Effects of interchange ratio on sludge reduction and microbial community structures in an anaerobic/anoxic/oxic process with combined anaerobic side-stream reactor


ABSTRACT

Oxic-settling-anaerobic (OSA) process is effective in minimizing sludge production, by inserting an anaerobic side-stream reactor (ASSR) in the recycling bypass. Interchange ratio (IR), the quantity ratio of sludge entering the ASSR to the sludge in the main stream reactors, is one of the most important parameters for OSA process. In the present study, a laboratory-scale anaerobic/anoxic/oxic (A2/O) process combined with an ASSR (A2/O-ASSR) was operated for 366 days in parallel with a conventional A2/O process to investigate the effects of IR on sludge reduction. IR was assigned values of 5%, 8%, 10%, and 15%, and the A2/O-ASSR process achieved 14.0%, 16.0%, 24.1%, and 13.7% of sludge reduction, respectively. At the optimum IR of 10%, high through-put sequencing analysis showed that the microbes responsible for pollutant removal and ubiquitous in wastewater treatment remained predominant in the two systems, and slow-growing microbes related to hydrolysis, nitrogen and phosphorus removal increased in the A2/O-ASSR process, which probably played a key role in sludge reduction. 40.6–58.6% of sludge reduction was caused by sludge decay in the ASSR. The tiny amount of extracellular polymeric substance released in the A2/O-ASSR process was subthreshold to cause remarkable sludge reduction.

Key words | anaerobic side-stream reactor, interchange ratio, mechanism, microbial community structure, oxic-settling-anaerobic process, sludge reduction

HIGHLIGHTS

- An anaerobic/anoxic/oxic (A2/O) system combined with an ASSR was operated for 366 days in a laboratory. A portion of external recycled sludge was diverted to the mainstream oxic reactor after being treated in the ASSR for some time, forming an adaptation of OSA process in the A2/O process.
- Interchange ratio ranging from 8–15% was adopted in the A2/O-ASSR system to investigate the effects of IRs on sludge reduction. IR refers to the quantity ratio of the amount of sludge exchanged to ASSR to the amount of sludge in the mainstream reactors.
- At IR of 10%, the A2/O-MOSA process reduced sludge production by 24.1%.
- Slow growing microbes related to nitrogen and phosphorus removal increased in the A2/O-ASSR process at IR of 10%, which probably played a key factor for sludge reduction in A2/O-ASSR process.
INTRODUCTION

Activated sludge process has become the most common and reliable process for wastewater treatment because of its advantages in effective pollutant removal, stability, and low operating cost. However, with the scale of sewage treatment expanding, huge volumes of excess sludge are generated and discharged, the treatment and ultimate disposal of which accounts for 25–65% of the total cost in operating wastewater treatment plants (Goel & Noguera 2006). The management of excess sludge produced from the activated sludge process has been one of the most severe problems restricting the development of the wastewater treatment industry. Reducing sludge production will relieve the wastewater treatment industry of the sludge management dilemma (Semblante et al. 2014).

The oxic-settling-anaerobic (OSA) process, which was first proposed in the 1970s and involved the addition of an anaerobic side-stream reactor (ASSR) in the recycling bypass of sludge between the settling tank and oxic reactor of the conventional activated sludge process, offered a potentially cost-efficient and low-impact alternative to sludge reduction (Wei et al. 2003). To avoid exclusive reactor volume for holding the whole external recycle in the conventional OSA process, the modified OSA process was proposed by diverting only a portion of external recycle to the ASSR, which also achieved considerable sludge reduction (Sun et al. 2015, 2016, 2018). As reviewed by Ferrentino et al. (2016), the most reported configuration in literature and in laboratory-scale application is the sequencing batch reactor (SBR)-OSA configuration which occupied less space without individual settling tank. Plenty of studies have proved that the SBR-OSA process performed well in sludge reduction and simultaneous carbon, nitrogen and phosphorus removal (Goel & Noguera 2006; Novak et al. 2007; Sun et al. 2010; Chon et al. 2011). However, as the most classical and common process for simultaneous nitrification, denitrification and phosphorus removal, anaerobic/anoxic/oxic (A2O) process was seldom modified into an OSA process probably due to the more complex configuration structures, as well as operating parameters, than SBRs.

As the core of the OSA process, many studies have been conducted to modify the structure and operation mode of the OSA process (Tang et al. 2011; Sun et al. 2015; Sun et al. 2018), and to promote sludge reduction on parameter optimization of the ASSR such as oxidation-reduction potential (ORP) (Saby et al. 2003), hydraulic retention times (HRTs) (Sun et al. 2016; Jiang et al. 2018) and solids retention times (SRTs) (Semblante et al. 2016b; Wang et al. 2016). The OSA process can reduce sludge production by approximately 30–80% depending on the aforementioned parameters (Wei et al. 2003).

