with flocculent sludge; ii) phase II: compare the effect of salt on nutrient removal in AGS seeded with mature granules and operated at moderate (2.5 mg/L) and high DO (8 mg/L) concentration; and iii) phase II: examine the effect of salt adaptation as a strategy to minimize the effect of salt on nutrient removal in AGS seeded with mature granules and operated at moderate DO concentration (2.5 mg/L).

2. Materials and methods

2.1. Experimental setup and operating conditions

Three identical sequencing batch reactors (SBRs) each with a working volume of 2.75 L, internal diameter of 5.6 cm and a total height of 150 cm were used in the study. The experiment was divided into two phases (Table 1). In phase I, SBR1a and SBR2 were inoculated with flocculent activated sludge collected from a local wastewater treatment plant (Al Ruwais district, Jeddah, KSA). Synthetic wastewater containing 0 and 15 g/L NaCl was fed to SBR1a and SBR2, respectively. In phase II, half of the biomass (mature granules) in SBR1a (0 g/L NaCl) was transferred into SBR3, and NaCl concentration was gradually increased from 2.5 g/L to 15 g/L with an increment of 2.5 g/L NaCl. The remaining half of the biomass in SBR1a (SBR1a was named SBR1b in phase II) was fed with a synthetic wastewater containing 15 g/L NaCl. The synthetic wastewater was prepared as described by de Kreuk et al. (2005) with a final composition of NaAc 6.3 mM, $MgSO_4 \cdot 7H_2O$ 0.36 mM, KCl 0.47 mM, NH_4Cl 3.54 mM, K_2HPO_4 0.42 mM, KH_2PO_4 0.21 mM and 1 mL/L trace element solution according to Vishniac and Santer (1957).

Table 1
Reactors and their operational phases according to salt concentration.

Phase I			Phase II		
Reactor	Day	NaCl (g/L)	Reactor	Day	NaCl (g/L)
SBR1a	1–330	0	SBR1b	331–493	15
SBR2	1–312	15	SBR3	331–493	2.5–15

All SBRs were operated in successive cycles of 3 h comprising four phases: 60 min anaerobic feeding from the bottom of the reactors in a plug-flow regimen through the settled bed, 112 min aeration, 3 min settling, and 5 min effluent withdrawal. During the first 42 days, the settling time was gradually reduced from 15 to 3 min; the aeration duration was correspondingly increased from 100 min to 112 min. The air flow was maintained at 4 L/min, pH at 7.0 ± 0.2, temperature at 22±2 °C, hydraulic retention time (HRT) at 5.6 h. DO concentration was kept at 2.5 ± 0.2 mg/L unless specified. The DO concentration was regulated by adding different proportions of compressed air and nitrogen gas, which were controlled by two mass flow controllers (Bronkhorst High-Tech, The Netherlands). The gas flow-rate was maintained at 4 L/min during the whole experiment. The exchange ratio of all SBRs was 0.545, corresponding to a HRT of 5.5 h. Sludge retention time (SRT) was not controlled in the first 135 days, but then was maintained at approximately 30 days by periodically removing sludge from the reactors.

2.2. Batch experiments

Batch experiments were conducted to evaluate the short-term effect of salt on nitrification/denitrification and P removal potential of mature granules at different salt concentrations (5, 10, 15, 20, 30 g/L NaCl). Mature granules taken from SBR1a at the end of phase I were used for this set of experiments. Granules collected from SBR1a were placed in a sieve and washed with tap water. Equal volume (50 mL) of washed granules was introduced into different 250 mL flasks filled with the corresponding medium solution (Table 2) and 0.1 M Tris-HCl buffer (pH 7.0). A concentration of 20 mg/L NO_2^--N was selected to conduct the batch experiments on the effect of salt on nitrite oxidation. This concentration is significantly lower than the lowest reported concentration (76 mg/L NO_2^--N) leading to 50% inhibition on the activity of NOB (Zhou et al., 2011). To determine nitrification rate, granules were collected from SBR1a at the end of a cycle (180 min) and incubated for 2 h in the flasks under aerobic condition by sparging air; to determine P uptake and denitrification rate, granules were collected immediately after anaerobic feeding (60 min) and incubated for 2 h under anaerobic condition by sparging nitrogen gas (Table 2). DO was almost at saturation level during air bubbling. Samples for chemical analysis were collected every 10–20 min. All calculations are the same as in Bassin et al. (2011).

2.3. Physical and chemical analysis

All liquid samples were filtered with 0.45 μm PVDF (polyvinylidine fluoride) filters prior to analysis. Ammonium nitrogen (NH_4^+-N), nitrite nitrogen (NO_2^--N), nitrate nitrogen (NO_3^--N) were measured by a flow injection analyzer (AutoAnalyzer 3, Germany). The TN was calculated by adding NH_4^+-N, NO_2^--N and NO_3^--N. Orthophosphate (PO_4^{3-}-P) and COD were quantified by HACH LANGE cuvette kits (Hach company, USA). The total phosphate in the

Table 2
Method of granule incubation and the composition of cultivation medium used in the batch experiments.

Conversion	Granule collection time	Incubation	Cultivation medium and redox condition
Ammonium oxidation	180 min	Aerobic[a]	NH_4Cl (50 mg/L NH_4^+-N); aerobic
Nitrite oxidation	180 min	Aerobic[a]	$NaNO_2$ (20 mg/L NO_2^--N); aerobic
Aerobic P uptake	60 min	Anaerobic with acetate as the carbon source[b]	K_2HPO_4 (60 mg/L PO_4^{3-}-P); aerobic
Nitrate reduction/Anoxic P uptake	60 min	Anaerobic with acetate as the carbon source[b]	K_2HPO_4 (60 mg/L PO_4^{3-}-P) & $NaNO_3$ (30 mg/L NO_3^--N); anoxic

[a] The aerobic incubation was to oxidize the ammonium and nitrite residue in the granules.
[b] The anaerobic incubation was to release the residual phosphate in the cells and to store poly-β-hydroxyalkanoates (PHA) and glycogen ins cells.

granules was measured according to the method described by Uhlmann et al. (1990). Acetate concentration was analyzed by a high-performance liquid chromatograph (HPLC) (Thermo Scientific, Accela, USA) equipped with a photo-diode array (210 nm) and an ultraviolet detector. Sludge volume index (SVI), mixed liquor volatile suspended solids (MLVSS), ash content and effluent suspended solid were determined according to a modified Standard Methods for the Examination of Water and Wastewater specifically for AGS (APHA, 2005; Pronk et al., 2014). The morphology and size distribution of granules were determined using optical microscopy (Morphologi G3, Malvern Instrument, UK). Granule settling velocity was measured according to Winkler et al. (2012). Briefly, a spoon of wet granules (approximately 20 granules) was released at once into a column filled with tap water and the time of the first and last granule passing a mark (herein 1.0 m distance) was recorded. The settling velocity was calculated from the average time recorded in five repetitions. Alginate-like exopolysaccharides (ALE) were extracted according to the methods described by Lin et al. (2010). Dried biomass (0.5 g) was homogenized and extracted in 80 ml 0.2 M Na_2CO_3 at 80° C for 1 h. The supernatant was collected after centrifugation (15,000 rpm, 20 min) and the pH was adjusted to 2 by adding 0.1 M HCl. The supernatant was centrifuged again (15,000 rpm, 30 min) and the pellet was washed with deionized water until the pH is 7. The pellet was then dissolved in 0.1 M NaOH. The ALE in the supernatant was precipitated by the addition of absolute ethanol to a final concentration of 80% (vol/vol). The precipitate was collected by centrifugation at 15,000 rpm for 30 min and then washed in absolute ethanol and lyophilized.

