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Interaction of organic carbon, reduced sulphur and nitrate in anaerobic baffled reactor for fresh leachate treatment

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ABSTRACT

Interaction of organic carbon, reduced sulphur and nitrate was examined using anaerobic baffled reactor for fresh leachate treatment by supplementing nitrate and/or sulphide to compartment 3. Nitrate was removed completely throughout the study mostly via denitrification (>80%) without nitrite accumulation. Besides carbon source, various reduced sulphur (e.g. sulphide, elemental sulphur and organic sulphur) could be involved in the nitrate reduction process via sulphur-based autotrophic denitrification when dissolved organic carbon/nitrate ratio decreased below 1.6. High sulphide concentration not only stimulated autotrophic denitrification, but it also inhibited heterotrophic denitrification, resulting in a shift (11–20%) from heterotrophic denitrification to dissimilatory nitrate reduction to ammonia. High-throughput 16S rRNA gene sequencing analysis further confirmed that sulphur-oxidizing nitrate-reducing bacteria were stimulated with increase in the proportion of bacterial population from 18.6% to 27.2% by high sulphide concentration, meanwhile, heterotrophic nitrate-reducing bacteria and fermentative bacteria were inhibited with 25.5% and 66.6% decrease in the bacterial population.

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KEYWORDS
Leachate; sulphur-based autotrophic denitrification; heterotrophic denitrification; DNRA; anaerobic reactor

1. Introduction

In developed countries, the amount of leachate produced in municipal solid waste incineration (MSWI) plant is relatively low due to characteristically low moisture content of municipal solid waste (MSW). The leachate is usually sprayed into the incinerator furnace, where the MSW has a high heating value (8400–17,000 kJ/kg). [1,2]. However, in China this is not feasible in most of the incineration plants due to large proportion of moisture-rich (60–70%) food wastes in MSW resulting in low heating value (4000–7000 kJ/kg) which generates a considerable amount of leachate before combustion.[3]

Leachate generated before incineration contains high concentrations of chemical oxygen demand (COD), ammonium ($NH_4^+ - N$), heavy metals, various sulphur species (e.g., sulphide, sulphate, elemental sulphur and organic sulphur) and other toxic compounds that pose a serious threat to the environment. Thus, treatment of fresh leachate from MSWI plants becomes necessary to meet the environmental discharge standards. Although landfill leachate treatment processes have been reported, and widely applied in different scales.[3–7] Only a few studies have been conducted to treat the fresh leachate before incineration in MSWI plants with an even higher COD than the landfill leachate.[8–10]

One approach to efficiently remove both carbon and nitrogen from leachate is to combine denitrification and anaerobic digestion in an integrated process coupled with a nitrification stage,[9] which removed up to 97.7% COD and 94.6% ammonia. Our previous study also examined the efficiency of an anaerobic baffled reactor-aerobic sludge (ABR-AS) system with nitrified effluent recirculation to compartment 3.[11]. Using MSWI leachate at an organic loading rate of 2.2–3.2 kg SCOD/(m^3·d) and a nitrogen loading rate (NLR) of 0.12–0.22 kg total nitrogen (TN)/(m^3·d), COD and TN removal efficiencies of 95% and 63%, respectively, were achieved. Interestingly, with low sulphide and sulphate level in the influent, high concentration of sulphate (200 mg S/L) was present in the effluent from ABR with recirculation; but no sulphate was detected in the effluent from ABR without recirculation. One possible reason for such result is that other reduced sulphur generated in the anaerobic environment was oxidized to sulphate through nitrate reduction.

Several studies examined the transformation of carbon and reduced sulphur compounds by microbial cultures in which nitrate served as an electron acceptor.[12–15] Based on stoichiometry, the oxidation of reduced sulphur by lithotrophic bacteria can lead to

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the formation of either elemental sulphur (S^0) or sulphate.

Since most of the studies reported so far involved batch assays using synthetic media such as acetate, butyrate, or dextrin/peptone, the mechanism of electron flow coupled with sulphur, carbon and nitrogen cycles, especially reduced sulphur oxidation in association with nitrate reduction in real scenarios, needs further investigation. Thus, it is important to understand the interactions of N and S cycles in continuously operating system using complex substrate such as leachate. The complex characteristics of leachate with various forms of sulphur, nitrogen and organic carbon compounds make it quite challenging to investigate the interactions of nitrate reduction pathways and reduced sulphur compounds.

Based on above rationale, the goal of this study was to examine the interaction of organic carbon, reduced sulphur and nitrate in a leachate-fed continuous-flow ABR by supplementing nitrate and/or sulphide. The study also investigated the bacterial community using high-throughput sequencing technology.

2. Materials and methods

2.1. Characteristics of the leachate

The leachate was collected from a refuse storage pit at the Lucheng MSWI plant (Jiangsu Province, China). Leachate composition was found to depend on the degree of compaction of the MSW and its composition, storage climate and moisture content. The important characteristics of leachate used in this study are listed in Table 1. The characteristics, however, fluctuated depending on the sampling time. For instance, the leachate used from the start-up stage to Stage II (60 days) had high sulphate than the leachate used in later Stages III–V (56 days), in which there was only a small amount of sulphate. In addition, from the start-up stage to Stage II, the leachate contained certain amount of elemental sulphur but little organic sulphur as evidenced by gas chromatography–mass spectrometry (GC–MS) analysis. And from Stages III to V, both elemental sulphur and high various organic forms of sulphur in the leachate were detected. The leachate was transported to the laboratory in sealed plastic barrels and refrigerated at 4°C before use.