Sludge interchange ratio (SIR or IR), the volume (or quantity) ratio of sludge returned from the external reactor into the main bioreactors of the OSA process, is a critical design parameter for OSA process to enhance sludge reduction. IR has been simulated from 4% to 100% in previous studies, different from each other in configurations, operative conditions, and design parameters, as reviewed by Ferrentino et al. (2016). However, IR in different studies had its own definition. Some research focused on IR referred to the volume ratio between sludge diverted into side-stream reactor (SSR) and sludge in the main stream reactor (MSR). According to Novak et al. (2007), the optimal IR was around 10% of the return sludge flow and resulted in around 60% of sludge reduction. Semblante et al. (2016b) found that the highest sludge reduction was achieved at the IR of 11% in an SBR-OSA system. Karlkanokvate-Balikci et al. (2019) found that the OSA process achieved around 58% sludge studies at IR of 7.7%. Usually, 10% of the settled volume is diverted to the side-stream reactor in plenty of the studies regarding the application of OSA strategy (Easwaran 2006; Huang et al. 2014; Yagci et al. 2015). Considering the diversity of process operations in various studies, it would be very difficult to estimate the exact amount of activated sludge circled in the ASSR, since the total amount of sludge is decided by not only the volume but also the concentration of biomass. Thus, there were also studies focused on IR referred to the quantity ratio between sludge diverted into side-stream reactor and sludge in the MSR. Ferrentino et al. (2018) found that a 100% interchange rate, corresponding to an anaerobic solid retention time of 2.5 days, led to 66% sludge reduction. Sun et al. (2010) achieved an enhanced sludge reduction (53–77%) by increasing the interchange frequency with IR of 10%.

Previous studies hypothesized that sludge reduction in the OSA process is driven by the selection of a distinct microbial community brought about by the exchange of sludge between different redox regimes (Semblante et al. 2014). Many slow growers related to phosphorus and nitrogen removal, and sulfate reduction, enriched in the OSA process and led to a decrease in sludge production (Semblante et al. 2017). Goel & Noguera (2006) studied the Cannibal-enhanced
biological phosphorus removal (EBPR) process and pointed out that there were abundant slow-growing microbes of phosphorus accumulating organisms (PAOs) and zymophytes. PAOs were also reported to be the dominant population in the CAS-OSA process (He & Wei 2018). Pang et al. (2018) found that ASSRs enriched the hydrolytic and fermentative bacteria in the membrane bioreactors coupled with SSRs with different dissolved oxygen (DO) levels. Zhou et al. (2015) revealed that the relative abundance of β-proteobacteria, especially anaerobic bacteria, such as Propionivibrio and Sulfuritalea enriched in the A + OSA system. Cheng et al. (2017) found that slow-growing microbes enriched at higher side-stream ratio, while hydrolytic and predatory bacteria increased with a lower side-stream ratio.

Other possible mechanisms for sludge reduction were proposed. Chen et al. (2003) thought that anaerobic sludge decay under a low ORP condition was the main reason for sludge reduction. Feng et al. (2011) confirmed that when microbes experienced anaerobic conditions, they used ATP as the energy source and then recovered ATP as soon as aerobic conditions returned at the expense of growth. Novak et al. (2007) and Chon et al. (2011) argued that the sludge reduction in OSA process was caused by the degradation of base-extractable extracellular polymeric substance (EPS) happened in both anaerobic and aerobic reactors.

In this study, an anaerobic/anoxic/oxic (A²/O) process combined with an ASSR (A²/O-ASSR) were built up in a lab. A portion of external recycle sludge from the settling tank was treated in the ASSR before being delivered to the mainstream oxic reactor, forming an adaptation of the modified OSA process in the A²/O process. The A²/O-ASSR process was operated for 366 days in parallel with a conventional A²/O process. The IR (Equation (1)), which refers to the quantity ratio of the amount of sludge diverted into the ASSR to the amount of sludge in the mainstream reactors, was assigned values of 5%, 8%, 10%, and 15% to investigate its effects on pollutant removal and sludge reduction in the A²/O-ASSR process. High-throughput sequencing was used to explore the changes of microbial community structures in the A²/O-ASSR and conventional A²/O process.

**METHODS**

**Reactor configurations and operating conditions**

To start the experiment, two A²/O systems (100 L/d) were created in laboratory and operated under room temperature, which fluctuated within a narrow range (20–30 °C) in most of the year. The mainstream configurations consisted of an anaerobic reactor (6.5 L), an anoxic reactor (11.2 L), an oxic reactor (25.4 L), and a settling tank (35.8 L). The designed value of SRT in the mainstream process for both systems was 16 days. The anaerobic side-stream reactor is a cylindrical container with a maximum effective volume of 4.6 L. A portion (dependent on the values of IR) of sludge in the external cycle was equally diverted into the ASSR four times each day. Intermittent stirring was carried out in the reactor. After a 5-day retention in the ASSR, mixed liquor was recycled into the main-stream oxic reactor once for every three times of liquid supernatant recycled in one day, as shown in Figure 1. Therefore, the ASSR was operated with an HRT of 5 days and SRT of 20 days. The ORP was maintained lower than −150 mV. The inoculated sludge in the two systems was taken from a local sewage treatment plant at the beginning of cultivation. To avoid the fluctuation and unexpected effects caused by real wastewater which can have high variations from day to day, synthetic wastewater based on the average of real influent in a local sewage treatment plant was continuously fed into each setup. The schematic diagrams of the reactor configuration are shown in Figure 1. The operation parameters and influent characteristics are shown in Table 1. The ratios of internal cycle and external cycle in the main streams were both 100%. The IR for the ASSR recycle, estimated by Equation (1), was adjusted to 5%, 8%, 10%, and 15%, respectively.