2.4. Microbial analysis

2.4.1. qPCR

Total genomic DNA was extracted from the samples using the Power soil DNA isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. The quality (A260/A280) and quantity (A260) of extracted genomic DNA was determined with a Nanodrop® 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The proportions of key functional groups, including AOB, NOB and PAOs, in the whole microbial community were determined by qPCR. Amplification was performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio Rad Laboratories Inc., Hercules, CA). All amplifications were performed in triplicate with a reaction volume of 25 μL containing 12.5 μL of iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 200 nmol/L of each primer and 5 ng of the template DNA. Primers, thermal cycling conditions and DNA used as the standard for qPCR calibration are listed in Table 3. For standard clone preparation, the PCR amplicons were first cloned into a TOPO cloning vector (pCR 2.1-Topo vector, Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Plasmids from transformed cells were extracted by the PureYield™ Plasmid Miniprep System (Promega, Madison, WI). Copy numbers per microliter were calculated from the concentration of extracted plasmid DNA using the known sequences of the vector and inserts. The accuracy of insert DNA was verified by amplification with gene-specific primers as described in Table 3. Standard template DNA was diluted in series and the C_t values for each dilution were plotted against the concentration of each dilution to construct the standard curve. All qPCR assays were performed in triplicate. No-template controls were included in all qPCR runs.

2.4.2. FISH

FISH was performed in this study to obtain the following information: 1) the spatial distribution of nitrifiers, PAOs and GAOs in a single granule; 2) the ratio of PAOs and GAOs in the whole community; and 3) the sub-lineages of PAOs. Fresh granule samples were fixed overnight with 4% (wt/vol) paraformaldehyde and then washed three times in 1 × PBS and re-suspended in ethanol-PBS solution (1:1) for storage at −20° C. For examining the spatial distribution of the different microbial groups, fixed granules were embedded in a tissue freezing medium (Leica Microsystems, Germany), hardened by freezing (−20° C) and cut in the frozen state with a microtome-cryostat (Leica CM1900-Cryostat, Germany) into 25 μm thin slices. The granule sections were collected on SuperFrost Plus microscope slides (Menzel-Glaser, Braunschweig, Germany). For the purpose of quantification, fixed granules were crushed and spread on gelatin-coated microscope slides. Both types of slides were dehydrated by sequential immersion for 3 min in 50%, 80% and 98% ethanol and air-dried. The hybridization and washing steps were according to the oligonucleotide probes used (Table 4). Detailed protocol can be found in Nielsen et al. (2009). Prepared slides were observed with a confocal laser scanning microscope (Leica DMI4000 B, Germany). To determine the relative abundance of PAOs and GAOs, at least 10 images from each sample were taken for the quantification analysis using the Measure/Count tool in Image Plus Pro 6.0 (Media Cybernetics,USA). In brief, the area of pixels contributed by PAO_{mix} or GAO_{mix} probes above a manually determined threshold was divided by the area of pixels contributed by the EUB_{mix} probes (also applying threshold) for each image.

3. Results

3.1. Granule characteristics and nutrient removal in phase I

3.1.1. Granule characteristics in SBR1a and SBR2

Flocculent activated sludge with SVI of 179 mL/g was used to inoculate both SBR1a and SBR2 (Table 1). During the first week of operation, SVI slightly increased to 191 mL/g in SBR1a; while it dropped to 135 mL/g in SBR2 on day 7. After that, the SVI in both reactors dropped quickly with the formation of granules. Visible granules were observed in SBR1a (0.73 mm) and SBR2 (0.94 mm) after 19 days of operation (Fig. S1). Mature granules characterized by constant size distribution were formed after approximately 70 days of operation in SBR1a and SBR2. The relatively long granulation period in the current study was probably due to 1) the long settling time (7 min) used during the first month of operation (Fig. S2); and the slightly bulking flocculent sludge (SVI of 179 mL/g) used to inoculate the reactors. The average equivalent diameter

Table 3
Primers, qPCR conditions and standards used in this study.

Primer	qPCR conditions	Standard	Reference
CTO 189f A/B & CTO 189f C CTO 654r	94°/2′ [94°/0.30′ 61°/1′ 72°/0.45′] × 30 52°/10′	*Nitrosomonas europaea*	(Kowalchuk et al., 1997)
Nspra 675f 746r	95°/10′ [95°/0.20′ 58°/1′ 72°/0.40′] × 40	*Nitrospira defluvii*	(Graham et al., 2007)
Nxrb 706f Nxrb 1431r	95°/5′ [95°/0.40′ 56°/0.30′ 72°/0.30′] × 35 72°/10′	*Nitrobacter* sp.[a]	(Bagchi et al., 2016)
Univ 518f PAO 846r	95°/3′ [94°/0.30′ 65°/0.45′ 72°/0.30′] × 45	EBPR sludge[b]	(He et al., 2007)
APAO 184f A445r	95°/2′ [95°/0.10′ 60°/0.15′ 72°/0.20′] × 50	*Tetrasphaera elongata*	(Okunuki et al., 2007)
Bac 338f Univ 518r	95°/5′ [94°/0.20′ 55°/0.20′ 72°/0.30′] × 40	EBPR sludge[b]	(Park and Crowley, 2005)

[a] *Nitrobacter* sp. enrichment culture provided by Dr. Eva Spieck at the University of Hamburg.
[b] Sludge was taken from SBR1a on Day 92.

Table 4
Oligonucleotide probes and their target microbial group.

