

Study of sulfate-reducing ammonium oxidation process and its microbial community composition

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ABSTRACT

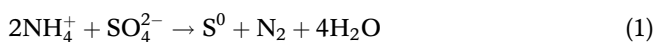
In this study, the simultaneous removal of ammonium and sulfate was detected in a self-designed circulating flow reactor, in which ammonium oxidation was combined with sulfate reduction. The highest removal efficiencies of $\text{NH}_4^+\text{-N}$ and SO_4^{2-}S were 92% and 59.2%. NO_2^- and NO_3^- appeared in the effluent, and experimental studies showed that increasing the proportion of N/S in the influent would increase the NO_2^- concentration in the effluent. However, N/S [$n(\text{NH}_4^+\text{-N})/n(\text{SO}_4^{2-}\text{S})$] conversion rates during the experiment were between 2.1 and 12.9, which may have been caused by the experiment's complex process. The microbial community in the sludge reactor included *Proteobacteria*, *Chloroflexi*, *Bacteroidetes*, *Chlorobi*, *Acidobacteria* and *Planctomycetes* after 187 days of operation. *Proteobacteria* bacteria had a more versatile metabolism. The sulfate-reducing ammonium oxidation (SRAO) was mainly due to the high performance of *Proteobacteria*. *Nitrospirae* has been identified as the dominant functional bacteria in several anammox reactors used for nitrogen removal. Approximately 12.4% of denitrifying bacteria were found in the sludge. These results show that a portion of the nitrogen was converted by nitrification-denitrification, and that traditional anammox proceeds simultaneously with SRAO.

Key words | ammonium and sulfate, anammox, microbial community, nitrification-denitrification

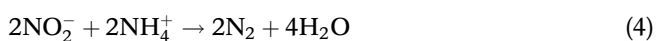
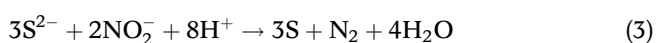
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INTRODUCTION

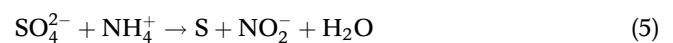
Fdz-Polanco *et al.* (2001b) proposed a novel process using sulfate as an electron acceptor to oxidize ammonium into nitrogen in a granular activated carbon (GAC) anaerobic fluidized-bed reactor. This sulfate-reducing ammonium oxidation (SRAO) process was condensed into one equation describing the two-stage process (Equation (1)).



In Equation (1) the end products are N_2 and S^0 , which are non-pollutants and so the process could offer great future potential as an energy-saving and environmentally sound wastewater treatment (Zhao *et al.* 2006; Ma *et al.* 2013). This process was combined with the conventional anammox reaction and the following steps were proposed the following steps (Fdz-Polanco *et al.* 2001a):



Liu *et al.* (2008) observed that the NO_2^- in a liquid began to decrease during the further oxidization of the remaining ammonium. At the same time, they observed that the actual, detected N_2 ratio was higher than the theoretical value of Equation (4) and proposed the SRAO reaction process under autotrophic conditions (Equations (4) and (5)).

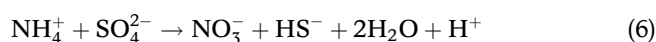


Yang *et al.* (2009) summarized the NO_2^- conversion methods in Equations (3) and (4). It is believed that some denitrification processes can be carried out by the reduction of NO_2^- to N_2 and accompanied by the oxidation of sulfide by autotrophic denitrifiers, as described in Equation (3) (Xu *et al.* 2014). Equation (4) is the reaction in the traditional anammox process. Therefore, the two anaerobic ammonium oxidation reactions were achieved during the SRAO process in Equations (3) and (4).