Table 1. Characteristics of leachate obtained from a refuse storage pit of a MSWI plant.

Operating stage	Start-up–II	III–V
In duration of study (Day)	0–60	61–116
pH	6.91 ± 0.08	7.74 ± 0.07
DOC (mg C/L)	6496 ± 280	6951 ± 212
Total nitrogen (mg N/L)	1886 ± 75	2545 ± 52
Ammonia (mg N/L)	1148 ± 103	1767 ± 159
Nitrate (mg N/L)	3.0 ± 2.2	3.5 ± 2.5
Sulfate (mg S/L)	590.7 ± 33.6	38.6 ± 13.4
Sulphide (mg S/L)	0.2 ± 0.1	6.7 ± 4.7

Note: Data are mean value of 20 samples from different days during each stage for each parameter.

2.2. Experimental set-up

The schematic diagram of the laboratory-scale ABR set-up is shown in Figure 1. A 15-L ABR reactor with a working volume of 10 L was fabricated using 10-mm thick transparent plexiglass with internal dimensions of 500 mm length × 100 mm width × 300 mm height. The ABR was divided into four compartments with an approximate volume ratio of 3:2:2:3. Vertical high/low baffles of 5-mm thickness were inserted to subdivide each compartment into upflow and downflow chamber. The

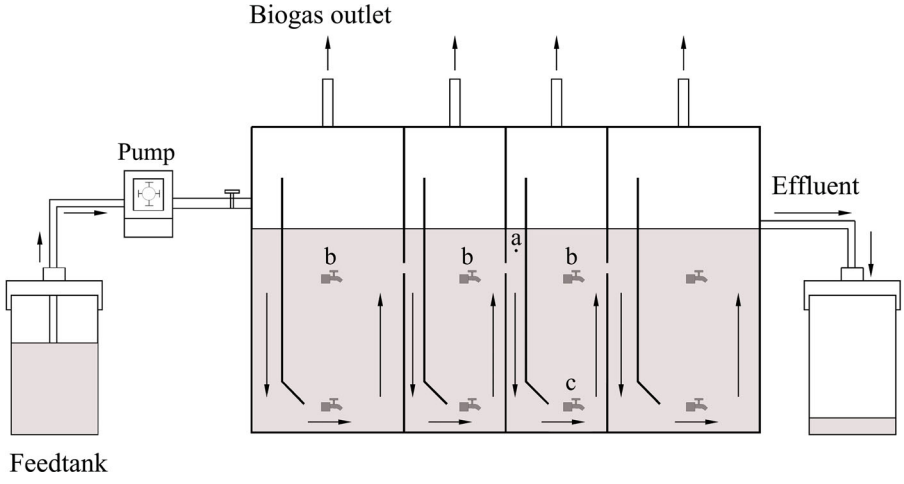

Figure 1. Experimental set-up of ABR: *a*-nitrate and/or sulphide dosing point; *b*-supernatant liquor sampling point; *c*-sludge sampling point.

lower portion of the baffles was bent 20-mm above the reactor bottom at an angle of 45° to facilitate the liquid flow towards the centre of the upflow chamber for effective mixing and contact between the substrate and the biomass. The ABR was enclosed in a water jacket to maintain a temperature of 35 ± 1°C throughout the study. Samples were collected from sampling ports located about 50 mm from the top of each compartment. The biomass (sludge) sampling ports were located 30 mm from the bottom of each compartment.

2.3. Reactor inoculation and operation

The ABR used in this study had been operated with nitrified effluent recycled to compartment 3 for nearly 350 days prior to the start of this study. The reactor was initially seeded with anaerobic granular sludge with a volatile suspended solids (VSSs) content of 37.6 g/L, collected from a full-scale up-flow anaerobic sludge bioreactor treating leachate. After inoculation, the reactor was sealed, and the headspace was purged with oxygen-free argon gas to strip off oxygen from the system. The ABR was operated at a hydraulic retention time (HRT) of 10 days. The operating conditions are summarized in Table 2. Nitrate was supplemented as potassium nitrate (500 mg N/L) continuously at the top of the downflow chamber of compartment 3 from the start-up stage until Stage V. Sulphide was supplemented as sodium sulphide (227 mg S/L) to the same compartment during Stages II and IV. At Stage V, the ABR was operated without nitrate and/or sulphide supplementation.

