Simultaneous nitrification, denitrification and phosphorus removal (SNDPR) in a full-scale water reclamation plant located in warm climate
Qin Yang, Nan Shen, Zarraz M.-P. Lee, Guangjing Xu, Yeshi Cao, Beehong Kwok, Winson Lay, Yu Liu and Yan Zhou

ABSTRACT
The combination of simultaneous nitrification-denitrification (SND) with enhanced biological phosphorus removal (EBPR) provides a more efficient and economically viable option for nutrient removal from municipal wastewater compared to conventional two-step nitrification-denitrification. This study analyzed the nutrients (N and P) profiles in a full-scale municipal wastewater reclamation plant (WRP) located in the tropical region, in which more than 90% of nitrogen was removed. Interestingly, average SND efficiency in aerobic zones was found to be up to 50%, whereas phosphorus profile displayed a clear cyclic release and uptake pattern with a phosphorus removal efficiency of up to 76%. The capability of sludge to perform SND and EBPR was further confirmed through a series of batch experiments. Microbial analysis revealed the presence of Accumulibacter and Tetrasphaera phosphate accumulating organisms in the plant, while few glycogen accumulating organisms (GAO) was observed. This study showed the significant occurrence of combined SND and EBPR, known as simultaneous nitrification, denitrification and phosphorus removal (SNDPR), in the studied WRP under warm climate. The possible causes behind the observed SNDPR were also discussed.

Key words | full-scale water reclamation plant (WRP), nitrite short-cut, SNDPR system, step-feed activated sludge (SFAS) process, warm climate

INTRODUCTION
Conventional biological nitrogen removal is achieved by nitrification and denitrification, which are two distinct metabolic pathways requiring different electron acceptors. The process involves two reaction basins, where nitrification occurs under aerobic condition while denitrification requires anoxic condition. In the nitrite short-cut process, aeration and carbon source cost can be reduced. The cost of nitrogen removal can be further reduced by simultaneous nitrification and denitrification (SND) in a low oxygen environment (Zhu et al. 2007). The scientific rationality of SND could be explained by two points of view: from the process engineering perspective, diffusion limit exists within sludge flocs or biofilms under low dissolved oxygen (DO) condition, creating a DO gradient which allows denitrification to occur in the anoxic zone; from a microbiology perspective, aerobic denitrification can occur but at a lower rate than anoxic denitrification (Frette et al. 1997).

Enhanced biological phosphorus removal (EBPR) is also accomplished by a two-stage process. Phosphate accumulating organisms (PAO) first store simple organic carbon as polyhydroxyalkanoates (PHA) anaerobically using intracellular polyphosphate as the energy source, then oxidize the stored PHA and uptake phosphorus in the subsequent aerobic phase. The required alternating
conditions for nitrogen and phosphorus removal offer the possibility for simultaneous nitrification, denitrification and phosphorus removal (SNDPR) in a single biological system. SNDPR indeed has substantial savings on aeration, carbon dosage, and reactor footprint.

Although the feasibility of SNDPR had been demonstrated in laboratory-scale systems at ambient temperatures of 18–25 °C (Meyer et al. 2005; Peng et al. 2008), SNDPR has not yet been reported in full-scale continuous flow activated sludge (AS) system under warm climate. The main challenges with SNDPR in a full-scale plant lies in (1) lack of precise process control for the simultaneous occurrence of three different biological processes and (2) low chemical oxygen demand/nitrogen (COD/N) ratio of municipal wastewater. The first challenge may induce microbial competition on certain substrates such as oxygen competition among nitrifiers, aerobic ordinary heterotroph organisms and PAO in the aerobic phase. The low COD/N ratio of municipal wastewater results in the difficulty to enrich PAO population. Furthermore, the competition between GAO and PAO can be intensified at high temperature (>20 °C). To the authors’ knowledge, only a few studies reported that phosphorus removal was achievable in high temperature conditions where abundance of GAO was lower than PAO (Ong et al. 2014).

This study aims to investigate SNDPR performance in a full-scale water reclamation plant (WRP) located in a tropical climate and to evaluate the operating conditions for process optimization. To investigate the performance, three rounds of sampling were carried out to obtain the nutrient profiles of each anoxic and aerobic zone. Microbial assessment was also performed for the sludge from each basin. In addition, a series of batch experiments were conducted in the laboratory to further validate the reaction kinetics.