In most other studies of SRAO (Fdz-Polanco *et al.* 2001c; Liu *et al.* 2008; Yang *et al.* 2009; Jing *et al.* 2010), NO_2^- was

found in the effluent, and their stoichiometric ratios were in accordance with Equation (1) or only slightly different, with the only notable exception being the experiments reported by Rikmann *et al.* (2012) and Sabumon (2008). Recent studies showed disproportionately higher removals of ammonium. At the same time, it should be noted that in most analyses the effluent contained more NO_3^- than the influent (Mahmood *et al.* 2007; Sabumon 2008; Rikmann *et al.* 2012).

Rikmann *et al.* (2014) believed that NO_2^- was converted to NO_3^- under anaerobic conditions due to O_2 entry from the tank and the drain provided in the reactor. At 30 °C and under normal pressure, ammonium can also be oxidised to NO_3^- (Schrum *et al.* 2009) and can be coupled with subsequent heterotrophic denitrification utilizing organics as an electron donor:



Strous *et al.* (2002) believed that some intermediates, such as NO_2^- , NO_3^- , S^{2-} and S, might affect the removal efficiencies of ammonium and sulfate. The SRAO process was heavily suppressed by increased NO_2^- concentrations. Peaks of NO_2^- in the influent (10 $\text{mg}\cdot\text{L}^{-1}$ or more) resulted in major disturbances of the SRAO process, thus showing a significant decrease in sulfate (Rikmann *et al.* 2012). A higher NO_3^- concentration also promotes sulfate resynthesis via sulfur-utilizing denitrification (Liu *et al.* 2015a).

In the simultaneous removal of ammonium and sulfate, $n(\text{NH}_4^+\text{-N})/n(\text{SO}_4^{2-})$ gave results of between 0.7 and 2 (Sabumon 2007; Liu *et al.* 2008; Yang *et al.* 2009; Rikmann *et al.* 2012), which made the mechanism of SRAO process unclear and complex (Zhang *et al.* 2013). Many researchers reached different conclusions in studying NO_3^- and NO_2^- , but they were all analysed from the angles of matrix concentrations and product changes. Few studies have focused on microbial community changes. Liu *et al.* (2008) isolated and described a new autotrophic *Planctomyces* bacterium, provisionally named *Anammoxoglobus sulfate*. SRAO was one of many possible metabolic pathways for this particular bacterium, with other pathways including the utilization of various NO_2^- and NO_3^- compounds. Hence, the SRAO process might be a more common metabolic pathway in nature and therefore may not be restricted to a few genera of bacteria.

This work adopts a self-designed anaerobic biological reactor, using ammonium and sulfate as the substrate to start SRAO, to explore wastewater treatment performance and the effect of various environmental factors. The end products of the SRAO reaction were tested, and the microbial

community was analysed to explain the mechanism. Moreover, the N/S ratio was explored to describe the structure of the products. A determination and observation of the SRAO process was carried out for the comprehensive treatment of wastewaters containing high levels of ammonium and sulfate.

MATERIALS AND METHODS

Reactor

As illustrated in Figure 1, a self-designed circulating flow reactor was used in this study. The reactor contained a round column made of plexiglas, which were 80 cm in height and 3 L in volume. Non-woven fabrics (100 cm × 12 cm) were fixed on the right side of the reactor to enhance microbial growth. On the left side, there was an axial agitator rotating at 120 rpm, installed to mix the substrates evenly. To