\[
IR (\%) = \frac{TSS_{SD}}{TSS_{MSR}} \times 100% = \frac{MLSS_{SD} \times Q_{SD} \times T_{SD} \times 4}{MLSS_{MSR} \times V_{MSR}} \times 100% \tag{1}
\]

wherein TSS_{SD} (mg) is the amount of sludge diverted into the ASSR each day; TSS_{MSR} (mg) is the average amount of total sludge in the main-stream reactors; MLSS_{SD} (mg/L) is the average mixed liquor suspended solids (MLSS) concentration of sludge diverted into ASSR from the settling tank; Q_{SD} (mL/min) is the flow of sludge diverted into ASSR each cycle; T_{SD} (min) is the time for diverting sludge into the ASSR in each cycle; there are four cycles for ASSR influent and effluent each day; MLSS_{MSR} (mg/L) is the average MLSS concentration of the main-stream reactors; V_{MSR} (L) is the effective volume of the main-stream reactors. The main-stream reactors consisted of the anaerobic reactor, anoxic reactor and oxic reactor, as shown in Figure 1.
In the present systems, the excess sludge discharged regularly each day to maintain relative stable thickening time of sludge in the settling tank, except for the infrequent manual intervention when the MLSS in mainstream reactors increased out of the expected range. Therefore, the MLSS in the external recycle and the mainstream reactors exhibited indistinctive variety. With the conditions of relative stability in MLSS mentioned above, no more modification

![Figure 1](https://example.com/figure1.png)

**Figure 1** | Schematic diagrams of the A²/O system (a) and the A²/O-ASSR system (b).

| Table 1 | Operation parameters and characteristics of the synthetic wastewater |
|---------|-------------|-------------|-------------|
| **Operation parameters** | **Value** | **Influent components** | **Value** |
| HRT in the anaerobic tank | 1.0 h | COD | 212.5 ± 10.9 mg/L |
| HRT in the anoxic tank | 2.4 h | TN | 31.3 ± 2.5 mg/L |
| HRT in the oxic tank | 5.7 h | NH₄-N | 15.8 ± 1.2 mg/L |
| MLSS in the oxic tank | 2,000–3,500 mg/L | TP | 3.5 ± 0.5 mg/L |
| DO in the oxic tank | 2–4 mg/L | SS | 50.5 ± 2.5 mg/L |
| SRT in the main stream process | 16 d | Fe | 3.8 ± 0.6 mg/L |
| HRT in the ASSR | 5 d | Al | 2.4 ± 0.3 mg/L |
| SRT in the ASSR | 20 d | Mg | 8.7 ± 0.4 mg/L |
| ORP in the ASSR | ≤−150 mV | Ca | 56.5 ± 3.7 mg/L |
| Number of the ASSR exchanges | 4 times per day | pH | 7.0 ± 0.1 |

In the present systems, the excess sludge discharged regularly each day to maintain relative stable thickening time of sludge in the settling tank, except for the infrequent manual intervention when the MLSS in mainstream reactors increased out of the expected range. Therefore, the MLSS in the external recycle and the mainstream reactors exhibited indistinctive variety. With the conditions of relative stability in MLSS mentioned above, no more modification
was made during the operation once the values of flow (QSD) and flowing time (TSD) were set to obtain expected IRs according to the average values of MLSSSD and MLSSMSR at the beginning of every period.

During the cultivation, the two systems operated in parallel, including HRT, SRT, MLSS, and dissolved oxygen (DO). After a 46-d cultivation, the A2/O (Conf. 1#) system was modified into an OSA system combining with an ASSR (HRT = 5 d, SRT = 20 d), while the A2/O (Conf. 2#) remained unmodified. Stepwise changes of IR (5%, 10%, 15%) were adopted to the A2/O-ASSR from day 47 to day 254. To narrow the best fit range of IR further, IR was set to be 8% from day 255 to day 304. During the last 61 days’ operation, IR was reset as 10% to investigate the changes of microbial community structures in the two systems. The whole operation of the two systems lasted for 366 days, and the timeline of the operation was shown in Table 2.

### Analytical procedure

#### Chemical analysis

During the operation, concentrations of pollutants in the influent and effluent, MLSS, MLVSS in the oxic tanks as well as in and out of the ASSR, DO in the oxic tanks, and ORP in the ASSR were monitored daily. The MLSS, MLVSS, ammonia nitrogen (NH4-N), total nitrogen (TN), total phosphorus (TP), and suspended solids (SS) in the influent and effluent were analyzed twice per week according to the standard methods (The State Environmental Protection Administration 2002). Metals and soluble protein were detected by inductively coupled plasma optical emission spectrometer (ICP-OES) and the Folin-phenol reagent method, respectively.