2.4. Chemical analysis

Liquor samples were collected every alternate day from the ABR inlet and from the four compartments (with the fourth compartment being the ABR outlet) for chemical analysis. Sulphide analysis was performed immediately after filtering through a 0.45-μm filter to minimize oxidation loss. The sulphide analysis was conducted using the methylene blue method.[16] Briefly, the samples were diluted with 6.9 mL of deionized water and reacted with 2 mL of zinc acetate, 1 mL of *N*,*N*-dimethyl-p-phenylenediamine dihydrochloride and 0.1 mL of ammonium ferric sulfate [$Fe(NH_4)(SO_4)_2{\cdot}12H_2O$]. Absorbance was then measured spectrophotometrically at 665 nm (Unico2100, USA). The liquid samples for the analysis of other parameters were treated with 0.2 mL of 2 M zinc chloride to precipitate the remaining sulphide and then centrifuged at 11,000×g for 10 min (Thermal Multifuge X1R, USA). The resulting supernatants were filtered through 0.22-μm filters and the filtrate was stored in 4°C before analysis. The elemental and organic sulphurs were examined by GC–MS (Shimadzu QP 2010, Japan) after liquid–liquid extraction with carbon disulphide (CS_2) as an extraction agent. Ammonium, nitrate, nitrite, thiosulphate, sulphite and sulphate ion concentrations were determined using Ion Chromatography (Dionex ICS-3000, Sunnyvale, CA, USA) as described in.[11] The dissolved organic carbon (DOC) and soluble TN were determined using a total organic carbon (TOC)/TN analyzer (Shimadzu, TOC-L CPN CN200, Japan) equipped with a platinum catalyst quartz tube. Metals were analysed using an inductively coupled plasma atomic emission spectrometry (ICP-AES, Agilent 720ES, USA). Volatile fatty acids (VFAs) were determined using a gas chromatography unit with a flame ionization detector (GC-FID, Agilent 6890N, USA). The precipitate in compartment 1 was analysed by X-ray diffraction scanning (XRD, RigakuD/max). All conventional parameters such as pH and total solids and VSSs were analysed according to Standard Methods.[17]

The biogas was collected in gas-tight aluminium bags daily from the upper part of each compartment. The biogas volume was measured by a syringe. Biogas composition, especially N_2, CH_4 and CO_2 were measured using a gas chromatography coupled with a thermal conductivity detector (GC-TCD) (Agilent 6890N, USA) using an analytical column (Supelco Hayesp Q, 80/100 mesh). The temperatures of the column, injector and detector were maintained at 50, 120 and 80°C, respectively. The hydrogen sulphide in the biogas was measured based on the capture of the gas formed in a trap solution of $Cd(OH)_2$, formation of methylene blue from *N*,*N*-dimethyl-p-phenylenediamine dihydrochloride and spectrophotometric measurement at 665 nm.

2.5. High-throughput 16S rRNA gene sequencing analysis

Sludge (biomass) samples were collected from compartment 3 at days 40 and 60, and kept in plastic sterilized

Table 2. Operating conditions of the ABR.

Operating stage		Start-up	I	II	III	IV	V
Time (Day)		0–25	26–40	41–60	61–80	81–100	101–116
Added into compartment 3	Nitrate(mgN/L)	506.1 ± 12.6	496.8 ± 0.6	497.3 ± 17.2	495.5 ± 4.2	487.3 ± 3.1	–
	Sulphide(mgS/L)	–	–	226.6 ± 12.7	–	227.0 ± 18.2	–
DOC/nitrate of compartment 3		2.52	1.48	1.55	1.54	1.59	–
Sulphide/nitrate of compartment 3		0.15	0.16	0.61	0.05	0.52	–

containers immediately. The containers were completely filled to maintain an anaerobic environment. The containers were transported in a cooler filled with ice blocks within 48 h and stored at –80°C until DNA extraction was carried out.

Total genomic DNA was extracted from each sample using the QIAamp DNA Stool Kit (catalog: 51504, Qiagen, Germany) according to the manufacturer's protocol. Polymerase chain reactions (PCRs) were performed via a Miseq sequencing platform. The V3 and V4 regions of bacterial 16S rRNA genes were amplified using the fusion primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) for the quantification of total bacteria. PCRs were run in triplicate in a 50-μL reaction mixture that consisted of 5 μl 10× PCR buffer, 0.5 μL each deoxynucleoside triphosphate (dNTP, 10 mM each), 10 ng Genomic DNA, 0.5 μL each forward and reverse primer (50 μM Bar-PCR primer F and 50 μM Primer R) and 0.5 μL Platinum® Taq DNA Polymerase (catalog: 10966–018, 5 U/μL, Life Technologies, CA, USA). The amplification programme consisted of an initial denaturation at 94°C for 3 min, followed by 5 cycles of denaturing at 94°C for 30 s, annealing at 45°C for 20 s, and extension at 65°C for 30 s, followed by 20 cycles of denaturing at 94°C for 20 s, annealing at 55°C for 20 s, and extension at 72 °C for 30 s, followed by a final extension at 72°C for 5 min prior to cooling at 10°C. Subsequently, PCR products were excised from a 1.5% (w/v) agarose gel purified by OMEGA Gel Extraction kit (cat: D2500–01, OMEGA, USA) following the manufacture's instruction, and quantified using the Qubitds DNA BR Assay Kit (catalog: Q32850, Life Technologies, CA, USA). The V3–V4 amplicons were pooled in equimolar concentrations and sequenced by Illumina Misequsing pair-end method with a MiSeq Reagent Kit v3 (600 cycle, catalog: MS-102–3001, Illumina, CA, USA). The average length of sequence reads was up to 450 bp.

The resulting reads were analysed using the PRINSEQ software (PRINSEQ-lite 0.19.5). The raw data were filtered with the removal of the joints and low-quality sequences to generate the clean data, followed by trimming the primer sequence from beginning and end of the clean data. FLASH software (v1.2.7) was used to merge the forward and reverse reads when a correct overlap was found. Then the tag sequences were classified into different files according to the barcodes of the samples, and removed chimaeric artefacts using UCHIME software with sequences in the SILVA database (http://www.arb-silva.de/) as a template before pre-clustering at 1/150 dissimilarity using the precluster function in Mothur software package to alleviate the per-base error rate of the sequencing platform. 'Clean' reads were clustered into operational taxonomic units (OTUs) in UCLUST software (v1.1.579) at a 97% similarity using the furthest neighbour clustering method.