**MATERIALS AND METHODS**

**Sampling**

The full-scale WRP has a treatment capacity of 800,000 m³ municipal wastewater per day. Each of the four identical treatment trains (reactors) contains five basins of equal volume in sequence with hydraulic retention time (HRT) of 1.65, 1.28, 1.05, 0.88 and 0.77 h, respectively. The designed solid retention time based on total volume is 5 days, with mixed liquor suspended solids (MLSS) concentration range from 3,700 mg/L to 2,000 mg/L in basins 1–5. The WRP adopts a step-feed activated sludge process. Mixed liquor passes through five basins of alternating anoxic and aerobic zones with the influent equally distributed into the anoxic zones of each basin (refer to Supplementary Information, Figure S1, available with the online version of this paper). DO level in aerobic zone was controlled in the range of 1.4–1.8 mg O₂/L, while in the range of 0.08–0.12 mg O₂/L in anoxic zone until 2013. Mainstream partial nitrification and anammox was reported previously by Cao et al. (2015). The anoxic and aerobic zones in five basins were marked as 1A–5A and 1O–5O, respectively. Three rounds of sampling were conducted within half a year in 2014.

**Batch experiment**

The aim of batch experiment is to verify SNDPR potential of WRP sludge in controlled DO environment. Batch experiments were conducted within 24 h of sampling. All medium contained 1.25 g/L NaHCO₃ as buffer and inorganic carbon source for nitrifiers, and 1.25 mL/L trace element solution as specified in the study of Suneethi & Joseph (2011). Nitrogen removal batch experiment was conducted for 1.5 h, while EBPR experiment was conducted for 3 h with 1.5 h each for anaerobic and aerobic phase.

Experiment 1 employed aerobic conditions with 30 mg NH₄⁺ N/L as nitrogen source under both high (2–4 mg O₂/L) and moderately low (1–2 mg O₂/L) DO levels, the latter was denoted as ‘low DO’ in the later discussion (Table 1). One set of the low DO experiment (1c) was provided with 100 mg COD/L of sodium acetate to verify aerobic denitrification capability by ordinary denitrifiers. Experiment 2 followed the same procedures of Experiment 1 with 30 mg NO₂⁻ N/L as nitrogen source under both high (2–4 mg O₂/L) and moderately low (1–2 mg O₂/L) DO levels, the latter was denoted as ‘low DO’ in the later discussion (Table 1).

Experiment 1 was denoted as 1A–5O, respectively. Three rounds of sampling were conducted within half a year in 2014.

**Glycogen and PHA determination**

Glycogen was extracted according the method of Zeng et al. (2005). Briefly, 5 mL of 0.6 M HCl was added to freeze-dried biomass then heated at 105°C. After 6 h, the glucose concentration in the supernatant was measured using Agilent 1200
series HPLC system (Agilent Technologies, Inc., Germany). PHA was represented by poly-β-hydroxybutyrate (PHB), poly-β-hydroxyvalerate (PHV) and poly-β-hydroxy-2-methylvalerate (PH2MV) in this study. PHA content was determined following the method of Oehmen et al. (2005). Freeze-dried samples were suspended with 3% H2SO4 acidified methanol and chloroform mixture. After 20 h heating at 100 °C, deionized water was added to remove the impurities and the organic portion was analyzed with Agilent 7890A GC system (Agilent Technologies, Inc., USA).

Chemical analysis

Nitrogenous compounds (NH4+-N, NO2-/NO3--N and PO43--P) were determined by colorimetric method using UV-1800 spectrophotometer (Shimadzu Co., Ltd, Kyoto, Japan). NH4+-N was measured with the Hach Nessler reagent set according to USEPA Nessler method. NO2-/NO3--N, PO43--P, MLSS and mixed liquor volatile suspended solids (MLVSS) concentration were determined according to standard methods.