### Sludge reduction analysis

The microbial yield coefficient (Yobs, kg MLSS/kg COD) was used to describe the sludge production. The total suspended solids (TSS) in the settling tanks were at a similar level and remain stable since the same sludge discharge mode was adopted to the two systems, and the amount of TSS in the ASSR could be considered as a relative stable value during long-term steady operation. Biomass growth mainly occurred in the mainstream reactors because of the higher metabolism activity with sufficient substrate. From this perspective, the biomass increased in the mainstream reactors and excess sludge discharged was mainly measured to investigate the sludge production. The values of Yobs in the two systems were estimated using Equation (2):

\[
Y_{obs} = \frac{(\text{MLSS}_2 - \text{MLSS}_1) \times V_{\text{MSR}} + U_{\text{elimination}}}{(\text{COD}_{\text{in}} - \text{COD}_{\text{out}}) \times Q \times T}
\]

wherein MLSS1 and MLSS2 are the initial and final concentrations of each period with different IRs, respectively; VMSR is the effective volume of the mainstream reactors including anaerobic, anoxic, and oxic reactors (40 L); Uelimination is the amount of sludge discharged, including the biomass losses in the effluent and all the losses during sampling and examining; CODin and CODout are the average concentrations of chemical oxygen demand (COD) in the influent and effluent; Q is the volume of treated wastewater per day (100 L); T is the operation time of each phase with different IRs (50 d).

The sludge reduction was measured by comparing the Yobs values of the A2/O-ASSR and the A2/O process, estimated by using Equation (3):

\[
\text{Sludge reduction rate (\%)} = \frac{Y_{obs(A2/O-OSA)} - Y_{obs(A2/O)}}{Y_{obs(A2/O)}} \times 100\%
\]

### Microbial community analysis

During day 304–366, activated sludge taken from the oxic tank of the A2/O system (2#-O), the oxic tank of the A2/O-ASSR (1#-O) and the ASSR of the A2/O-ASSR (1#-ASSR) was sampled to analyze the microbial community by high-throughput sequencing on the Illumina MiSeq platform, which has clear superiority in determining microbial community characteristics and diversity analysis.
RESULTS AND DISCUSSION

Effect of IRs on sludge reduction

Both systems were operated as A2/O process under the same condition for 46 days before one of the two systems were refitted into A2/O-ASSR process. The observed \( Y_{\text{obs}} \) for both systems during the 46-day cultivation were 0.3862 and 0.3852 g COD/g MLSS, respectively, indicating that both systems remained comparable during operation. As shown in Table 3, the substrate utilization and accumulative sludge production data under different IRs were summarized to estimate observed sludge production. According to Equation (2), the \( Y_{\text{obs}} \) values for A2/O-ASSR were 0.2468, 0.2770, 0.2697, and 0.2705 kg COD/kg MLSS at IRs of 5%, 8%, 10%, and 15%, respectively. The maximum corresponding sludge reduction of 24.1% was achieved at IR of 10%, while lower sludge reductions (14.0%, 16.0%, and 13.7%) were revealed at IRs of 5%, 8%, and 15%, respectively.

The results indicated that IR was a critical design parameter for the A2/O-ASSR system, in where the efficiency of sludge reduction decreased at IRs either too high or too low. These results were consistent in good agreement with other studies, which have shown a similar trend at different sludge interchange ratios (Semblante et al. 2010a; Karlikanovaite-Balikci et al. 2013)

The predominant death phase in the ASSR at high retention times, and higher endogenous decay rate due to the introduction of a higher amount of active biomass with higher recirculation rate were the possible reasons for the results (Yagci et al. 2018).

<table>
<thead>
<tr>
<th>Operational period</th>
<th>Conf. 1#</th>
<th>Conf. 2#</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR (%)</td>
<td>I 5</td>
<td>II 10</td>
</tr>
<tr>
<td>COD_{in} (mg/L)</td>
<td>210.9 ± 16.9</td>
<td>210.9 ± 16.9</td>
</tr>
<tr>
<td>COD_{out} (mg/L)</td>
<td>20.1 ± 6.7</td>
<td>22.5 ± 9.8</td>
</tr>
<tr>
<td>MLSS1 (mg/L)</td>
<td>2,178</td>
<td>3,195</td>
</tr>
<tr>
<td>MLSS2 (mg/L)</td>
<td>3,518</td>
<td>2,979</td>
</tr>
<tr>
<td>U_{elimination} (g)</td>
<td>202.25</td>
<td>259.19</td>
</tr>
<tr>
<td>Y_{obs} (kg MLSS/kg COD)</td>
<td>0.2468</td>
<td>0.2697</td>
</tr>
<tr>
<td>Sludge reduction (%)</td>
<td>14.0</td>
<td>24.1</td>
</tr>
</tbody>
</table>

Microbial community and sludge reduction mechanisms

Microbial diversity

Alpha diversity was used to describe the species richness and diversity in the samples, shown in Table 4. The greater microbial richness was detected in the oxic tank of the A2/O process and the ASSR due to the higher Chao 1 estimators in 2#-O (2992.9374) and 1#-ASSR (2988.4853), followed by 1#-O (2717.1241). The highest Shannon indexes and Simpson indexes in 1#-ASSR also indicated that microbial communities in the ASSR were more diverse than that in the oxic tanks of the A2/O and A2/O-ASSR processes.

Development of microbial community in oxygen-deficient external reactors was also found in other studies (Niu et al. 2016; Semblante et al. 2017).