The alpha diversity of bacterial population in each biomass sample was generated using the MOTHUR programme. The 3% dissimilarity cutoff value was used for assigning an OTU. Good's coverage was calculated as $1–n/N$, where n is the number of singletons (the OTUs with only 1 sequence) and N is the total number of sequences in the sample. In this study, Good's coverage of each individual sample was similar at about 95 ± 1%, indicating that the majority of bacterial populations present in the samples were likely to have been identified.

2.6. Statistical analysis

Differences in the means of chemical variable concentrations in different stages in this study were evaluated by t-test. An analysis of variance (ANOVA) was used to test the significance of results, and differences at the level $p < .05$ were considered to be statistically significant.

3. Results and discussion

3.1. Biochemical reactions occurring in different compartments of the ABR: methane production, nitrate reduction and sulphate reproduction

The influent was fed to the ABR continuously at a HRT of 10 days, with nitrate supplementation of 500 mg N/L to compartment 3 from the start-up stage until Stage V. As shown in Figure 2, the added nitrate was completely removed in compartment 3 without nitrite accumulation (data not shown here) from the start-up stage to Stage II. However, without sulphide supplementation at Stage III, nitrate was partially removed (~65%) in compartment 3 and was completely eliminated in compartment 4. A further addition of sulphide (~227 mg S/L) in Stage IV accelerated the nitrate reduction process in compartment 3. The interaction between nitrate reduction and reduced sulphur is discussed later.

Figure 3 shows the change in DOC concentration with time in different compartments throughout the study period. With the influent DOC concentrations of leachate ranging from 6000 to 7500 mg C/L, the DOC removal efficiency of the ABR was stable at about 92–93% after the start-up stage. Most of the influent DOC (> 80%) was removed in compartment 1 of the ABR, where methane gas was the major component of biogas production (more than 70%) (Table S1 in Supplementary Data), indicating that the first compartment was mainly

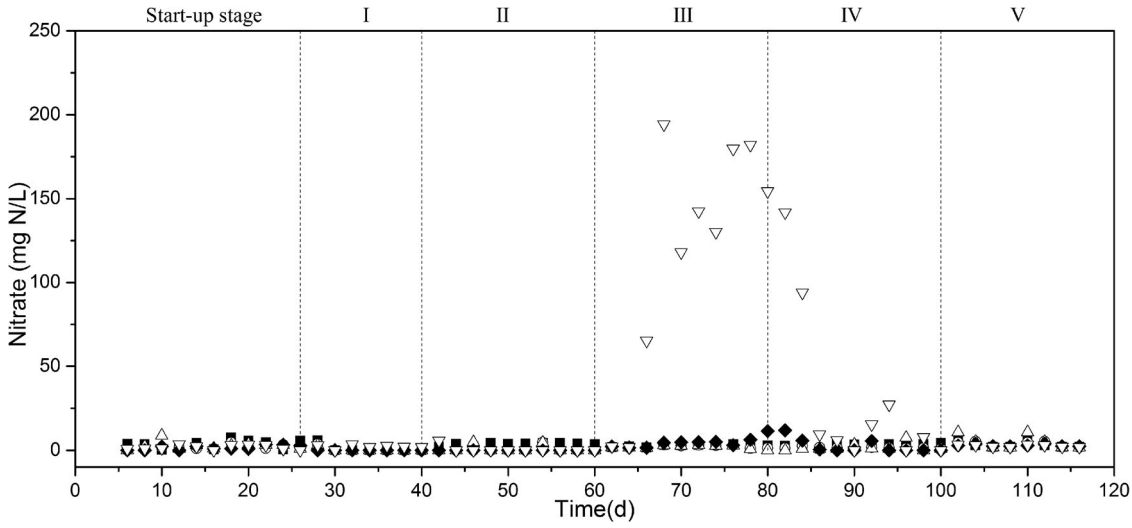

Figure 2. Nitrate profile: ■, influent; ○, compartment 1; △, compartment 2; ▽, compartment 3; ◆, effluent of the ABR.

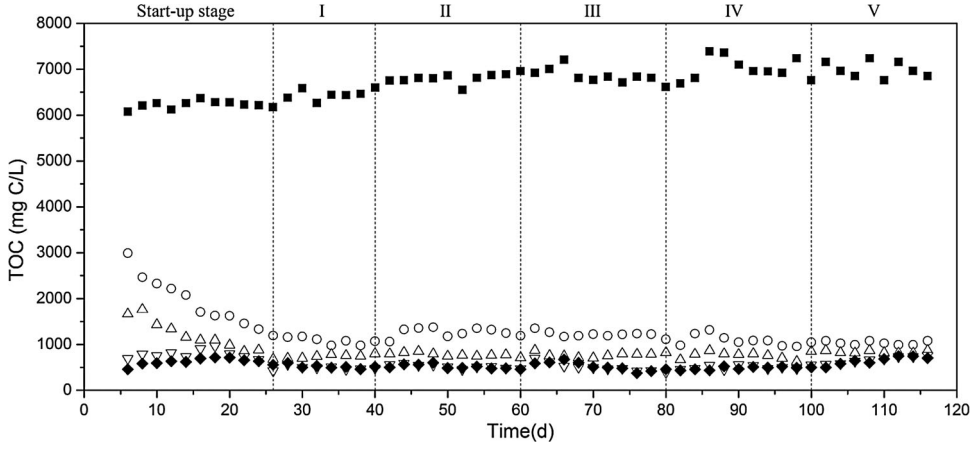

Figure 3. DOC profile: ■, influent; ○, compartment 1; △, compartment 2; ▽, compartment 3; ◆, effluent of the ABR.

associated with methanogenesis of influent organic carbon. DOC removal continued in compartment 2 that resulted in a relatively low DOC/nitrate ratio in compartment 3, which ranged from 1.5 to 1.6 from Stages I to V. The further decrease in DOC concentration in compartments 3 and 4 should be due to residual organic carbon consumption (mainly acetates, as shown in Table S2 in Supplementary Data) for the nitrate reduction process.