Probe	Sequence (5′-3′)	Target group	Mix	Reference
PAO 462	CCGTCATCTACWCAGGGTATTAAC	Most *Accumulibacter*	PAOmix	(Crocetti et al., 2000)
PAO 651	CCCTCTGCCAAACTCCAG	Most *Accumulibacter*	PAOmix	(Crocetti et al., 2000)
PAO 846	GTTAGCTACGGCACTAAAAGG	Most *Accumulibacter*	PAOmix	(Crocetti et al., 2000)
Acc-I-444	CCCAAGCAATTTCTTCCCC	Clade IA and other Type I clades		(Flowers et al., 2009)
Acc-II-444	CCCGTGCAATTTCTTCCCC	Clade IIA, IIC and IID		(Flowers et al., 2009)
GAO Q431	TCCCCGCCTAAAGGGCCT	Some *Competibacter*	GAOmix	(Crocetti et al., 2002)
GAO Q989	TTCCCCGGATGTCAAGGC	Some *Competibacter*	GAOmix	(Crocetti et al., 2002)
Nso 1225	CGCCATTGTATTACGTGTGA	Betaproteobacterial AOB		(Mobarry et al., 1996)
Nso 190	CGATCCCCTGCTTTTCTCC	Betaproteobacterial AOB		(Mobarry et al., 1996)
Ntspa662	GGAATTCCGCGCTCCTCT	Genus *Nitrospira*		(Daims et al., 2001)
NIT3	CCTGTGCTCCATGCTCCG	Genus *Nitrobacter*		(Wagner et al., 1996)
EUB 338 I	GCTGCCTCCCGTAGGAGT	Most bacteria	EUBmix	(Amann et al., 1990)
EUB 338 II	GCAGCCACCCGTAGGTGT	*Planctomycetes*	EUBmix	(Daims et al., 1999)
EUB 338 III	GCTGCCACCCGTAGGTGT	*Verrucomicrobiales*	EUBmix	(Daims et al., 1999)

of the mature granules in SBR1a and SBR2 was 2.06 ± 0.24 mm and 2.44 ± 0.20 mm, respectively (Table 5). Two types of granules (white and yellow) that are circular and smooth were observed in SBR1a (Fig. 1a). In contrast, the granules in SBR2 were yellowish-brown and irregularly shaped (Fig. 1b). The biomass concentration in SBR1a was almost 64% higher than that in SBR2, suggesting that microbial growth was inhibited by salt addition in SBR2 (Table 5; Fig. S3). The amount of ALE isolated from the granules in both reactors was comparable (Table 5).

3.1.2. Nutrient removal in SBR1a and SBR2

Nitrification was deteriorated during the granulation period, since a large amount of biomass was discharged with the effluent when short settling time was applied to force granulation, and this might have caused a reduction in the population number of nitrifiers. Complete removal of N and P was achieved in SBR1a after 70 days of operation when granules formed and when the proportions of nitrifiers and PAOs in the whole community significantly increased based on qPCR (Fig. 2a). On day 135, PO_4^{3-}-P was detected in the effluent due to poly-P saturation in PAOs (Gonzalez-Gil and Holliger, 2011). P removal efficiency was recovered by periodically discharging granules to control the SRT around 30 days. On the contrary, N and P removal were not established in SBR2 and these results agreed with the qPCR results where the proportions of nitrifiers and PAOs in the whole community were low or below the detection limit throughout the duration of the experiment (Fig. 2b). On day 186, both reactors underwent an unintentional high DO (saturation level) disturbance for 20 days. During this period, the granule size increased to ~4 mm in diameter in both reactors. Nitrite accumulation occurred in SBR1a, and this in turn affected phosphorus removal efficiency. When the DO was later reduced to pre-disturbance condition (i.e. 2.5 mg/L), large granules were broken in SBR1a and a significant amount of biomass was discharged in the effluent resulting in a decrease of biomass concentration (Fig. S3a). Complete removal of N and P was recovered in SBR1a after four months from the removal of disturbance. While in

Table 5
Physical and chemical properties of the mature granules in the different reactors.[a]

Reactor	Mean size (mm)	Circularity	MLVSS ($g\ l^{-1}$)	ALE ($mg\ g^{-1}$ VSS)	Ash (%)	Settling velocity (m/h)	SVI_8[b] ($ml\ g^{-1}$)
SBR1a	2.06 ± 0.24	0.77 ± 0.02	16.90 ± 1.02	148 ± 6	23.8 ± 3.1	71.9 ± 3.4	18.1 ± 1.5
SBR2	2.44 ± 0.20	0.64 ± 0.07	10.28 ± 0.83	162 ± 14	9.0 ± 1.8	59.1 ± 3.9	15.6 ± 1.1
SBR1b	3.35 ± 0.47	0.75 ± 0.02	13.76 ± 0.31	n.d.[c]	13.8 ± 1.3	78.3 ± 1.1	19.9 ± 1.1
SBR3	2.63 ± 0.34	0.74 ± 0.03	14.50 ± 0.52	n.d.[c]	8.8 ± 0.7	79.1 ± 2.3	16.0 ± 0.5

[a] All data in the table were averaged from the samples collected during the pseudo steady-state of each reactor.
[b] SVI_8 = Sludge volume index measured after 8 min settling.
[c] n.d = not determined.

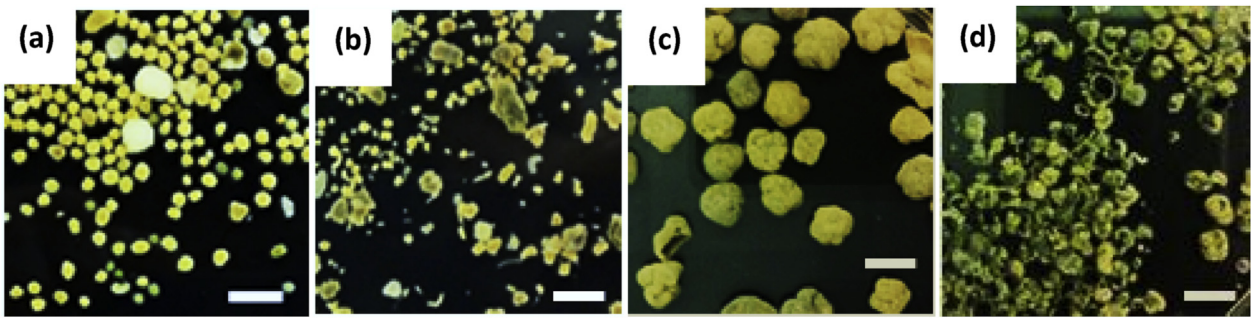

Fig. 1. Granules cultivated from the different reactors: a) SBR1a, b) SBR2, c) SBR1b, and d) SBR3. Bar = 5 mm. Samples from SBR1a and SBR2 were collected on day 99; Samples from SBR1b and SBR2 were collected on day 493.