Table 1 | Batch experiment design for verification of SND and EBPR potential

<table>
<thead>
<tr>
<th>Test conditions</th>
<th>Nitri-</th>
<th>Denitrifi-</th>
<th>EBPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch experiment</td>
<td>cation</td>
<td>cation</td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>Experiment 2</td>
<td>Experiment 3</td>
<td>Experiment 4</td>
</tr>
<tr>
<td>DO level</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Organic carbon</td>
<td>–</td>
<td>–</td>
<td>100 mg/L as COD</td>
</tr>
<tr>
<td>Nutrient</td>
<td>30 mg/L NH4+-N</td>
<td>30 mg/L NO2--N</td>
<td>30 mg/L NO3--N</td>
</tr>
</tbody>
</table>

Table 2 | Primers for qPCR verification of Accumulibacter PAO and denitrifiers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene</th>
<th>Target</th>
<th>Sequence</th>
<th>Annealing temp. (°C)</th>
<th>Amplification efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTO189f</td>
<td>16S rRNA</td>
<td>β-Subdivision AOB</td>
<td>5'-CTAGCYTTGTAGTTTCAAACGC-3'</td>
<td>55</td>
<td>99</td>
</tr>
<tr>
<td>CTO654r</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGPS872</td>
<td>16S rRNA</td>
<td>Nitrobacter</td>
<td>5'-CTAAAACTCAAAGGAATTGA-3'</td>
<td>55</td>
<td>91</td>
</tr>
<tr>
<td>FGPS1269</td>
<td></td>
<td></td>
<td>5'-TTTTTTGAGATTTGCTAG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSR1113f</td>
<td>16S rRNA</td>
<td>Nitrospira</td>
<td>5'-CCTGCTTTCACTTCCAGATTTGTAG-3'</td>
<td>55</td>
<td>98</td>
</tr>
<tr>
<td>NSR1264r</td>
<td></td>
<td></td>
<td>5'-GTTTGCAGCGCTTTGTACCG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nosZF</td>
<td>nosZ</td>
<td>Denitrifiers</td>
<td>5'-CGYTGTTCMTCGACAGCC-3'</td>
<td>55</td>
<td>100</td>
</tr>
<tr>
<td>nosZ1622R</td>
<td></td>
<td></td>
<td>5'-CGSACCTTSTTGCCSTYGCG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>518f</td>
<td>rs</td>
<td>Accumulibacter</td>
<td>5'-CCACGACGCCGCCGTAAT-3'</td>
<td>61</td>
<td>95.7</td>
</tr>
<tr>
<td>PAO846r</td>
<td></td>
<td></td>
<td>5'-GTTAGCTACGGCGACTAAAAAG-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Floc size determination

Mixed liquor floc size distribution was measured by Shimadzu SALD-3101 Laser Diffraction Particle Size Analyzer (Shimadzu Co., Ltd, Kyoto, Japan), which was able to detect particles ranging from 50 nm to 3,000 μm.

qPCR and FISH

Improved Griffiths method was adopted for DNA extraction (Towe et al. 2011). The abundance of target microbial communities was determined by SYBR Green based quantitative polymerase chain reaction (qPCR) using the primers listed in Table 2. The presence of PAO and GAO in the sludge samples was also analyzed by fluorescence in situ hybridization (FISH) according to Amann & Fuchs (2008). The probes used for the hybridization are EUBMIX (equimolar of EUB338, EUB338 II and EUB338 III) targeting all bacteria, PAOmix (equimolar of PAO462, PAO651 and PAO846) targeting Accumulibacter-type PAO, Actino221
and Actino658 targeting Tetrasphaera-type PAO, GAOmixture (equimolar of GAOQ431 and GAOQ989) targeting Competibacter-type GAO, DFIi_mix (equimolar TFO_DF218 and TFO_DF618) targeting Cluster I Defluvicoccus-type GAO and DFIi_mix (equimolar of DEF988 and DF1020) targeting Cluster II Defluvicoccus-type GAO. Images were visualized with Nikon A1R confocal laser scanning microscope and analyzed with NIS Elements v4.10 by thresholding.