Figure 4 shows the change in sulphide and sulphate concentration with time in different compartments. As shown in the figure, from the start-up stage to Stage II, the sulphide concentration increased to about 80 mg S/L in compartment 1 in spite of low sulphide level (0.2 mg S/L) in the influent. The occurrence of sulphate reduction in the anaerobic environment contributed to the increased sulphide level. However, it is important to note that when the sulphate in the influent was about 600 mg S/L, the total amount of sulphur (sulphate and sulphide) detected in compartment 1 was no more than 100 mg S/L. As significant decline (>90%) in metal concentrations was observed in the effluent of compartment 1 (Table S3 in Supplementary Data), sulphate could be precipitated as $CaSO_4$ and some of the generated sulphide (with a theoretical value of 232.3 ± 5.1 S mg/L) was likely precipitated as metal-bound sulphide (mainly FeS, ZnS and CuS) in compartment 1, thus resulting in the loss of sulphur. At the same time, Ca was also precipitated as $CaCO_3$ as evident by XRD analysis, by consumption of generated CO_2 during methanogenesis, leaving a large proportion of methane gas (70–80%) in biogas production.

In the start-up stage, sulphide was not removed initially in compartment 3 (abundant of organic matter with a DOC/nitrate ratio >1.6) while sulphide was removed in compartment 4 (with a DOC/nitrate ratio

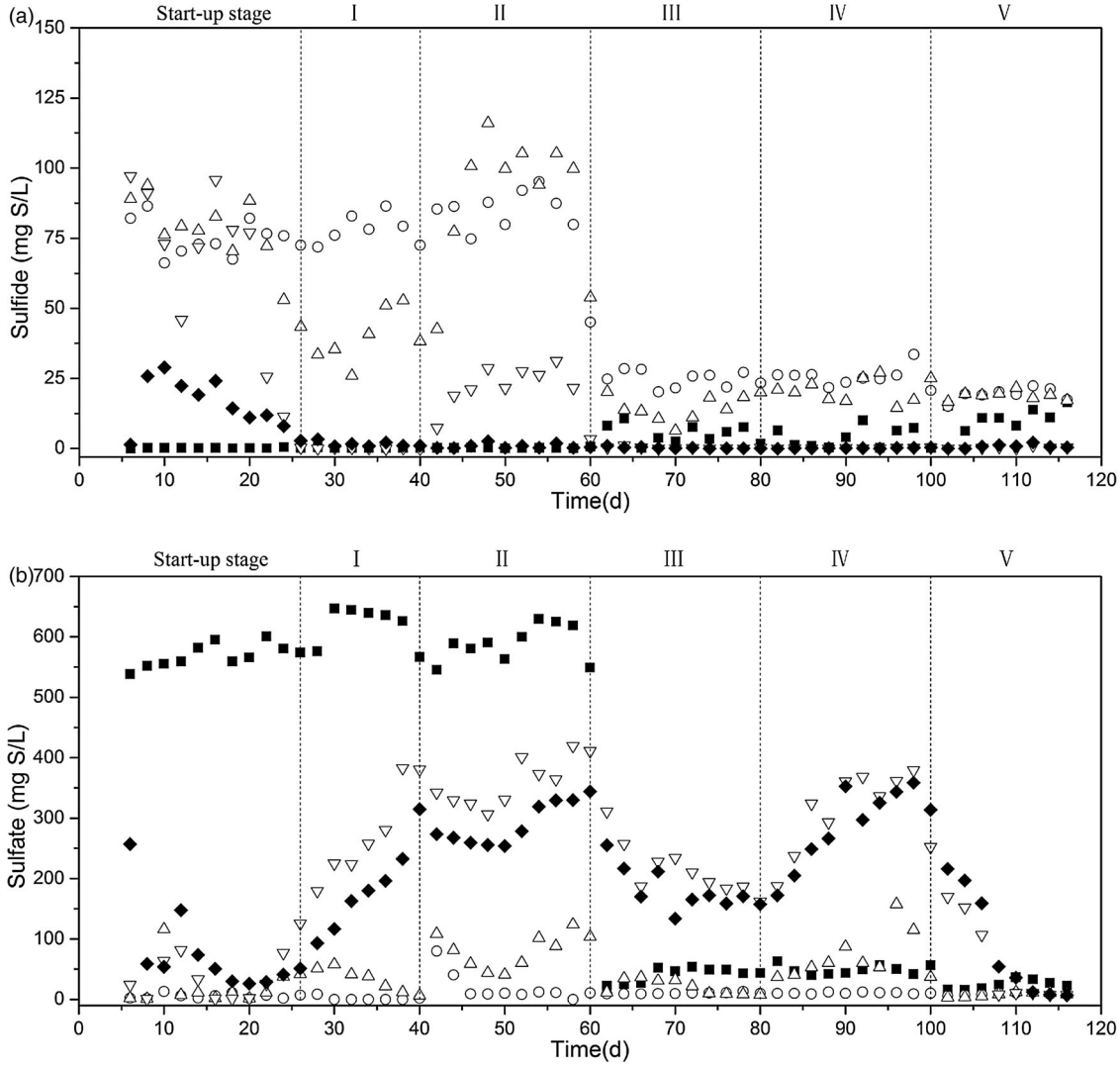

Figure 4. (a) Sulphide and (b) sulphate profiles: ■, influent; ○, compartment 1; △, compartment 2; ▽, compartment 3; ◆, effluent of the ABR.