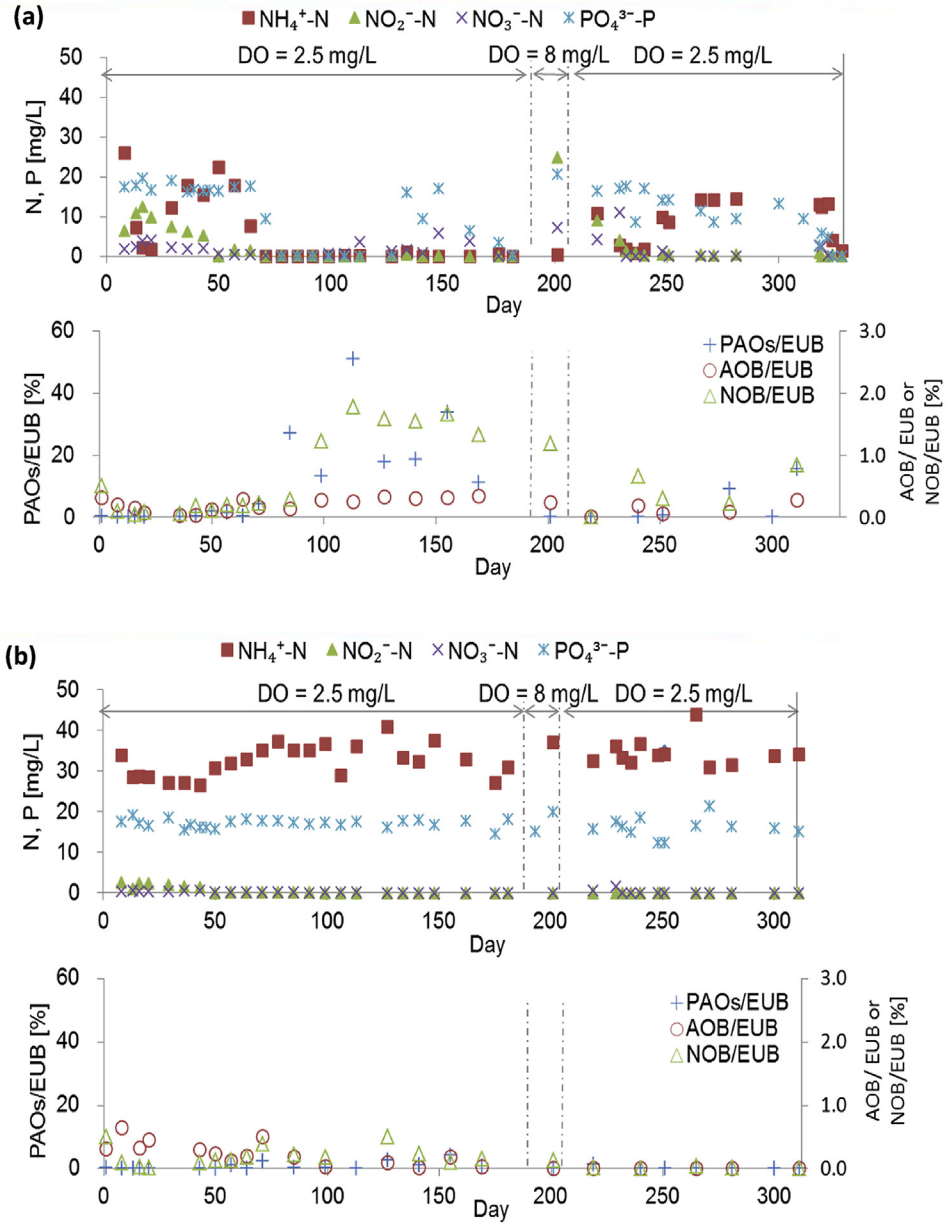

Fig. 2. Effluent concentrations of NH_4^+-N, NO_3^--N, NO_2^--N and PO_4^{3-}-P and the proportions of AOB, NOB and PAOs in the whole community in a) SBR1a and b) SBR2. The influent concentrations of NH_4^+-N and PO_4^{3-}-P were 50 mg/L and 20 mg/L, respectively.

SBR2, the reduced DO led to filamentous bulking and biomass washout. The MLVSS (Fig. S3b) and sludge settleability gradually recovered in SBR2 accompanied by complete anaerobic acetate uptake on day 270. COD removal was unstable during the

granulation period, but reached to 93.5 ± 2.5% in SBR1a and 89.2 ± 2.3% in SBR2 once mature granules were formed. Acetate was not detected in the effluent, and the remaining COD was possibly due to the presence of non-biodegradable compounds such as EDTA (50 mg/L EDTA, which is equivalent to 47 mg/L COD was added in the influent).

3.2. Short-term effect of salt on nutrient removal

Batch experiments (Table 2) were conducted to evaluate the short-term effect of salt on nitrification/denitrification and phosphate removal of the granules cultivated in SBR1a (i.e. salt-free reactor). In aerobic tests, both the specific ammonium oxidation and phosphate uptake rate were significantly reduced by ~72% after addition of 10 g/L of NaCl (Fig. 3). Adding higher salt concentrations (>10 g/L of NaCl) resulted in additional decrease in the specific ammonium oxidation rate, but no further decrease in phosphorus uptake rate was observed. Surprisingly, nitrite oxidation was not significantly affected by salt addition. The maximum specific nitrite oxidation rate (1.44 mg N/(gVSS·h) occurred at 5 g/L NaCl. In anoxic tests, specific nitrate reduction rate slightly increased at salinity of 5 g/L NaCl, but reduced to 40% of its maximum rate at salinity of 15 g/L. Anoxic phosphate uptake was not observed in the batch tests, which suggests that GAOs rather than PAOs catalyzed the denitrification process in the batch experiments. Since no external carbon source was added in the batch tests, the contribution of other denitrifiers can be excluded. This is also true for the reactor where all added COD was sequestered in the anaerobic period.

3.3. Granule characteristics and nutrient removal in phase II

3.3.1. Granule characteristics in SBR1b and SBR3

In phase II, half of the biomass (mature granules) in SBR1a (salt-free reactor) was transferred into SBR3 and the remaining biomass in SBR1a (named SBR1b in phase II) was fed with synthetic wastewater containing 15 g/L NaCl. The NaCl concentration was gradually increased in SBR3 from 2.5 g/L to 15 g/L while maintaining the DO at 2.5 mg/L, whereas the DO was increased from 2.5 mg/L to 8 mg/L in SBR1b. White granules gradually disappeared and instead yellowish-brown granules appeared in both SBR1b and SBR3. But unlike the granules in SBR2, granules in SBR1b and SBR3 were still round and smooth (Fig. 1c and d). The size of granules in SBR1b increased to 3.35 ± 0.47 mm in diameter at high DO concentration (8 mg/L), whereas the granules in SBR3 (DO = 2.5 mg/L) were 2.63 ± 0.34 mm (Table 5). Due to the sudden exposure of granules in SBR1b and SBR3 to salt, a small proportion of the granules floated and subsequently washed out from the reactors leading to a decrease in biomass concentration (Fig. S4). The two reactors retained similar amount of MLVSS after 3 months of operation, which are higher than that in SBR2 but lower than that in SBR1a (Table 5).

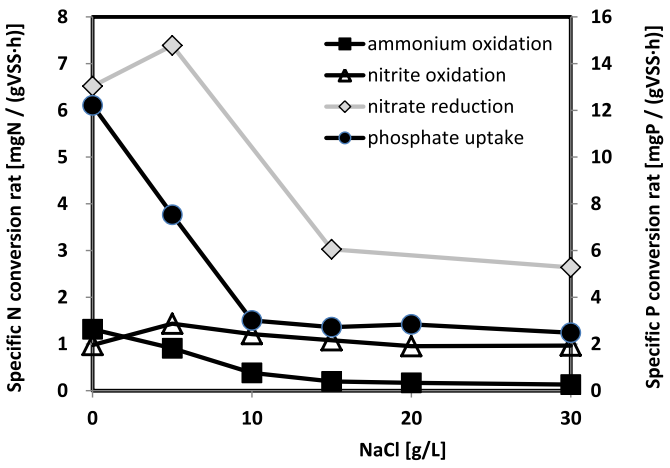

Fig. 3. Short-term effect of salt on specific nitrogen and phosphorus conversion rates (Granules were collected from SBR1a at steady state).