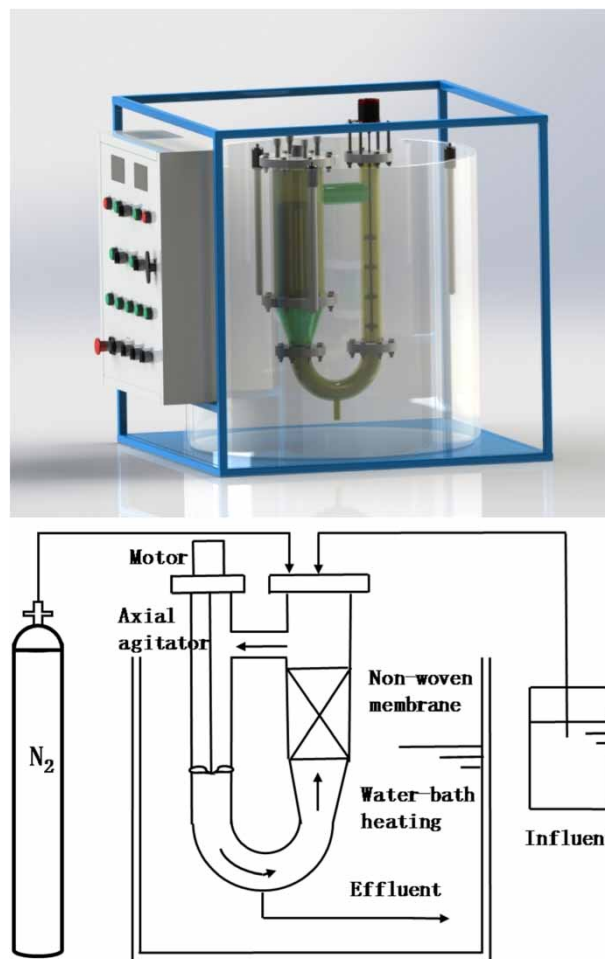


Figure 1 | Scheme of the reactor performing the SRAO process.

maintain anaerobic conditions, nitrogen was flushed into the reactor for 10 min before every test. The reactor was kept at 30 ± 1 °C using a water bath and was covered by a black cloth to protect the bacteria from light and algal growth (Liu *et al.* 2012). The inlet was at the top of the reactor and was used to introduce influent into the tank. The outlet of the silicone tube was located at the bottom of the reactor, and discharge was released from the outlet.

The reactor was seeded with 0.8 L mixed sludge, which consisted of 0.2 L anaerobic granular sludge that was obtained from a municipal wastewater plant and 0.6 L denitrification sludge from a continuous stirred-tank reactor (CSTR). The volatile suspended solids (VSS) of the seed sludge was $3.0 \text{ g}\cdot\text{L}^{-1}$.

Synthetic wastewater

Inorganic synthetic wastewater including ammonium and sulfate was the main source for the microorganisms as well as trace elements that were introduced as the influent to the reactor. Ammonium and sulfate were added at a molar ratio of 2:1 and 4:1, respectively, in the form of $(\text{NH}_4)_2\text{SO}_4$ and NH_4Cl , respectively. The pH of the reactor was maintained at 8.1–8.6 by adding Na_2CO_3 and KHCO_3 , which also served as the bicarbonate source. The composition of the mineral medium was based on that of Yuan *et al.* (2013).

Chemical analysis

$\text{NH}_4^+\text{-N}$, SO_4^{2-} , $\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$ were measured using an ion chromatograph (ICS-2000, DIONEX). The pH was measured with a pH meter (FE28, METTLER). The concentration of sulfide, including HS^- , S^{2-} and $\text{H}_2\text{S}_{(\text{aq})}$, was measured by the methylene blue method. All liquid samples were filtered through a $0.45 \mu\text{m}$ membrane before analysis.

Microbial community structure analysis

The activated sludge samples were taken from the reactor by using centrifuge tubes (10 mL) on days 62 and 187 and were stored at -20 °C. A total DNA extraction ($2 \mu\text{L}$) from the samples was performed using a PowerSoil DNA Isolation Kit (MoBio, USA) according to the instructions (Zhang *et al.* 2009). The quality of the extract DNA was examined by electrophoresis using a 1% agarose gel, and the concentration was tested with a UV-V is spectrophotometer (NanoDrop 2000, USA). The V3-V5 region of the 16S rRNA gene was amplified using the bacterial primers 515F (GTGCCAGCMGCCGCGG) and 907R (CCGTC AATTCMTTTRAGTTT), and polymerase

chain reaction (PCR) amplification was performed according to Chen *et al.* (2010). The purified amplicon was quantified by a QuantiFluor-ST Fluorometer (Promega, USA) and the PCR results were analysed on an Illumina MiSeq platform at Major Bio-Pharm Technology Co, Ltd (Shanghai, China). Trimmomatic and FLASH methods were used for the sequencing data analysis.