of about 1.5). In addition, sulphide oxidization with sulphate generation in compartment 3 was observed once the DOC/nitrate ratio in compartment 3 decreased below 1.6 in Stage I, suggesting that sulphide utilization was dependent on the DOC/nitrate ratio. That is, with sufficient carbon source in the compartment, sulphide-oxidizing bacteria were unlikely dominated the system while carbon-limiting conditions stimulated the sulphide-oxidizing bacteria to use sulphide as an alternative electron donor in this continuous study. However, in other batch studies using butyrate as a substrate, [13,14], sulphide utilization was found to be independent of the C/N ratio. It is also important to note that sulphide was not removed after compartment 3 until propionate was completely consumed (Table S2 in Supplementary Data). It was speculated that propionate might have inhibited sulphide-oxidizing bacteria. Additionally, under steady-state conditions in Stage I, in which sulphide and sulphate concentrations in the effluent of compartment 1 were below 80 and 10 mg S/L, respectively, sulphide concentration in the ABR effluent was below detectable limit while sulphate concentration increased to over 200 mg S/L. The leachate contained certain amount of elemental sulphur from the start-up stage to Stage II as evident by the GC–MS analysis. Thus, besides sulphide, the elemental sulphur was likely involved in nitrate reduction, as an electron donor. Metal-bound sulphides were not responsible for nitrate reduction because most of metal sulphides precipitated in compartment 1 and no increase in metal concentration was observed in compartment 3. To confirm nitrate reduction using reduced sulphur as an electron donor, reduced sulphur in the form of sodium sulphide (~227 mg S/L) was added to compartment 3 in Stage II. The consumption of sulphide associated with nitrate reduction further supported the use of sulphide as an

electron donor during nitrate reduction in the system. However, the amount of sulphate generated (with complete oxidation of 227 mg S/L sulphide) was only about 100 mg S/L, demonstrating that no more than half of the added sulphide was oxidized to sulphate and the remaining sulphide was only partially oxidized into elemental sulphur. It was also observed by Reyes-Avila et al.[12] that sulphide oxidation proceeded in two steps: sulphide was first oxidized to elemental sulphur, then oxidized further to sulphate in the second step. And the first step was faster than the second one. Therefore, it was speculated that the sulphate formation from intermediates (elemental sulphur) is the bottleneck of the autotrophic denitrification. The complete oxidation of sulphide to sulphate was generated only when there are enough electron acceptors. In addition, during the Stages I–II, a small amount of sulphate (about 50 mg S/L) was reduced in compartment 4 where anaerobic conditions resumed.

In this study, from Stage III, since the characteristics of influent changed, the sulphide and sulphate concentrations in the influent leachate were lower (about 0.2 and 40 mg S/L, respectively) than that of the influent prior to Stage III (Figure 4(a) and (b)). A small amount of sulphide (~25 mg S/L), excluding the sulphur loss due to precipitation as mentioned before, was generated in compartment 1. However, without the addition of sulphide in Stage III, the sulphate concentration in the effluent of compartment 3 remained fairly constant at about 200 mg S/L, suggesting the oxidation of the reduced sulphur, both elemental sulphur and various organic forms of sulphur in the leachate (e.g. methyl mercaptan, dimethyl sulphide, dimethyl disulphide and dimethyl trisulphide) which were detected (during the GC–MS analysis) in the nitrate reduction process, thus resulting in increase in sulphate concentration. A further addition of sulphide (~227 mg S/L) in Stage IV accelerated the nitrate reduction process in compartment 3, where nitrate was only partially removed in Stage III (Figure 2). This also suggests that the added sulphide was consumed in nitrate reduction, thereby resulting in increase in sulphate generation and elemental sulphur precipitation. When neither nitrate nor sulphide was added to compartment 3 in Stage V, the sulphate concentration in the ABR effluent dropped subsequently, showing that sulphur oxidation did not occur without nitrate addition, which further indicated that reduced sulphur compounds were consumed in nitrate reduction. Several researchers isolated denitrifying bacterial strains that anaerobically oxidized inorganic sulphur compounds, such as sulphide, sulphur and thiosulfate, by using nitrate as an electron acceptor.[18–20] In this study, the end products were SO_4^{2-}, S^0, N_2, CO_2 and CH_4 (Table S1 in Supplementary Data), which suggests the co-existence of autotrophic denitrification and methanogenesis in the anaerobic reactor.