RESULTS AND DISCUSSION

SNDPR performance in full-scale WRP

Over a period of 5 months, influent nitrogen and phosphorus concentrations were in the range of 30–35 mg NH₄⁺-N /L and 5–7 mg PO₄³⁻-P/L, respectively, with negligible NO₂⁻ and NO₃⁻. Influent soluble COD (sCOD) was in the range of 80–150 mg COD/L (Table 3). An average temperature of 28–30 °C in the mixed liquor was recorded over the period of study. It was found that more than 70% of sCOD, 91.7% of total nitrogen and 76% of phosphorus were removed in the WRP. These suggest an excellent biological nutrient removal performance in the full-scale WRP. Figure 1(a) further shows concentration profiles of nutrient in basin 1A to 5O and an overall decreasing trend is found. The synthesis and degradation of PHA and glycogen in different anoxic and aerobic zones is displayed in Figure 1(b). As PH2MV was not detected, only PHB and PHV are plotted in Figure 1(b). Cyclic PO₄³⁻ release and PHA synthesis in anoxic zones, PO₄³⁻ uptake and PHA consumption in aerobic zones with an overall PO₄³⁻ decreasing indicated a typical EBPR pattern by PAO. However, the activity of GAO, represented by the change of glycogen content from basins 1–5, was insignificant.

The respective specific substrate utilization rates for nitrification, denitrification and phosphorus removal in each oxic and anoxic zone is summarized in Figure 2. Some ammonium increase was observed in the first and second anoxic zones, which was likely due to the hydrolysis of particulate matters from the influent. It is noteworthy that NH₄⁺ removal rates in all aerobic zones were higher than NOx production rates (Figure 2(b)). After biomass growth on NH₄⁺ consumption is considered, the removal rates of NH₄⁺ are still significantly higher. This indicates that part of NO₂⁻ and/or NO₃⁻ produced by nitrification may be denitrified in the aerobic basins, indicating the occurrence of simultaneous NH₄⁺ oxidation and NOx removal (SND). Equation (1) (Third et al. 2003) defined the SND efficiency at aerobic stage. On average, 43.4% of nitrogen was removed through SND in the aerobic zones during the period of study. This can be partially attributed to low DO of 0.7–1.1 mg/L in the aerobic zone during the sampling period. In fact, a DO concentration of 0.5–1.5 mg/L had been reported to favor SND (Ruscalleda Beylier et al. 2011). Floc size was also reported to be a key parameter that affected SND efficiency. The median diameter of sludge flocs in this WRP was 76 μm (Supplementary Figure S2, available with the online version of this paper), which was in the lower range of that in conventional AS plants (median 70–300 μm) (Zhang et al. 1997). In the study of Pochana & Keller (1999), 52% SND removal efficiency was achieved with similar floc size (median 80 μm) and SND efficiency reduced to only 20% when median floc size was 40 μm. Gómez-Silván et al. (2014) also reported similar transcription level of nosZ gene in aerobic and anoxic conditions, indicating the denitrification capability of AS in aerobic condition. In addition, Zhu et al. (2007) found a linear relationship between DO concentration and the ratio of NOx production rate to NH₄⁺ removal rate, i.e. lower DO led to higher proportion of denitrification to nitrification in aerobic environment.

<table>
<thead>
<tr>
<th>Nutrient concentration (mg/L)</th>
<th>NH₄⁺-N</th>
<th>NO₂⁻-N</th>
<th>NO₃⁻-N</th>
<th>sCOD</th>
<th>PO₄³⁻-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influent</td>
<td>30–35</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>80–150</td>
<td>3–7</td>
</tr>
<tr>
<td>Effluent</td>
<td>0.5–3</td>
<td>0–0.6</td>
<td>0–1</td>
<td>15–35</td>
<td>1–2</td>
</tr>
</tbody>
</table>
Nitrogen removal pathway in the studied WRP was consistent with previous reports of denitrification at low DO.

\[
\text{SND efficiency} = \left( 1 - \frac{\text{NO}_{2,e} - \text{NO}_{2,i}}{\text{NH}_4,i - \text{NH}_4,e} + \frac{\text{NO}_{3,e} - \text{NO}_3,i}{\text{NH}_4,i - \text{NH}_4,e} \right) \times 100\% \tag{1}
\]

where \( \text{NH}_4,i \) is the \( \text{NH}_4^- \)-N concentration at the end of anaerobic zone, mg \( \text{NH}_4^- \)-N/L; \( \text{NH}_4,e \) is the \( \text{NH}_4^- \)-N concentration at the end of aerobic zone, mg \( \text{NH}_4^- \)-N/L; likewise for \( \text{NO}_2 \) and \( \text{NO}_3 \).