RESULTS AND DISCUSSION

Performance of the reactor

The concentration change of each matrix during the operation of the reactor is shown in Figure 2. The seed sludge was fed with synthetic wastewater at an $\text{NH}_4^+\text{-N}$ concentration of $50.0 \text{ mg}\cdot\text{L}^{-1}$, an $\text{NH}_4^+\text{-N}$ to SO_4^{2-}S molar ratio (N/S) of 2, a hydraulic retention time (HRT) of 48 hours, a pH of 8.1–8.6, and a temperature of 30 ± 1 °C for the first 26 days, when some NH_4^+ and SO_4^{2-} were converted and some heterotrophic microorganisms began to die due to the difference in environmental conditions. The removal of NH_4^+ and SO_4^{2-} was clearly observed as the experiment continued. After 62 days, the HRT was decreased to 24 hours and the concentration of $\text{NH}_4^+\text{-N}$ was gradually increased to $120.0 \text{ mg}\cdot\text{L}^{-1}$ to maintain an N/S of 2. The removal efficiencies of $\text{NH}_4^+\text{-N}$ and SO_4^{2-}S were up to 92.0% and 30.5%, respectively, and the minimum concentrations achieved in the effluent were $8.4 \text{ mg}\cdot\text{L}^{-1}$ and $67.3 \text{ mg}\cdot\text{L}^{-1}$, respectively. NO_2^- and NO_3^- could be observed in the effluent at the average concentrations of $4.5 \text{ mg}\cdot\text{L}^{-1}$ and $9.6 \text{ mg}\cdot\text{L}^{-1}$, respectively. In this reactor, after being inoculated with inorganic synthetic wastewater and flushed with nitrogen before testing, the NO_2^- and NO_3^- were excluded during the nitrification process; thus, only SO_4^{2-} acted as an electron donor to NH_4^+ . From days 188 to 213, the influent $\text{NH}_4^+\text{-N}$ concentration was maintained at $180.0 \text{ mg}\cdot\text{L}^{-1}$ and the SO_4^{2-}S concentration was $360 \text{ mg}\cdot\text{L}^{-1}$. A clear decline in the NH_4^+ and SO_4^{2-} removal efficiencies appeared. The $\text{NH}_4^+\text{-N}$ removal efficiency in this period decreased from 59.2% to 23.4% and was stable at approximately 20% during the rest of the experiment. The SO_4^{2-}S in the effluent increased from approximately $66.7 \text{ mg}\cdot\text{L}^{-1}$ to $111.5 \text{ mg}\cdot\text{L}^{-1}$, and then to $269.8 \text{ mg}\cdot\text{L}^{-1}$ after increasing the influent SO_4^{2-} concentration. At the end of the experiment, SO_4^{2-} was hardly converted. During the whole operation, sulfide from the effluent was detected only at the early stage. As a result, sulfate-reducing bacteria used the remaining organics to reduce SO_4^{2-} in the reactor.