3.3.2. Nutrient removal in SBR1b and SBR3

SBR1b was initially used to test whether mature granules in SBR1a (salt-free reactor) with good nutrient removal capabilities are resistant to salt (15 g/L NaCl) at moderate DO (2.5 mg/L). Nutrient removal capabilities deteriorated within 1 week after the addition of salt (Fig. 4a). PO_4^{3-}-P in the effluent was higher than the influent (20 mg/L) at the beginning of phase II after adding 15 g/L NaCl, which indicated that P uptake was more susceptible to salt than P release. By comparing the P removal profiles during the cycles before and after adding 15 g/L NaCl on day 331, the specific aerobic PO_4^{3-}-P uptake rate decreased by 68%, whereas the

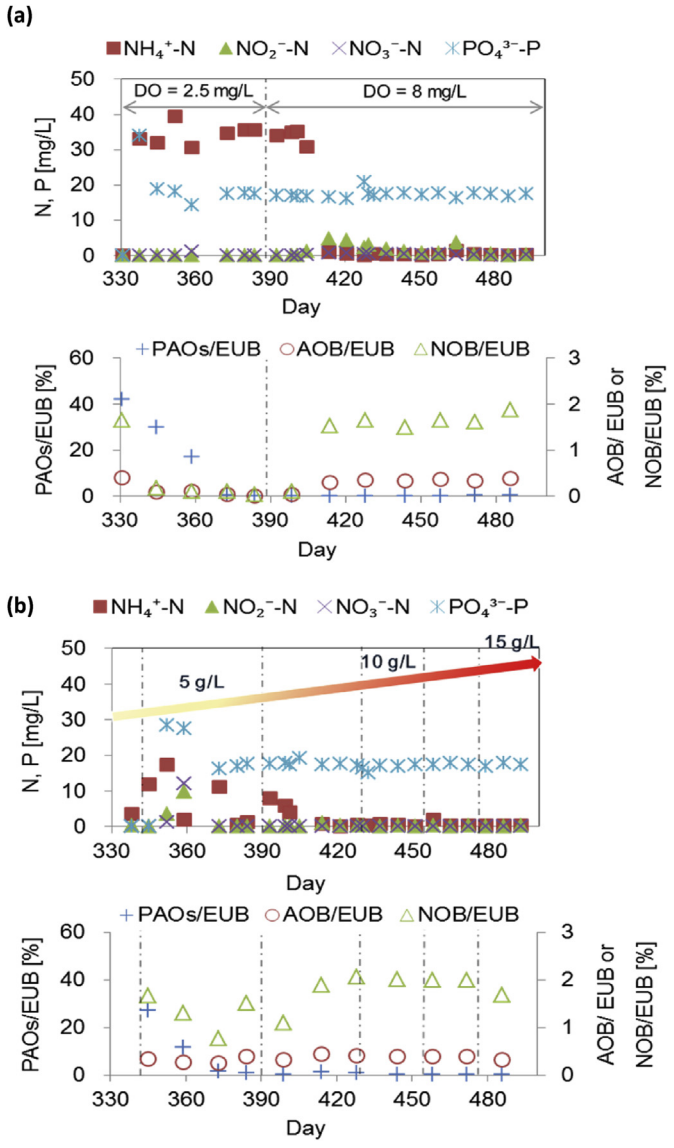

Fig. 4. Effluent concentrations of NH_4^+-N, NO_3^--N, NO_2^--N and PO_4^{3-}-P and the proportions of AOB, NOB and PAOs in the whole community in a) SBR1b and b) SBR3. The influent concentrations of NH_4^+-N and PO_4^{3-}-P were 50 mg/L and 20 mg/L, respectively.

anaerobic PO_4^{3-}-P release rate remained the same (Fig. S5). The proportions of AOB, NOB and PAOs in the whole community decreased sharply after the addition of salt (Fig. 4a). COD removal also decreased to 73.5 ± 7.6% in the first 3 weeks after salt addition, but gradually increased up to 90% for the remainder of the experiment. Pronk et al. (2014) studied the effect of elevated salt concentrations on nutrient removal in AGS operated at high DO (up to 90% saturation). In their study, the specific ammonia oxidation rate was still high even at Cl^- concentration of 20 g/L (33 g/L NaCl), and P removal activity was almost not affected at salinity up to 13 g/L Cl^- (22 g/L NaCl) in the absence of nitrite. It should be noted that nitrite was also absent in the current study. It is possible that the difference between their study and the current study was due to the difference in the DO level. To test this, DO in SBR1b was increased to 8 mg/L on day 388, and after 25 days of operation nitrification efficiency was fully recovered (Fig. 4a). This was also supported by the increase in the proportions of nitrifiers measured by qPCR (Fig. 4a). Denitrification was not significantly affected by the high DO level. Limited nitrite (less than 5 mg/L N) was occasionally detected in the effluent, but in most of the times the effluent NO_x^--N (NO_2^--N + NO_3^--N) was below 1 mg/L. In contrast, phosphorus removal was not recovered at high DO (8 mg/L) levels.

SBR3 was used to test whether gradually adapting mature granules to increasing salt concentrations can alleviate the adverse effect of salt on nutrient removal. Results showed that the effluent NH_4^+-N concentration increased immediately after each increase in salt concentration, then gradually decreased to undetectable levels (Fig. 4b). Nitrite and nitrate were detected in the effluent at a salt concentration of 5 g/L NaCl, with the highest concentrations measured were 9.7 mg/L NO_2^--N and 12.0 mg/L NO_3^--N, respectively. But they were not detected after 50 d of operation in phase II, and the total nitrogen (TN) removal was maintained over 98% even at the highest salinity tested (i.e. 15 g/L NaCl). In contrast, phosphorus removal was deteriorated even at a salt concentration of 5 g/L NaCl, and it did not recover during the whole experimental period (Fig. 4b). COD removal slightly decreased (85.7 ± 4.5%) at a salt concentration of 5 g/L NaCl, but it soon recovered for the remainder of the experiment.

3.4. Microbial community

The change in the proportions of the different functional groups in the whole community measured by qPCR agreed with the nutrient removal performance in all the reactors during the whole experimental period of phase I (Fig. 2) and phase II (Fig. 4). When the reactors were performing well in terms of nitrification and P removal, nitrifiers (AOB and NOB) accounted for 1–3% of the whole microbial community and the relative abundance of PAOs ranged from 11% to 51%. *Nitrospira* and *Accumulibacter* were the dominant NOB and PAOs in this study, respectively. *Nitrobacter* and *Actinobacterial* PAO were not detected by qPCR in all the reactors.