3.2. Nitrogen and sulphur transformation in the ABR

Table 3 shows the data used to calculate nitrogen transformations in the ABR. The proportion of denitrification in nitrate reduction pathway was calculated by the generated nitrogen gas. As shown in the table, nitrate was removed nearly 100%, without nitrite accumulation throughout the reactor. It has been found that in the anaerobic matrix with nitrate amendment, the ratio of DNRA to denitrification was proportional to the ratio of COD/nitrate.[11]. However, in this study the low DOC/nitrate ratio of about 1.5 leads to denitrification as the main nitrate reduction pathway (>80%) at all sulphide/nitrate ratios tested. The sulphide/nitrate ratio was also shown to influence nitrate reduction pathways. As presented in Table 3, when only nitrate was added (Stages I and III, with sulphide/nitrate ratios <0.2), about 94% (Stage I) and 97% (Stage III) of the nitrate was consumed for denitrification while only 6% (Stage I) and 3% (Stage III) for dissimilatory nitrate reduction to ammonia (DNRA). The addition of sulphide (Stages II and IV, with sulphide/nitrate ratios >0.5) caused a small (11–20%) shift in the nitrate reduction pathway from denitrification to DNRA, leaving 80% and 89% in Stages II and IV, respectively, for denitrification pathway, which provided further evidence of the important role that sulphide/nitrate ratios played in nitrate reduction pathway. Sulphide-mediated stimulation of DNRA has also been reported in freshwater sediments and in butyrate synthetic wastewater.[13,14,21] Stately, it is electron donors/nitrate ratios that determine nitrate utilization pathway.

Table 4 shows the conversion of sulphide and the generation of sulphate in the compartments 3–4 of the bioreactor, without taking into account the loss of hydrogen sulphide (not detected). As intermediate products, thiosulphate and sulphite were ignored in sulphate reduction/sulphide oxidation processes in this study, with their detection at negligible levels in the ABR. As evident from the table, the sulphide removal efficiency was as high as 99% at different sulphide/nitrate ratios. Firstly this could be attributed to the precipitation of part of sulphide as metal sulphide (mainly FeS, ZnS and CuS) present in the leachate; secondly, some denitrifying bacteria, as discussed in Section 3.1, are lithoautotrophic and use reduced sulphur compounds such as thiosulphate, sulphite, S^0 or sulphide as electron donors for nitrate reduction at low C/N ratios.[22–24] The overall biochemical reaction of lithoautotrophic denitrification

Table 3. Nitrogen transformations in the ABR.[a]

Operating stage	Sulphide/nitrate in compartment 3	Initial N (mg N/L)		Final N (mg N/L)			Nitrate removal	Nitrate reduction pathway	
		Nitrate[b]	Ammonia	Nitrate	Ammonia	N_2		Denitrification	DNRA
I	0.16	504.3 ± 0.6	1058.9 ± 70.3	0.4 ± 0.1	1379.0 ± 86.8	474.3 ± 13.8	99.9%	94.1%	5.9%
II	0.61	501.0 ± 17.2	1189.8 ± 79.8	0.0 ± 0.0	1696.4 ± 128.6	402.7 ± 7.4	100%	80.4%	19.6%
III	0.05	498.2 ± 4.2	1683.7 ± 107.2	3.7 ± 1.7	1723.8 ± 68.6	482.7 ± 4.2	99.3%	96.9%	3.1%
IV	0.52	490.2 ± 3.1	1821.8 ± 153.0	3.9 ± 2.5	2027.0 ± 127.6	435.8 ± 6.5	99.2%	88.9%	11.1%

Note: DNRA, dissimilatory nitrate reduction to ammonia.
[a]Data represent mean values of 10 samples for each parameter obtained from steady-state conditions ± standard deviation.
[b]The added nitrate was included in the initial N.

in which sulphide serves as an electron donor is shown by Equations (1)–(3) [25]:

$$HS^- + 0.4NO_3^- + 1.4H^+ \rightarrow S^0 + 0.2N_2 + 1.2H_2O \ \Delta G^\theta = -191 \text{ kJ/reaction}, \quad (1)$$

$$S^0 + 1.2NO_3^- + 0.4H_2O \rightarrow SO_4^{2-} + 0.6N_2 + 0.8H^+ \ \Delta G^\theta = -547.6 \text{ kJ/reaction}, \quad (2)$$

$$HS^- + 1.6NO_3^- + 0.6H^+ \rightarrow SO_4^{2-} + 0.8N_2 + 0.8H_2O \ \Delta G^\theta = -738.6 \text{ kJ/reaction}. \quad (3)$$

Under excess nitrate condition, complete oxidation of sulphide and S^0 to sulphate could be achieved; whereas under nitrate limited condition, sulphide is partially oxidized to S^0. Interestingly, as shown in Table 4, increases in total S (only sulphide and sulphate were included) were observed in compartments 3–4 at different sulphide/nitrate ratios, suggesting that besides sulphide, some other species of reduced sulphur in the ABR were likely oxidized to sulphate. In addition, both elemental and organic sulphurs were present in the influent leachate while they disappeared in the effluent, as evident from the GC–MS analysis. It further proved the involvement of different kinds of reduced sulphur in the nitrate reduction process via autotrophic denitrification.

According to the stoichiometry (Equations (1)–(3)), the contributions of sulphide and sulphur to the denitrification pathway showed that in the ABR, sulphur-based autotrophic denitrification occurred together with heterotrophic denitrification (Table 4). The contribution of reduced sulphur to nitrate reduction pathway in compartment 3 was elucidated based on the following assumptions: (1) sulphide was completely oxidized to sulphate while elemental sulphur was only partly oxidized, considering that the generated sulphate concentration was higher than the sulphide concentration in the influent of compartment 3; (2) the contribution of non-denitrifying sulphur oxidizers was negligible, which accounted for low bacterial population in the ABR (<0.3%), for example, *Thiohalobacter*, *Thiohalocapsa* and *Thiobacter*; (3) metal-bound sulphides were not responsible for nitrate reduction because most of metal sulphides precipitated in compartment 1 and no increase in metal concentration was observed in compartment 3.