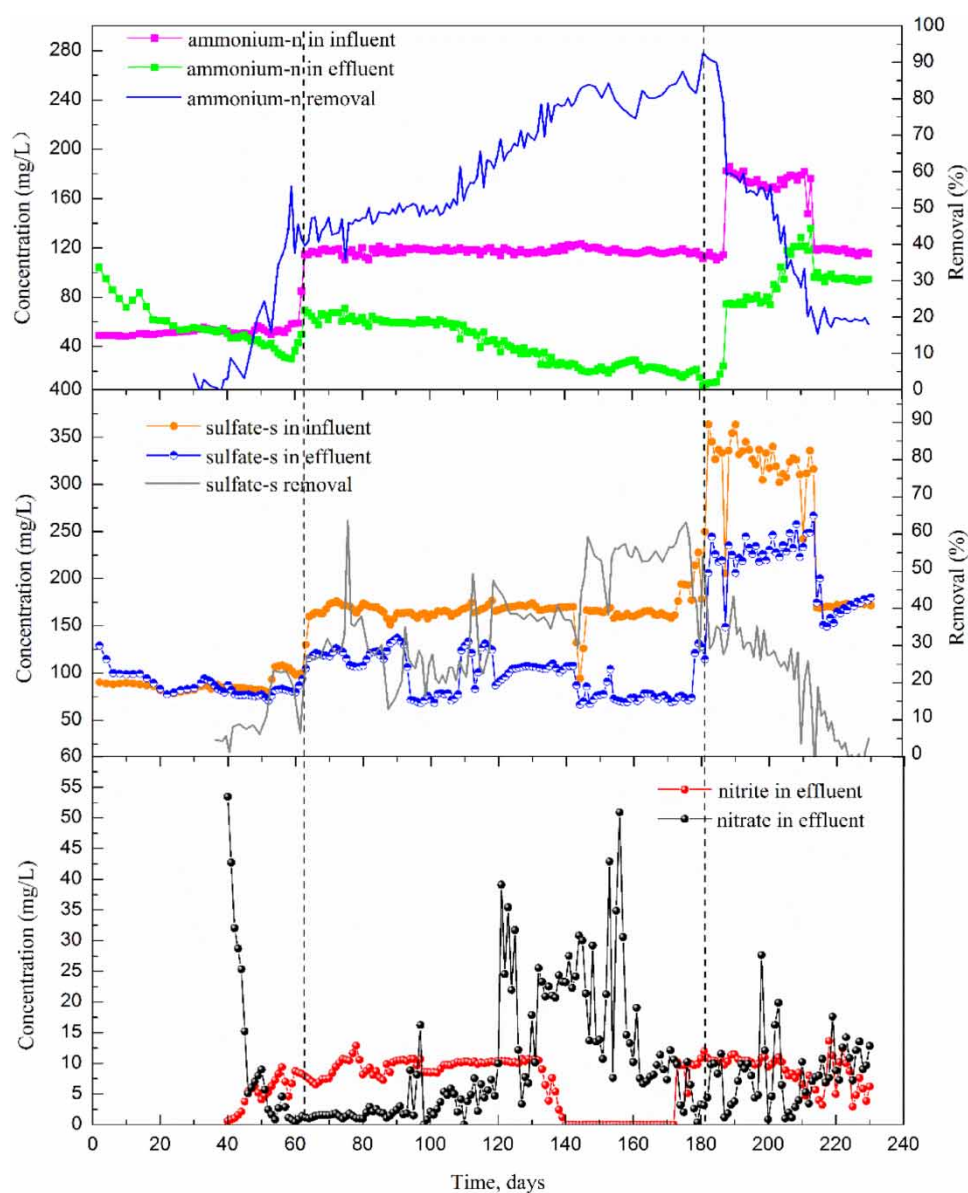


Figure 2 | Influent and effluent $\text{NH}_4^+\text{-N}$, SO_4^{2-}S , NO_2^- and NO_3^- concentrations, $\text{NH}_4^+\text{-N}$ and SO_4^{2-}S removal efficiency.

The sludge was observed to change from black to yellow in the reactor, as compared to the small amount of yellow granular effluent (Figure 3). We determined whether the yellow matter was S^0 by using organic solvents as referenced in Liu *et al.* (2015b). The result showed that S^0 was produced during the process.