The maximum P-release rate of \( 7.3 \pm 1.3 \text{ mg P/g VSS·hr} \) was observed in the first anoxic zone where the carbon sources were likely mainly consumed by PAOs for PHA synthesis. Although no volatile fatty acids (VFAs) were detected (data not shown), about \( 25-30 \text{ mg PO}_4^-\text{P}/\text{L} \) was found in the return sludge stream, which was higher than the P concentrations in all basins. Moreover, the storage of PHA (i.e. mainly PHB and PHV) in the return sludge was significantly higher than the last aerobic zone (5O) (6.52 mg PHA/g VSS vs 3.51 mg PHA/g VSS). Therefore, it is reasonable to consider that fermentation and/or hydrolysis of sludge might occur in the final settling tank (FST), in which soluble carbon sources could be generated and was then consumed by PAO. A recent study also showed that return sludge hydrolysis in a side-stream system or a longer anaerobic period would favor PAO against GAO (Stokholm-Bjerregaard et al. 2015). Further study will be needed to investigate the competition between PAO and GAO with continuous carbon supply at low concentrations under warm climate.

P-uptake rates in aerobic zones showed close correlation with denitrification rates in anoxic zones, where denitrification rates were increased from 1A to 4A (0.05 ± 0.2 to 4.9 ± 1.0 mg N/g VSS-hr) and P-uptake rates decreased from 1O to 5O (4.1 ± 0.6 to 1.5 ± 0.8 mg P/g VSS-hr). The correlation corroborated with the decrease of internal carbon storage (PHA) of PAOs from 1A to 4A (i.e. 10.39 to 3.78 mg PHA/g VSS), which is due to carbon source competition. Generally, the P-uptake rate was faster than P-release rate within the same basin, which resulted in net P-removal. The concurrent nitrification, denitrification and phosphorus removal clearly showed a typical SNDPR in the WRP studied.

**Batch experiment**

**SND potential**

SND potential of mixed liquor taken from the full-scale WRP was verified in three batch experiments (Experiments 1–3). Under high DO condition (Experiment 1a), \( \text{NH}_4^- \) was fully oxidized to NOx whose production rates were higher than \( \text{NH}_4^- \) consumption rates (4.0 mg N/g VSS-hr vs 3.5 mg N/g VSS-hr). The difference may be attributed to \( \text{NH}_4^- \) released from cell lysis since no carbon was provided in these batch experiments. The DO level of 2–4 mg O_2/L may completely penetrate the flocs, so the possibility of aerobic denitrification was low. Nitrification rates measured as \( \text{NH}_4^- \) removal rates were reduced by 16.8 ± 4.9% when low DO condition was applied (Figure 3, Experiment 1b). The NOx accumulation rates at low DO were reduced by 63.7 ± 4.8% compared to high DO condition. It is clear that NOx accumulation rates were lower than \( \text{NH}_4^- \) reduction rates. The difference between \( \text{NH}_4^- \) reduction and NOx accumulation strongly indicated that SND occurred whereby some NOx was consumed aerobically.

The SND efficiency for batch experiment is defined by Equation (2), according to which the SND efficiency for Experiment 1b was estimated at 50.1 ± 14.4%, which was comparable with that (43.4%) determined in the full-scale
WRP. With no addition of external carbon in the feed medium of Experiment 1b, it could be assumed that the carbon source for denitrification was from endogenous metabolism or intracellular storage. In order to evaluate sludge’s full aerobic denitrification potential, external carbon source was added in Experiment 1c. External carbon source addition slightly inhibited nitritation. This may be due to competition from heterotrophs for O₂ and NH₄⁺, and the impaired NH₄⁺ affinity of ammonia oxidizing bacteria (AOB). Denitrification rates on the other hand were enhanced by 36.2%.

SND efficiency = \left( 1 - \frac{NOx \text{ production rate}}{NH4 \text{ reduction rate}} \right) \times 100\% \quad (2)

Aerobic denitrification potential was further verified by Experiment 2 with NO₂⁻ as nitrogen substrate. This experiment aimed to investigate the correlation between nitratation and aerobic denitrification. Under high DO conditions, it was interestingly noted that NO₃⁻ accumulation rates were much higher than NO₂⁻ consumption rates. This confirmed that there was continuous release of ammonium from endogenous metabolism or cell lysis. With low DO, NO₂⁻ reduction rates were 2.0 times higher than with high DO, and 2.7 times higher than NO₃⁻ accumulation rates. Higher NO₂⁻ reduction rates demonstrate that nitratation and denitrification occurred simultaneously. With external carbon source, NO₂⁻ reduction rates were further increased (Figure 3, Experiment 2c).