Beta diversity was used to describe the species richness and diversity among the samples. According to 24.94% and 74.33% of the main components, there was noteworthy difference among the three samples (Figure 2), indicating that the microbial communities in the three reactors differ a lot. The microbes with different dissolved oxygen demand differ in the oxic tanks (2#-O and 1#-O) and the ASSR (1#-ASSR). The microbial community in the ASSR would evolve toward anaerobic or facultative microbes with long-term operation as an adaptation in the anaerobic environment, which indicates the possibility of high variety between microbial community structures in the ASSR and the oxic reactors. The noteworthy difference between 2#-O and 1#-O indicated microbial community diversity in the oxic reactor changed significantly after retaining in the ASSR. The result indicated that the microbial community...
structure in the mainstream reactors in the A²/O-ASSR process and that in the A²/O process would evolve toward different directions with long-term operation due to the insertion of the ASSR.

**Taxonomic complexity of the bacterial community**

In order to identify the phylogenetic diversity of bacterial communities in the two systems, the relative abundance at the phylum classification was investigated (Figure 3(a)). *Proteobacteria* (24.2–45.8%), *Bacteroidetes* (22.0–26.1%) and *Chloroflexi* (13.2–30.7%) were detected as predominant in all samples, which was in accordance to the results reported by Cheng et al. (2014a), Sun et al. (2016) and Karlkanovaite-Balikci et al. (2019). *Proteobacteria* and *Bacteroidetes* decreased in the A²/O-ASSR process because of the lower relative abundance in 1#-O and 1#-ASSR. *Chloroflexi*, showed the highest abundance in the oxic tank of the A²/O process. *Acidobacteria* enriched in the oxic tank as well as the ASSR. *Planctomycetes* was enriched in the ASSR with a highest relative abundance of 11.5%. *Verrucomicrobia*, *Gemmatimonadetes*, *Actinobacteria*, *Firmicutes*, and *Chlorobi* were greatly enriched in the ASSR, accounting for 11.3% in

### Table 4 | Statistics of alpha indexes of the samples taken from the oxic tank of A²/O (2#-O), the oxic tank of A²/O-ASSR (1#-O), and the ASSR of A²/O-ASSR (1#-ASSR)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Observed species</th>
<th>Chao 1</th>
<th>Shannon</th>
<th>Simpson</th>
</tr>
</thead>
<tbody>
<tr>
<td>2#-O</td>
<td>1,916</td>
<td>2,992.9374</td>
<td>8.2473</td>
<td>0.9843</td>
</tr>
<tr>
<td>1#-O</td>
<td>1,892</td>
<td>2,717.1241</td>
<td>7.9506</td>
<td>0.9676</td>
</tr>
<tr>
<td>1#-ASSR</td>
<td>2,152</td>
<td>2,988.4835</td>
<td>9.3199</td>
<td>0.9949</td>
</tr>
</tbody>
</table>

**Figure 2 | Microbial diversity of the two systems.**

**Figure 3 | The relative abundances of species in (a) phylum, (b) family and (c) genus classification levels.**
total. *Caldiserica*, *OD1* and *OP8* were detected in the ASSR with a low abundance.

Further comparison down to the family level was conducted to reveal more information on the microbial community evolution among 2#O, 1#O, and 1#ASSR (Figure 3(b)). *Chitimonopasgaceae* maintained a similar abundance in all samples. The relative abundance of *Comamonadaceae*, *Caldilineaceae* and *Rhodocyclaceae* decreased in the A2/O-ASSR system as shown in sample 1#O and 1#ASSR. *Comamonadaceae* and *Rhodocyclaceae* were related to nitrogen and phosphorus removal (Heylen et al. 2006; Mao 2009). *Kouleothrixaceae* was greatly enriched in the 1#O. The relative abundances of *Hyphomicrobiaceae*, *Pirellulaceae*, *Saprospiraceae* and *Thiotrichaceae* were higher in the 1#O and 1#ASSR. *Hyphomicrobiaceae* could live on C1 compounds and was considered to be responsible for denitrifying (Chen et al. 1992). *Saprospiraceae*, which was responsible for denitrification in anoxic and transitory anaerobic environments, and became more active in oxic zones, used the energy generated from amino acids for proteolysis (Xia et al. 2008).

Figure 3(c) depicts relative abundances of microbial communities of three samples from the A2/O and A2/O-ASSR systems in genus level. *Caldilinea*, *Dechloromonas* and *Rhodobacter* decreased in the ASSR and oxic tank of the A2/O-ASSR process. *Kouleothrix* became predominant in the A2/O-ASSR system because of the high abundance in 1#O and 1#ASSR. *Thiotrix* significantly increased in the oxic tank of A2/O-ASSR system, and exhibited low abundance in 2#O and 1#ASSR. The results were in accordance to those of Karlikanovaite-Balikci et al. (2019) who found *Thiothrix* boosted in the OSA trials. *Hyphomicrobium* and *Gemmatina* was of the family *Planctomyetes*, many of which were anammox bacteria.