The proportion of PAOs (*Accumulibacter*) in the whole microbial community was compared to GAOs (*Competibacter*) using FISH (Fig. 5). Samples were taken from the different reactors at the end of each phase. Almost similar proportions of PAOs (21.7 ± 7.5%) and GAOs (23.5 ± 7.3%) were detected in granules cultivated in salt-free wastewater (i.e. SBR1a), whereas only GAOs were enriched in granules cultivated in SBRs fed with saline wastewater (i.e. SBR1b, SBR2 and SBR3). The low relative abundance of GAOs in SBR1b compared to SBR2 and SBR3 might result from DO remaining in the feeding phase of SBR1b, which inhibited the anaerobic acetate uptake by GAOs, but gave an advantage to the growth of ordinary heterotrophic organisms (OHOs) (Henze 2008). The relative abundance of PAOs and GAOs in the white granules were 46.4% and 6.3%, respectively, and in the yellow granules were 5.3% and 34.9%, respectively. Similar results were reported by Barr et al. (2010) where white granules had higher percentage of PAOs (97.5%) than yellow granules (12.3%). This is in line with the disappearance of white granules in SBR1b and SBR3, where PAOs were not detected after 50 days of salt exposure.

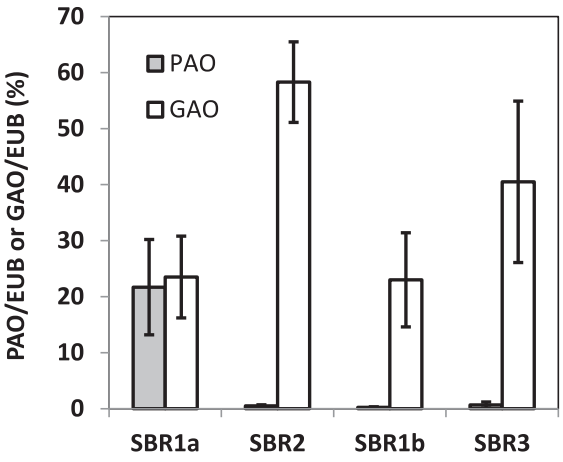

Fig. 5. Proportions of PAOs and GAOs in the whole microbial community assessed by FISH. Samples were taken at the end of each phase from the different reactors. Values represent the average of at least 10 images from each sample as described in the Materials and Methods.

FISH images on a sliced granule taken from SBR1a revealed that nitrifiers and PAOs were mainly located on the outer layer, while GAOs were located at both the outer layer and inner part (Fig. 6a). Accumulibacter Type II (PAO II) was the dominant PAO clade detected at the end of phase I in SBR1a, whereas Accumulibater Type I (PAO I) was scarcely observed in the same sample (Fig. 6b).

4. Discussion

4.1. Effect of salt on granule formation and nutrient removal at moderate DO level (phase I)

Salt inhibited bacterial growth as indicated by a lower biomass concentration in SBR2 than SBR1a. A lower growth yield is usually the result of extra substrate consumption for maintenance processes (i.e. to keep the sodium outside the cell). Addition of salt affected the settling velocity of granules. It is known that salt can increase the water density, which will have an effect on the settling behaviour of the granules (Winkler et al., 2012). Moreover, granules in SBR1a contained much higher proportion of PAOs than SBR2, where GAOs dominated (Fig. 5). Additionally, the phosphate content of granules in SBR1a (0.154 g P/g VSS) was 3 times higher than SBR2 (0.045 g P/g VSS). Phosphate precipitation might occur inside the granules in SBR1a due to the high phosphate content (de Kreuk et al., 2005). This possibility was supported by the high ash content of the granules in SBR1a (Table 5). Winkler et al. (2013) revealed that 1) PAOs had a higher density than GAOs; and 2) a small increase in the volume fraction of precipitates (1–5%) strongly increased the granule density. As a result, the settling velocity of granules in SBR1a (0 g/L NaCl) was higher than SBR2 (15 g/L NaCl) (Table 5). Similar amount of ALE, a valuable material (van Loosdrecht and Brdjanovic, 2014), was extracted from granules cultivated in SBR1a and SBR2, despite differences in microbial community (PAOII in SBR1a, GAO in SBR2). Actually, different communities tend to produce different amount of ALE. For example, Pronk et al. (2015) observed different GAO species produced different amount of ALE. Further studies are needed to

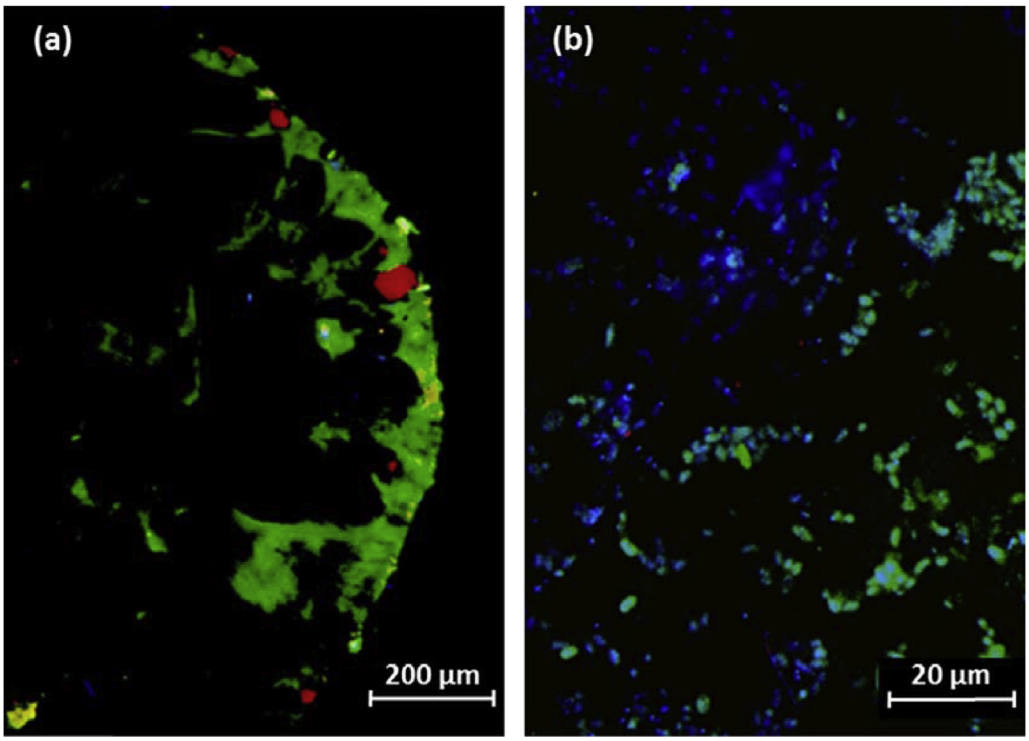

Fig. 6. a) FISH image of a sliced granule taken from SBR1a: PAO (red), GAO (green), AOB and NOB (blue); b) FISH image of crushed granules taken from SBR1a: PAO I (red), PAO II (green), EUB (blue). Samples were collected on day 327. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

explore the relationship of microbial communities and ALE production.