Experiment 3 was designed to compare the anoxic and aerobic denitrification rates with external carbon sources. From the batch study, average anoxic denitrification rates were 10.9 ± 0.3 mg N/g VSS-hr, which was nearly two times of aerobic NOx reduction rates (5.0 ± 0.6 mg N/g VSS-hr). Although the denitrification rate was lower in aerobic zones, it appeared that denitrifiers were not inhibited significantly by periodical exposure to aerobic conditions.

**SNDPR performance in batch experiment**

Experiment 4 was designed to verify the SNDPR performance of the AS. Clear EBPR phenotype is displayed in Figure 4. The batch experiment showed an average P-release rate of 5.7 mg P/g VSS-hr and aerobic P-uptake rate of 14.3 mg P/g VSS-hr. The P-uptake rate in batch experiment was higher while P-release rate was slower than the plant data obtained in basin 1A (Table 4).

It should be noted that under low DO condition, there could be oxygen competition among various microbial populations. As shown in Figure 4, P uptake was distinct (from 49.7 to 50.4 mg/L), while NH₄⁺ decreased slightly (from 24.2 to 23.2 mg/L) in the first 20 minutes of aerobic phase. It was reported that the oxygen half saturation coefficients of AOB and NOB were in the range 0.2–1.1 mg O₂/L (Rongsayamanont et al. 2010) while that was only 0.002–0.32 mg O₂/L for PAO (Carvalheira et al. 2014). Hence, it was expected that PAO could outcompete nitrifiers at the initial stage of aerobic phase. When PHA content of PAOs decreased to a lower level, the oxygen consumption rate by PAO was reduced, allowing nitrifiers to uptake oxygen. Nitrification delay was also observed in a SNDPR system operated in sequencing batch reactor (SBR) mode (Meyer...
et al. 2005). The above discussion, however, did not take ordinary heterotrophs into consideration. In the last 70 min of aerobic phase, \( \text{NH}_4^- \) decreased from 23.2 mg N/L to 5.2 mg N/L while NOx increased from 3.9 mg N/L to 13.9 mg/L. The 8 mg N/L difference between NH4 consumption and NOx production confirmed the existence of SND.

**Functional microbial communities**

The abundance of microbial population responsible for nitrogen and phosphorus removal in the full-scale WRP was investigated using culture-independent qPCR approach. It was found that the abundance of nitrifying population turned out to be highly dynamic, while co-existed with a larger NOB population (Figure 5) during the sampling period in 2014. However, even in such a situation, the nitrite accumulation was still observed in the aerobic zones. Moreover, it should be pointed out that *Nitrospira* appeared to be the dominant NOB as shown in Figure 5 at a reduced DO concentration in the aerobic zone. The significant increase in *Nitrosira* population eventually led to the decrease in nitrite accumulation (data not shown). The selection of *Nitrospira* may be attributed to the low concentration of standing NO2-N in the aerobic zones and the survivability of *Nitrospira* at low DO in the anoxic zones. *Nitrospira* had been known as a K strategist, i.e. having higher affinity for nitrite compared to *Nitrobacter* (Nowka et al. 2015), whereas it was found to be dominant in both continuous and batch cultures operated at DO as low as 0.074 mg/L (Sliqueers et al. 2005). It should be noted that the role of *Nitrospira* in nitrification and SND still remains unclear and further study shall be needed.