**Correlation of microbial community with sludge reduction**

Low ORP was cited as the sole reason for sludge reduction in the OSA for accelerating sludge hydrolysis in the ASSR (Chen et al. 2003; Saby et al. 2005). *Proteobacteria*, *Bacteroidetes* and *Chloroflexi*, which were ubiquitous in sewage treatment and responsible for pollutant removal (Reed et al. 2006; Xie et al. 2014), were predominant microbes in the A2/O and the A2/O-ASSR process. *Proteobacteria* and *Chloroflexi* members could help releasing intracellular compounds, after which *Bacteroidetes* could grow by using the secondary substrate produced by hydrolytic fermentation (Albers & Siebers 2014; Cheng et al. 2018b). *Caldiserica* and *OP8* bacteria emerged in the ASSR with low abundances, which were ubiquitous in sufficient simple organics (Tao et al. 2008). *Anaerolineaceae* also increased in the ASSR and was considered likely in a co-metabolic relationship with methanogens (Zhang et al. 2015). In the present study, the ASSR was not considered as an ideal reactor for methane production since experiments were carried out in room temperature with operating parameters as follow: the MLSS of influent was below 20,000 mg/L; the SRT in the ASSR was 20 days; the ORP in the ASSR was below −150 mV. According to the study by Park et al. (2018), methane yield of 14-day digestion exhibited a very low value in an ASSR operated at 21–22 °C.

The enriched fermentative bacteria in the A2/O-ASSR system proved that sludge hydrolysis had important contribution to sludge reduction, consistent with previous studies argued for sludge hydrolysis or sludge reduction (Chen et al. 2003; Wang et al. 2008; Pang et al. 2018). Relative abundance of microbes related to nitrogen and phosphorus removal were found to be enriched in the A2/O-ASSR system, which was considered likely to have a key role for sludge reduction (Goel & Noguera 2006; Semblante et al. 2014; Zhou et al. 2015; Semblante et al. 2017). The abundance of *Acidobacteria*, *Kouleothrix*, *Thiothrix*, *Saprospiraceae*, *Hyphomicrobiota* and *Gemmatina* increased in the oxic reactor of the A2/O-ASSR process. *Acidobacteria*, generally considered to be related to phosphorus removal and characterized slow growth (Jean et al. 2005). *Thiothrix* species were able to grow heterotrophically, mixotrophically and lithoautotrophically with thiosulfate or sulfide as the sole energy source (Chernousova et al. 2009), which was considered likely to have a key role in OSA system (Karlikanovaite-Balikci et al. 2019). *Hyphomicrobiota* could grow in oxic zones or live on nitrate in anoxic zones, and shows slower growth than other microbes in solid media. The relative abundances of *Planctomyces*, *Verrucomicrobia*, *Gemmatimonadetes*, *Actinobacteria*, *Firmicutes* and *Chlorobi* were increased in the ASSR. *Planctomyces* and *Chlorobi* were reported to be responsible for anammox (Zhang 2014). *Verrucomicrobia* was a newly classified mesophilic hydrocarbon-degrading phylum of bacteria that performs nitrogen removal in anoxic zones (Freitag & Prosser 2003). *Thiothrix* and *Saprospiraceae* were responsible for denitrification in low oxygen environment (Yan et al. 2019), increased in the oxic reactor of A2/O-ASSR system. Microbes using nitrate or nitrite as electron acceptors generate less energy and have a slower growth rate (Copp & Dold 1998; Rittmann & McCarty 2012). The enriched abundance of slow growing microbes responsible for nitrogen and
phosphorus removal indicated that slow growing microbes may have a key role in sludge reduction in A2/O-ASSR process (Sun et al. 2016; Semblante et al. 2017).

Other possible causes of sludge reduction

Correlation of sludge decay with sludge reduction

Sludge decay was considered to be a main contributor to sludge reduction by causing cell dissolution and sludge hydrolysis, which resulted in direct reduction of biomass (Chen et al. 2005; Chu et al. 2009). Microbes would conduct endogenous metabolism in the ASSR due to the lack of substrate (Yan et al. 2018). A portion of microbes use the intracellular organic matter produced by cell lysis as substrate to generate energy for living activities, thus the total biomass delivered out of the ASSR would be less than that into the ASSR. Therefore, the proportion of TSS reduction in the ASSR in the whole TSS reduction in the A2/O-ASSR process indicates the contribution of sludge decay to sludge reduction in the A2/O-ASSR process. The accumulated amounts of TSS reduced in the ASSR and the overall A2/O-ASSR process with different IRs were summarized in Table 5. The amount of TSS reduced in the ASSR was measured by taking the difference between the total amount of TSS in and out of the ASSR. The amount of TSS reduced in the A2/O-ASSR process was measured by taking the difference between sludge production in the A2/O-ASSR and A2/O process. The results showed that accumulated TSS reduction in the ASSR accounted for a large proportion (40.6–58.6%) of the overall sludge reduction. It was in accordance to Wang et al. (2008) who found that sludge decay was the main cause (accounting for two-thirds reduction) of sludge reduction in the OSA system. The result indicated that sludge decay was an important factor contributing to sludge reduction in A2/O-ASSR process, consistent with previous studies that argue for sludge decay hypothesis. However, the contribution increased at IR of 5%, 8%, and 15%, where the corresponding efficiency of sludge reduction were relatively lower (i.e. less amount of TSS were reduced in the A2/O-ASSR system). The most likely explanation for the results was that higher retention times and higher recirculation of active biomass accelerated endogenous decay and suppressed sludge reduction (Yagci et al. 2018). The analysis results suggested that sludge decay tended to be a less important reason for more efficient sludge reduction.