Impacts of the ammonium to sulfate ratio

At an HRT of 24 hours and with N/S ratios of 2:1 and 4:1, three levels of $\text{NH}_4^+\text{-N}$ concentrations were investigated in the reactor (Figure 4(a) and 4(b)). At influent concentration

of approximately $118 \text{ mg}\cdot\text{L}^{-1}$, the $\text{NH}_4^+\text{-N}$ removal efficiency at an N/S of 2:1 was 56.4%, which was lower than that at an N/S of 4:1. The nitrogen conversion was 44.8% when the N/S ratio was 4:1, which was also higher than at the N/S ratio of 2:1. However, approximately 16.7% of NO_2^- was produced when the N/S ratio was 2:1. When the N/S ratio was 4:1, the NO_2^- yield was 22.6% and the effluent concentration was 10.46 mg/L , which might be toxic to microorganisms in the reactor. At an influent $\text{NH}_4^+\text{-N}$ concentration of approximately $178 \text{ mg}\cdot\text{L}^{-1}$, a regular pattern that improved the influent N/S ratio enhanced the $\text{NH}_4^+\text{-N}$ removal efficiency and nitrogen



Figure 3 | Colour change of the sludge. Please refer to the online version of this paper to see this figure in colour: <http://dx.doi.org/10.2166/wst.2019.027>.

conversion but accumulated more NO_2^- . When the N/S ratio was 2:1, the NO_3^- product was approximately 7.2%, although the product was far more than when the N/S was 4.

In contrast with these two situations, when the N/S of the influent was improved, the $\text{NH}_4^+\text{-N}$ in the conversion ratio was higher and the NO_2^- formation was higher. However, the NO_3^- formation was lower. The scenario described above also applied to high levels of substrates.

According to Figure 4(c), there was no apparent N/S permanent conversion ratio, which was between 2.1 and 12.9, meaning that the sulfate-reducing anammox process was a multi-step reaction.

Microbial community structure

High-throughput 16S rRNA gene sequencing technology was used to identify the microbial communities at influent $\text{NH}_4^+\text{-N}$ concentrations of $50 \text{ mg}\cdot\text{L}^{-1}$ and $120 \text{ mg}\cdot\text{L}^{-1}$. The phylogenetic relationship and classification of the bacterial 16S rRNA sequence from the sludge samples extracted on days 62 and 187 with relative abundances above 1% are shown by phylum in Figure 5. The results showed that *Proteobacteria* (39.9%, 30.9%) were the most abundant, followed by *Chloroflexi* (11.1%, 16.1%), *Bacteroidetes* (10.4%, 13.9%), *Chlorobi* (7.8%, 14.5), *Acidobacteria* (5.3%, 5.2%) and *Planctomycetes* (3.2%, 4.4%) in the two samples. The conclusion was that the dominant bacterial communities were comparatively stable, but their relative abundances changed over the operation of the reactor. Relative abundance increases in *Acidobacteria*, *Nitrospirae*, *Armatimonadetes* and *Proteobacteria* observed in the samples could indicate that bacteria played a more important role in high