The overall granulation speed in the salt-free (SBR1a) and saline (SBR2) wastewater was similar. Initial granules were formed within 3 weeks in both reactors, and it took 70 days for mature granules to form. Figueroa et al. (2008) tested the feasibility of AGS technology in the treatment of fish-canning effluent. Using flocculent activated sludge as inoculum, it took 75 days for mature granules to form at salinity of 11–15 g/L NaCl. Li and Wang (2008) reported that granules grew faster and larger at high (50 g/L NaCl) than low salinity (10 g/L NaCl). This disagreement in the above results could be attributed to the difference in the operational mode, salt concentration and substrates composition.

The addition of salt affected the morphology of the granules in the reactors seeded with flocculent sludge (Fig. 1). For example, the granules in SBR2 were fluffy and irregularly shaped compared to round and smooth granules in the salt-free reactor (i.e. SBR1a). This result agrees with the results reported by Taheri et al. (2012) and Figueroa et al. (2008) at salt concentrations of 5–10 g/L NaCl and11-15 g/L NaCl, respectively. However, Li and Wang (2008) observed that the surface of granules were more regular and smoother at high (50 g/L NaCl) than low (10 g/L NaCl) salinity.

Starting with flocculent sludge followed by reducing the settling time to cultivate granules resulted in biomass washout and decreased SRT in both SBRs. However, biomass washout was more severe in SBR2. Slow growing bacteria (i.e. nitrifiers and PAOs) were unable to proliferate in the two SBRs. When mature granules were formed and the SRT was maintained at or higher than 30 d, nitrifiers and PAOs increased in abundance and excellent nutrient removal efficiency was achieved in SBR1a (salt-free reactor) (Fig. 2a). In contrast, no significant ammonium removal was observed in SBR2 (15 g/L NaCl) during the whole experimental period and AOB were below the detection limit of qPCR after 100 days of operation (Fig. 2b). Figueroa et al. (2008) reported complete failure of nitrification during the granulation period followed by a successful ammonium removal when mature granules were formed. In their study, they have used fish canning effluent as the feed, and operated the reactors at high DO level (6.5–8.0 mg/L). The moderate DO (2.5 mg/L) used in the current study exacerbated by severe biomass washout might have affected the recovery of nitrification in SBR2.

P removal was also not established in SBR2. The relative abundance of PAOs was very low (varied from 0.1% to 4.3%) compared to GAOs (58.3% ± 7.2%) (Fig. 5) which underlines the failure of P removal in SBR2. Bassin et al. (2011) reported that high salinity levels (33 g/L NaCl) favored GAOs over PAOs in their long-term experiment. Welles et al. (2014) conducted a short-term experiment and suggested that GAOs gain advantage over PAOs at salinity higher than 6 g/L NaCl.

4.2. Effect of salt adaptation and DO on nitrogen removal in mature granules (phase II)

In SBR1b and SBR3, seeded with mature granules from SBR1a (i.e. salt-free reactor), the morphology of granules was not obviously changed after salt addition. These results are similar to the observation by Pronk et al. (2014), who operated their reactor at salinity of 11–33 g/L NaCl.

Complete nitrogen removal was achieved in SBR3 at DO of 2.5 mg/L where salinity was gradually increased (Fig. 4b). Similar results were reported by Bassin et al. (2011), where salt exerted a negative effect on ammonium oxidation in short-term batch experiment. But in their long-term experiment (SRT was also maintained at 30 days), and with the aid of long period of acclimation at 11 g/L NaCl, the ammonium removal rates were not affected by the increase in salt concentrations up to 33 g/L NaCl. As mentioned earlier, gradually adapting the microbial community to increasing salt concentrations successfully mitigated the adverse

effect of salt on nitrification in a flocculent sludge system (Bassin et al., 2012). Here, we demonstrated that this strategy is also successful in mitigating the adverse effect of salt on nitrification in granular sludge system operated at moderate DO level.

No significant nitrogen removal was observed in SBR1b when operated at DO of 2.5 mg/L and at a salt concentration of 15 g/L NaCl (Fig. 4a). However, nitrification was recovered when the DO was increased to 8.0 mg/L at the same salinity level. This may be attributed to the following reasons: 1) High DO could increase the specific ammonium oxidation rate, which indicates a faster growth rate of AOB. In the current study, the specific ammonium oxidation rate was increased by 38% when the DO was increased from 2.5 mg/L to saturation level (by comparing the rate in SBR1a where DO was 2.5 mg/L with the rate in the batch test where DO was at saturation level); 2) Nitrifiers have a lower SRT than other biomass in aerobic granules because they colonize the outer layer (aerobic) of granules, which is more rapidly eroded and subsequently washed out from the reactor compared to the interior layer (anoxic/anaerobic) (Winkler et al., 2012). At high DO level, the oxygen penetration depth is larger than at low DO level. Thus, nitrifiers could inhabit a deeper layer of granules, which reduced their loss due to surface erosion, and this increased their effective SRT; and 3) In SBR1b, the granule size increased from 2.33 mm to 3.35 mm (data not shown) when the DO was increased from 2.5 mg/L to 8.0 mg/L. Large granules have a smaller surface to volume ratio and thus they suffer less from surface erosion than smaller granules. The effluent suspended solid of SBR1b decreased from 44 ± 4.6 mg MLSS/L to 15 ± 1.4 mg MLSS/L (data not shown) when DO was increased from 2.5 mg/L to 8 mg/L. This further reduced the loss of nitrifiers. In summary, increasing the DO to 8 mg/L promoted growth of nitrifiers and reduced their loss from the reactor which alleviated the negative effect of salt on nitrification. However, since the saturated DO level and oxygen transfer rate decrease with the increase in salinity (Bassin et al., 2011), higher aeration rates are required to maintain the same DO concentration at saline versus salt-free conditions. Collectively, both salt adaptation at moderate DO level and operation at high DO were successful in recovering nitrification.

Based on the batch experiments, which were designed to evaluate the short-term effect of salt on mature granules at saturation DO level, the biomass specific ammonium oxidation rate dropped with the increase in salinity (Fig. 3). Unlike AOB, NOB were more tolerant to salt in the current study. The specific nitrite oxidation rate was not affected by the addition of salt up to 30 g/L NaCl. Using pure (*Nitrobacter Agilis*) and mixed culture of NOB, Hunik et al. (1993) and Moussa et al. (2006) reported also that NOB were less affected by salt stress than AOB. In contrast, several studies reported that NOB are more sensitive to salt (Vredenbregt et al., 1997; Figueroa et al., 2008; Cui et al., 2009; Bassin et al., 2011; Pronk et al., 2014). It is not clear why NOB were less sensitive to salt in the current study. Koops and Pommerening-Röser (2001) reported that both *Nitrobacter* and *Nitrospira* contain halophilic or halotolerant sublineages. It is possible that the NOB enriched in the current study were more tolerant to salt than the NOB enriched in the above studies.