Correlation of extracellular polymeric substance dissociation with sludge reduction

Extracellular polymeric substance is mainly composed of polysaccharides, proteins and nucleic acid, connected to the cell surface by metal ions. According to the study by Novak et al. (2003), protein, polysaccharide, and ions bound to those protein and polysaccharide would be released into the supernatant when EPS was dissociated. To analyze the effects of EPS dissociation on sludge reduction, the amounts of soluble protein and soluble metals in the two systems were measured when IR was 10%, shown as Table 6 and Table 7.

According to Table 6, a portion of protein was released after retaining in the ASSR because of the slight increase (0.89 mg/L) in the effluent of the ASSR. The concentrations of soluble protein in the oxic reactors of both systems were similar, since the protein was released by only a small part of sludge in the overall configuration. In accordance with the analysis of soluble protein, the concentrations of soluble metals detected in the supernatant (as shown in Table 7)

**Table 5** | The comparison of the amounts of TSS reduced in the ASSR and the overall A2/O-ASSR system at different IRs

<table>
<thead>
<tr>
<th>IR</th>
<th>TSS reduced in the ASSR (mg)</th>
<th>TSS reduced in the A2/O-ASSR system (mg)</th>
<th>Proportion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
<td>4,805.91</td>
<td>8,201.3</td>
<td>58.6%</td>
</tr>
<tr>
<td>8%</td>
<td>14,676.76</td>
<td>29,650</td>
<td>49.2%</td>
</tr>
<tr>
<td>10%</td>
<td>27,355.25</td>
<td>42,760</td>
<td>40.6%</td>
</tr>
<tr>
<td>15%</td>
<td>8,905.26</td>
<td>15,340</td>
<td>58.1%</td>
</tr>
</tbody>
</table>

**Table 6** | The concentrations of soluble protein in the oxic reactors and the influent and effluent of the ASSR

<table>
<thead>
<tr>
<th>Reactors</th>
<th>A2/O system (mg/L)</th>
<th>A2/O-ASSR system (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxic reactor</td>
<td>18.2 ± 0.1</td>
<td>18.0 ± 0.09</td>
</tr>
<tr>
<td>Influent of the ASSR</td>
<td>/</td>
<td>17.8 ± 0.1</td>
</tr>
<tr>
<td>Effluent of the ASSR</td>
<td>/</td>
<td>18.7 ± 0.1</td>
</tr>
</tbody>
</table>

**Table 7** | The concentrations of soluble metals in the A2/O-ASSR system

<table>
<thead>
<tr>
<th>Metal ion (mg/L)</th>
<th>Influent</th>
<th>Effluent</th>
<th>Oxic reactor</th>
<th>ASSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al</td>
<td>2.41 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>Ca</td>
<td>56.70 ± 0.02</td>
<td>55.74 ± 0.02</td>
<td>57.24 ± 1.0</td>
<td>35.48 ± 1.1</td>
</tr>
<tr>
<td>Fe</td>
<td>3.79 ± 0.02</td>
<td>0.22 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>Mg</td>
<td>8.72 ± 0.02</td>
<td>8.73 ± 0.02</td>
<td>8.86 ± 0.03</td>
<td>7.09 ± 0.01</td>
</tr>
</tbody>
</table>

*The concentrations of Al in the oxic reactor and the ASSR were below detection.*
were not very high, indicating that only a small portion of EPS was dissociated in the ASSR. The concentrations of soluble metals decreased significantly in the effluent due to the microbial utilization for EPS formation (Novak et al. 2003). Soluble Al was barely detected in the ASSR and oxic reactor probably due to its being tightly bound to organic material (Park et al. 2006a). Fe was detected in higher concentrations in the ASSR and effluent than in the oxic reactor, because the protein associated with Fe was degraded in anaerobic conditions (n.b. the sludge holding tank in the settler could provide anaerobic condition; Park et al. 2006b). The concentrations of Ca and Mg were higher in the oxic reactor than in the ASSR, which was due to the release of lectin-like proteins that were linked to polysaccharides and bridged by Ca and Mg (Novak et al. 2003). The chemical precipitation was not considered in the present study since the chemical precipitation might not be evident at such low concentrations of soluble metals. Even if chemical precipitation occurred in the ASSR, too few metal ions were reduced by precipitation to have a noticeable effect. The above changes of soluble protein and metals indicated that a tiny portion of EPS bound to Fe, Ca and Mg was released in the ASSR and oxic reactors, but the amount was subthreshold to cause remarkable sludge reduction in the A².O-ASSR process.