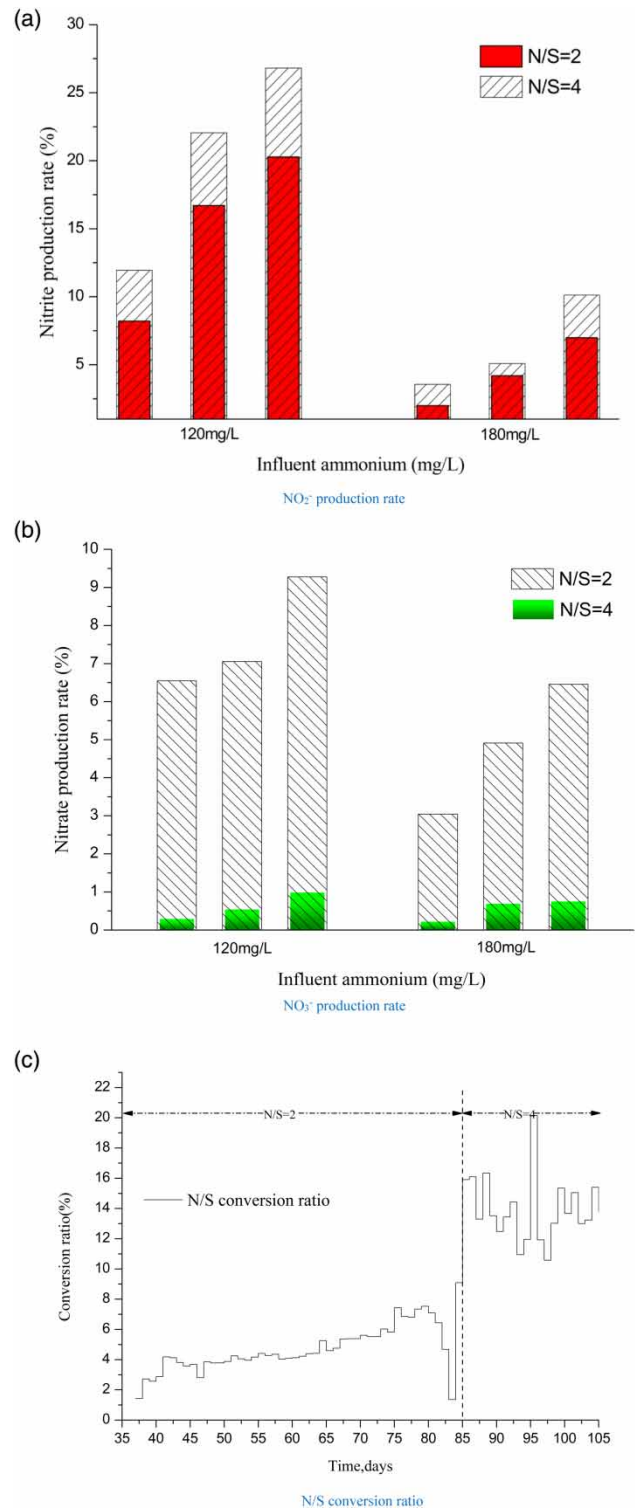


Figure 4 | The influent N/S ratio effect. (a) NO_2^- production rate, (b) NO_3^- production rate, (c) N/S conversion ratio.

ammonium and sulfate environments than in low ammonium and sulfate environments. *Acidobacteria*

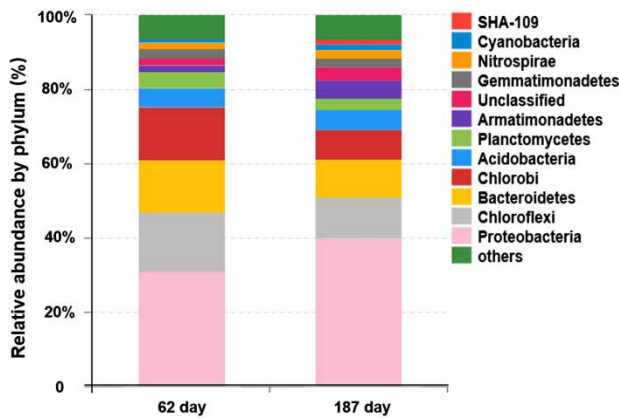


Figure 5 | Microbial community structure of sludge on days 62 and 187 at the phylum level. Please refer to the online version of this paper to see this figure in colour: <http://dx.doi.org/10.2166/wst.2019.027>.

could use multiple electron acceptors to reduce iron or single carbon compounds. *Nitrospirae* was previously identified as the dominant functional bacteria in several anammox reactors used for nitrogen removal (Zhang et al. 2013). *Proteobacteria* had a more versatile metabolism. The large proportion of *Proteobacteria* and *Armatimonadetes* present in this reactor contributed to the partial nitrification process and NO_2^- accumulation (Liu et al. 2012; Mi et al. 2017). Thus, the SRAO process could be a more common metabolic pathway in nature rather than just being restricted to a few genera of bacteria.

Moreover, no traditional sulfate-reducing bacteria, such as *Sulfurimonas* (Takai 2004) and *Desulfobacter*, were found in this reactor, thus indicating that sulphur removal was carried out by other bacteria.