The presence of PAOs was quantified by primers targeting *Accumulibacter*-specific 16S rRNA gene (Table 5). In fact, *Candidatus* ‘Accumulibacter phosphatis’ (later referred to as *Accumulibacter*) has been well known as the major PAO species responsible for P-removal (Oehmen et al. 2007). The abundance of *Accumulibacter* PAO was comparable to denitrifiers, and higher than AOB and NOB in the full-scale WRP studied. If each *Accumulibacter* PAO carried two copies of 16S rRNA gene, the abundance of PAO accounted for 9–23% of the denitrifiers population in the full-scale WRP (Table 5). The presence of *Accumulibacter* PAO was further confirmed by FISH using PAO mix probe (Figure 6(a)). From a total of 15 fields of view, *Accumulibacter* PAO represent 6.0 ± 3.2% of total EUBmix stained area on average. Rod-shaped *Tetrasphaera*, a PAO candidate often found in full-scale EBPR plants, was also detected by FISH (Figure 6(b)). *Tetrasphaera* PAO represents an average of 4.0 ± 1.9% of total EUBmix stained area. In addition, polyP granules were mainly observed in cells in the aerobic zones (Figures 7(a) and 7(b)) instead of anoxic zones (Figure 7(c)).

However, in the WRP studied, surprisingly, GAO was rarely detected (data not shown), which was unusual. High temperature may enhance hydrolysis of particulate COD. With a sludge return ratio of 50%, sCOD in the return sludge can be used for EBPR, and sludge hydrolysis may also take place in anoxic tanks where sCOD can be slowly released to bulk liquid. This was partially supported by the observed release of ammonia in the anoxic tanks. As very low-concentration VFA was detected in all the anoxic tanks and returned sludge, it is highly possible that the carbon source produced from sludge hydrolysis was taken up by PAO immediately. This, in turn, favored the growth of PAO against GAO. Second, total HRT for anoxic and aerobic phases in the five reaction basins were equal at the level of 2.8 h each. It should also be realized that the FST was essentially operated in anaerobic condition with a HRT of 4.3 h, thus, this may add in an additional anaerobic phase to the overall treatment process. Generally, anaerobic or anoxic phase for PHA formation is much shorter than aerobic phase. A recent study revealed that extended anaerobic phase can strengthen PHA storage through efficient carbon source usage. This may further encourage the growth of PHA accumulators such as PAO.

![Figure 5](image-url) Abundance of 16S rRNA gene representing AOB and NOB (NBT: Nitrobacter, NSR: Nitrospira). Gene abundance was determined by qPCR using taxa-specific primers.

### Table 5
Gene abundance of denitrifiers and *Accumulibacter* PAO in anoxic zones from three different sample collections. All values are reported as \(10^{10}\) copies/g VSS. Each value represents the average of five basins.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Denitrifiers (nosZ)</th>
<th>All <em>Accumulibacter</em> (rrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>22.0 ± 12.5</td>
<td>5.6 ± 1.3</td>
</tr>
<tr>
<td>Sample 2</td>
<td>9.5 ± 2.8</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td>Sample 3</td>
<td>18.1 ± 10.9</td>
<td>8.4 ± 2.0</td>
</tr>
</tbody>
</table>

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CONCLUSIONS

Through full-scale nutrient profiling and batch experiment, a municipal WRP operated under temperatures ranging from 29 to 32 °C performed effective nitrogen and phosphorus removal. SNDPR process played an important role. Although the population of nitrifiers was highly dynamic, the nitrification performance was relatively stable. It was interesting to note that the abundance of *Accumulibacter* PAO was comparable with denitrifier while GAO population was hardly detected. The hypothesized major contributing factors for SNDPR were low DO environment in aerobic zones, which would favor both aerobic denitrification and aerobic/anoxic P-uptake, and warm temperature that may lead to additional carbon source generation for P-removal. The slowly released sCOD in FST may provide further advantage for PAO over GAO on competitive carbon accumulation prior to main biological treatment process. Anoxic step-feed significantly reduced carbon loss in the aerobic phase so that carbon source in the wastewater can be efficiently used by both denitrifiers and PAO. This is the first full-scale plant operated under a warm climate that is able to achieve SNDPR performance. While the potential negative effects from such process will require further study, more investigation is certainly required to better understand the microbial interactions in order to develop system manipulation strategies and propose optimal operating conditions.

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Figure 6 | Representative FISH image from anoxic zone AS hybridized with (a) PAOMix (Cy3, blue) or (b) Actino_658 (Cy3, blue) and EUB338mix (Cy5, red). Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wst.2016.214.

Figure 7 | PolyP staining of AS from aerobic zones (a), (b) and anoxic zone (c) at ×1000 magnification. The scale bar = 10 μm.
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