Compared with nitrification, denitrifying capability was robust to the addition of salt in both short- or long-term experiments. Based on the batch experiments, denitrification was mainly performed by GAOs in this study and the abundance of GAOs was high in all the reactors based on FISH analysis (Fig. 5). Additionally, high DO did not reduce the denitrifying efficiency in the current study due to the formation of large granules where a suitable ratio of aerobic/anoxic zone was maintained. Di Bella and Torregrossa (2013) also found that simultaneous nitrification and denitrification can be achieved at high DO (7–8 mg/L) in AGS systems.

4.3. Effect of salt adaptation and DO on phosphorus removal in mature granules (phase II)

Like AOB, the activity of PAOs in terms of phosphate uptake rate was substantially inhibited at the salinity level equal to or higher than 10 g/L NaCl in the batch tests (Fig. 3). Also, biological phosphorus removal was not established in SBR1b where mature granules with good phosphorous removal performance were exposed for the first time to 15 g/L NaCl, regardless of the DO concentration (i.e. 2.5 or 8 mg/L) (Fig. 4a). Similarly, phosphorus removal was not established in SBR3 (DO = 2.5 mg/L), seeded with mature granules with good phosphorous removal performance, where salt concentration was gradually increased from 2.5 to 15 g/L NaCl (Fig. 4b). These results suggest that long-term exposure to high salinity (SBR1b) and salt adaptation (SBR3) did not help in the acclimatization of PAOs to salinity.

Pronk et al. (2014) reported that the deterioration of phosphorus removal in granules was mainly due to nitrite accumulation caused by the inhibition of salt on NOB activities rather than the salt itself when the concentration of Cl^- was below 13 g/L (22 g/L NaCl). The inhibition of nitrite on bio-P removal was also demonstrated by many other researchers (Intrasungkha et al., 1999; Wu et al., 2008; Cui et al., 2009; Pijuan et al., 2010), and in many cases, it was due to incomplete nitrification caused by salt. However, these previous studies fail to explain the observation in the current study since no nitrite accumulation occurred in all the reactors. This discrepancy might be due to the different PAO clades enriched in the different experiments. Different PAO clades may have different tolerance or response to salinity. In the current study, the granules used in the batch tests, SBR3 and SBR1b were from SBR1a (salt-free reactor), which was enriched by PAO II based on FISH analysis; while in Pronk et al. (2014), all PAOs belonged to PAO I. In a recent study focused on the short-term effect of salt on enriched PAO II culture, Welles et al. (2015) found that at salinity of 1.8 g/L NaCl, the corrected PO_4^{3-} uptake rate decreased by 46%; and above 3.5 g/L NaCl, PO_4^{3-} released. These results might suggest that for salt water systems inoculation with sludge containing the correct PAO clade is something to consider. A systematic study comparing the salinity tolerance of enriched PAO I and PAO II cultures is needed to further elucidate the effect of salt on PAOs. Currently, the main factors affecting the accumulation of PAO I or PAO II is not very well understood. However, it seems that PAO I tend to be enriched at low temperature and low SRT (Tian et al., 2013). Welles et al. (2015) showed that PAO II had a strong competitive advantage over PAO I when Poly-P is stoichiometrically limiting the VFA uptake, suggesting that PAO I can be enriched by feeding with high P/COD ratio medium. Genomic analysis revealed that Clade IA are more susceptible to phages than PAO IIA (Flowers et al., 2013) and different PAO clades had different denitrification capability (Skennerton et al., 2015). It should be noted that the granular sludge (taken from SBR1a) used in SBR1b and SBR3 was enriched with PAO II (21.7%) and GAOs (23.5%) based on FISH analysis. After exposure to salt, the abundance of PAOs in SBR1b and SBR3 significantly decreased to very low levels, whereas GAOs remained abundant (Fig. 5). These results suggest that GAOs were more tolerant to salt than PAO II.

The effect of DO and salt on PAOs and GAOs was recently studied (Carvalheira et al., 2014; Welles et al., 2014). Carvalheira et al. (2014) showed that *Accumulibacter* PAOs had an advantage over *Competibacter* GAOs at low DO levels (2 mg/L), since PAOs have higher affinity for oxygen, and thus maintained their aerobic activity at low DO levels. In contrast, high DO levels (8 mg/L) led to higher growth and activity of GAOs. These results could explain the decreased P-removal when the DO was increased in order to stimulate nitrification under saline conditions. Welles et al. (2014)

conducted short-term batch experiments to compare the acetate uptake rate of PAOs, including PAOII, and GAOs at different salinities. They showed that PAOs had higher acetate uptake rates at salinity ≤0.6%, whereas GAOs had higher uptake rates at salinity >0.6%, under the same operational conditions (pH = 7, T = 20 °C). In the current study, PAOII was outcompeted by GAO at 15 g/L NaCl (1.5% salinity) in the long-term experiments.

4.4. Practical implications and future research

Based on the current study, the nutrient removal capability of AGS decreased when exposed to saline conditions at DO of 2.5 mg/L. In addition, none of the previous studies have successfully achieved complete nitrogen and phosphorus removal at the highest salinity levels tested (Table S1). However, these studies also shed light on how to manipulate the AGS system under high salinity level. Gradually adapting granules to increasing salt concentration at moderate DO level could help in mitigating the adverse effect of salt on nitrification. However, unlike laboratory experiments, it is not practical to dilute the salt concentration in the influent to the full-scale wastewater treatment plant. Alternatively, increasing the DO to promote nitrification is a more practical approach especially after a temporal salinity shock. Denitrification efficiency will not be impacted by increasing the DO due to the formation of large granules as shown in the current study and by Di Bella and Torregrossa (2013). Once nitrification is restored after a shock load, the DO should be gradually reduced to a moderate level since a sharp DO decrease might lead to granule breakup or filamentous bulking. As for phosphorus removal, GAOs seem to be more tolerant to salinity than PAO II. The tolerance of different PAO clades to salinity should be tested in future studies.

5. Conclusions

The effect of salt on granule formation and nutrient removal at moderate DO level (phase I), and the effect of salt adaptation and DO on nutrient removal in mature granules (phase II) were investigated in this study. The following conclusions could be drawn:

- Phase I: addition of 15 g/L NaCl at DO of 2.5 mg/L did not affect the granulation process, but it significantly affected ammonium and phosphorous removal due to inhibition of AOB and PAOs.
- Phase II: increasing the DO to 8 mg/L or adapting mature granules by gradually increasing the salt concentration minimized the adverse effect of salt on nitrification. However, these strategies were not helpful for phosphorus removal.
- Nitrite oxidation and denitrification were relatively robust at all salt concentrations tested in this study.
- GAOs were more tolerant to salt than PAO II.

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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.2017.08.026.

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