Bacteria with denitrifying-function

Sludge samples extracted from the reactor were used to identify the microbial community structures when the 62 and 187 day samples contained ammonium oxidation bacteria, denitrifying bacteria and anaerobic ammonium oxidation bacteria (Table 1). The results provided a comprehensive and deeper insight into the microbial community compositions in the different influent concentrations, which could be valuable for studying NO_3^- and NO_2^- sources. The increase in NH_4^+ -N oxidizing bacteria (AOB), known as a type of chemolithotroph that could convert NH_4^+ -N to NO_2^- , included *Nitrosomonas* and *Nitrosomonadaceae*, which were also responsible for part of the NO_2^- produced by the ammonium-oxidizing process. Representing a miniscule portion of the NO_2^- oxidizing bacteria (NOB), only *Nitrospira* was detected in the sludge samples. NO_2^- can be oxidized to NO_3^- to prove that there is a certain amount of NO_3^- in the reactor. Since NH_4^+ -N had a relative high removal efficiency and there was a small amount of AOB, there must have been a novel way to consume ammonium during the process.

The potential functional bacteria that oxidized ammonium belong to *Planctomycetes* including *Pla4_lineage*, *SM1A02*, and *Planctomycetes*, whose proportion increased from 1.5% to 3.0%. The bacteria of *Planctomycetes* certainly constituted a new genus in the anammox line, but there is no related research that analyses their biochemical performance.

Approximately 12.4% of the denitrifying bacteria were found in sludge sample 2, which showed a 1.2% increase compared to sample 1. The results showed that part of nitrogen

Table 1 | Quantity of nitrogen removal functional bacteria at the level of phylum and genus

Bacteria	Phylum	Genus	Sample 1 (%) (N/S = 2)	Sample 2 (%) (N/S = 4)
Ammonium-oxidizing bacteria	<i>Proteobacteria</i>	<i>Nitrosomonas</i>	1.2	1.6
		<i>Nitrosomonadaceae</i>	1.0	1.0
		Total	2.2	2.6
Denitrifying bacteria	<i>Proteobacteria</i>	<i>Denitratisoma</i>	2.7	4.0
		<i>Nitrosomonas</i>	0.9	0.6
		<i>Thiobacillus</i>	2.0	1.6
		<i>Rhodanobacter</i>	5.6	6.2
		Total	11.2	12.4
Anaerobic ammonium-oxidating bacteria	<i>Planctomycetes</i>	<i>Pla4_lineage</i>	0.7	1.7
		<i>SM1A02</i>	0.3	0.6
		<i>Planctomycetaceae</i>	0.5	0.7
		Total	1.5	3.0

was converted by the nitrification-denitrification process. Traditional denitrification occurs simultaneously with SRAO, which leads to an immense boost in SRAO functional bacteria and thus improves the removal efficiency of ammonium and sulfate.

CONCLUSION

Simultaneous reduction of ammonium and sulfate was achieved in a self-designed reactor; the removal efficiency of $\text{NH}_4^+\text{-N}$ was 92.0% and that of $\text{SO}_4^{2-}\text{-S}$ was 59.2%. NO_2^- and NO_3^- were found in substantial amounts in the effluent of the reactor. A higher influent N/S ratio could increase the N/S conversion ratio and accelerate NO_2^- and nitrogen production. The sludge acclimated in the reactor had *Proteobacteria*, *Chloroflexi*, *Bacteroidetes*, *Chlorobi*, *Acidobacteria* and *Planctomycetes* after the reactor had run for 187 days. Three kinds of *Planctomycetes* were found: *Pla4_lineage*, *SM1A02*, *Planctomycetaceae*; all were found to have increased their percentages compared to the initial amount. Since denitrifying bacteria and AOB had also acclimated after the operation of the reactor, traditional denitrification was observed to be occurring simultaneously with SRAO, which offers a substantial biotechnological potential for the complete removal of ammonium and sulfate, and should therefore be used more widely. Future work will focus on isolating the functional bacteria and avoiding the production of excess nitrate and nitrite.

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