**Process performance**

**Pollutant removal**

Variation of pollutants in the effluent and removal efficiency of the A²/O and A²/O-ASSR systems are illustrated in Figure 4. The two systems exhibited similar performance of pollutant removal throughout the operation, and were barely affected by different IRs. As shown in Figure 4(a), Conf. 1# and Conf. 2# achieved similar COD removal efficiency of 89.1 ± 4.5% and 86.2 ± 5.1% with average concentrations in the effluent of 23.1 ± 9.5 and 28.4 ± 10.9 mg/L, in accordance with results reported by Semblante et al. (2016a). The average ammonia nitrogen concentrations in the effluent of Conf. 1# and Conf. 2# were 1.5 ± 2.7 and 0.9 ± 1.9 mg/L, achieving removal efficiency of 92.6 ± 17.3% and 95.8 ± 12.1%, respectively (Figure 4(c)). The average TN concentrations in the effluent of Conf. 1# and Conf. 2#, except for the sludge bulking period, were 12.8 ± 1.1 and 11.8 ± 0.8 mg/L, achieving removal efficiency of 58.8 ± 8.0% and 61.7 ± 5.8%, respectively (Figure 4(d)). The TN removal efficiency was comparable to the results reported by Huang et al. (2019), and relatively lower than that reported by Ferrentino et al. (2018). The possible reason for the relatively low TN removal was the insufficient carbon or nitrate being recycled to the anoxic reactor caused by a low internal recycle of 100% (as shown in Figure 1), resulting in limited denitrification. As shown in Figure 4(e), Conf. #1 exhibited high removal efficiency (93.9 ± 6.3%) of TP, slightly better than Conf. 2# (92.9 ± 4.0%), with concentrations of 0.2 ± 0.2 mg/L and 0.2 ± 0.1 mg/L, respectively, in the effluent except for the sludge bulking period. According to the content analysis of phosphorus in sludge, more phosphorus was accumulated in sludge in Conf. 1# (0.42 g TP/g MLSS) than Conf. 2# (0.39 g TP/g MLSS), which was possibly due to the increased abundance of microbes responsible for phosphorus removal in A²/O-ASSR system (shown in Figure 3; Chen et al. 2005). During the sludge bulking period, the declined settleability led to an increase in concentration of TP in the effluent since the SS is an important contributor to particulate phosphorus (Fernández et al. 2003). Concentrations of ammonia nitrogen and TN in the effluent increased in both systems, and several possible explanations were given as follow. Firstly, the activated sludge in the reactors might gather into a cluster due to the excessive growth of filamentous bacteria. A portion of microorganisms inside the cluster were put into anoxic condition and the nitrification could not effectively proceed (Xia et al. 2016). Secondly, the abundance of ammonia-oxidizing bacteria, nitrite-oxidizing bacteria and denitrifying bacteria decreased during sludge bulking, resulting in the limited nitrogen removal (Wang et al. 2014). The decrease of biomass concentration in the reactors might also lead to the lack of nitrification.

**Sludge characteristics**

Figure 5 shows the changes in MLSS and the ratio of MLVSS to MLSS (VSS/SS) in the two systems. The excess sludge from both systems discharged regularly, except for manual intervention when the MLSS was beyond the expected range infrequently. Although fluctuation appeared some time, the A²/O-ASSR system showed trends similar to the A²/O system throughout the operation, and the values of VSS/SS both ranged from 0.6 to 0.8. For the A²/O and A²/O-ASSR process, the difference in MLSS between the two systems could be attributed to the ASSR since both systems have the same way of excess sludge discharging. As shown in Figure 5, the mean values of MLSS in the A²/O-ASSR system (2733.6 ± 868.4 mg/L) was slightly lower than that in the A²/O system (3188.4 ± 946.9 mg/L), indicating less biomass in the configuration, probably due to the lower Yobs in the A²/O-ASSR system.
CONCLUSION

The A²/O-ASSR process performed high pollutant removal efficiency throughout the operation at different IRs. When the IR was 10%, the A²/O-ASSR process achieved the maximum sludge reduction of 24.1%. The insertion of the ASSR increased the microbial diversity in the A²/O-ASSR system. *Proteobacteria, Bacteroidetes* and *Chloroflexi*, which were ubiquitous in sewage treatment and responsible for pollutant removal, were predominant microbes in the A²/O and the A²/O-ASSR process. The microbes increased in the A²/O-ASSR system (including the oxic reactor and the ASSR) were facultative anaerobes or anaerobic bacteria; they were mostly slow growers related to hydrolysis, ammonia, denitrifying, and phosphorus removal. 40.6–58.6% of sludge reduction was caused by sludge decay in the ASSR.

Figure 4 | Concentrations of (a) chemical oxygen demand (COD), (b) suspended solids (SS), (c) total nitrogen (TN), (d) ammonia nitrogen (NH₄⁺-N), and (e) total phosphorus (TP) in the A²/O and A²/O-ASSR systems throughout the operation.
but the lower contribution was exhibited in higher sludge reduction in overall system. The results indicated that the ASSR in A2/O-ASSR process acted as a biological selector to retain the slow-growing bacteria with nitrogen and phosphorus removal functions, which was more likely to be the dominant factor for more prominent sludge reduction.

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REFERENCES

Easwaran, S. P. 2006 Developing A Mechanistic Understanding and Optimization of the Cannibal Process Phase II. Master’s Thesis, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA.


Mao, Y. J. 2009 *Structural and Functional Analysis of Thauera Genus in Wastewater Treatment Plants*. Shanghai Jiao Tong University, Shanghai, China.


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