When only nitrate was added in Stages I and III, significant amount of nitrate was heterotrophically reduced to nitrogen gas (56.6% and 79.1%, respectively). However, sulphide addition (Stages II and IV) not only stimulated autotrophic denitrification, but also inhibited heterotrophic denitrification (43.2% and 52.8%, respectively) thereby resulting in a shift from heterotrophic denitrification to heterotrophic DNRA. Chen et al.[26] also concluded that heterotrophic denitrifiers were markedly inhibited at sulphide concentrations exceeding 200 mg S/L. Several studies suggested sulphide and acetate oxidation pathway in the order of denitrification reaction rate (q) as $qS^{2-} > q\text{Acetate} > qS^0$ due to inhibitory effect of sulphide on heterotrophic denitrifiers, thereby favouring sulphur-based autotrophic denitrifiers.[12,27] In this study, the preferred electron donor for denitrification could not be identified, because of the complexity of the organic carbon present in the leachate. Furthermore, since the theoretical amount of acetate required for the portion of heterotrophic denitrification (based on Equation (4)) was higher than that present in compartment 3 (Table S2 in Supplementary Data), acidogenesis was likely taking place with denitrification.

$$NO_3^- + 0.625CH_3COO^- + 0.375CO_2 \rightarrow 0.5N_2 + 0.125H_2O + 1.625HCO_3^-. \quad (4)$$

3.3. The bacterial population in compartment 3 of the ABR

To compare the bacterial communities without and with sulphide supplement, biomass samples from compartment 3 at days 40 and 60 (Stages I and II) were collected for analyses. After quality control processes filtered out reads containing incorrect primer or barcode sequences and sequences that were shorter than 250 nucleotides or with more than one ambiguous base, high-quality sequences were obtained from the biomass sample. As

Table 4. Sulphur transformations in compartments 3–4.[a]

Operating stage	Sulphide/nitrate ratio in compartment 3	Initial S in the influent of compartment 3 (mg S/L)			Final S in the effluent of ABR (mg S/L)			Sulphide removal	Contribution to denitrification		
		Sulphide[b]	Sulphate	Total	Sulphide	Sulphate	Total		Sulphide →Sulphate	S^0/org S→Sulphate	Heterotrophic
I	0.16	77.4 ± 5.3	2.2 ± 0.5	79.6 ± 5.8	0.0 ± 0.0	381.9 ± 58.5	381.9 ± 58.5	100%	9.8%	31.7%	56.6%
II	0.61	312.7 ± 18.8	9.1 ± 3.6	321.8 ± 22.4	0.8 ± 0.3	393.8 ± 34.2	394.6 ± 34.5	99.7%	46.3%	8.4%	43.2%
III	0.05	24.1 ± 3.8	10.0 ± 1.4	34.1 ± 5.2	0.3 ± 0.3	197.1 ± 15.7	197.4 ± 16.0	98.8%	3.0%	17.3%	79.1%
IV	0.52	252.3 ± 21.3	10.4 ± 1.1	262.7 ± 22.4	0.1 ± 0.1	356.9 ± 24.8	357.0 ± 24.9	100%	35.7%	10.2%	52.8%

[a]Data are the mean values of 10 samples for each parameter obtained from steady-state conditions ± standard deviation. Organic sulphur and elemental sulphur in the liquor were untested.
[b]The added sulphide was included in the initial S.

shown in Table S4 in Supplementary Data, there were a total of 46,154 and 37,506 high-quality sequences in the biomass sample at Stages I and II, respectively. With more high-quality sequences in the sample at Stage I, however, the number of observed OTUs was lower at 3061 compared with 3382 in the sample at Stage II, which indicating that more bacterial species were detected at Stage I. The OTU assignment was also used to estimate several species diversity estimates such as the Abundance-based Coverage Estimator (ACE) index,[28] the Chao1 index [29] and the Shannon diversity index.[30] The number of OTUs in each sample estimated by ACE and Chao1 richness estimator was considerably higher than the number of observed OTUs (covering 23–26% and 37–40% of the estimated richness, respectively). The richness indexes of Chao 1 and ACE of biomass during Stage II were significantly higher than those during Stage I. That is, the diversity of bacteria was richer with sulphide supplement. The bacterial diversity could also be estimated by the Shannon diversity index from the OTU data for each sample. The rarefaction analysis of the Shannon diversity index (Figure S1 in Supplementary Data) revealed that the diversity of each sample had reached a stable value. As shown in Table S4, the Shannon diversity of biomass at Stage I was slightly higher than that at Stage II. These results together indicate that the abundance of bacteria was increased by sulphide addition. In addition, the Shannon diversity index accounts for both the richness and evenness of OTUs, such that the index can be increased by either having additional unique species or by having greater species evenness. It was speculated that more specific bacteria concerning sulphide utilization was developed during Stage II.

Figure 5 graphically illustrates the phylum and functional genus-level distributions of bacterial OTUs involved in compartment 3 during Stages I and II of the ABR. The OTUs were then functionally taxonomically identified using the Ribosomal Database Project (RDP) classifier software based on Bergey's taxonomy and Naïve Bayesian assignment algorithm. As seen in Figure 5(a), most bacteria belonged to *Proteobacteria*, *Bacteroidetes* and *Firmicutes* (36.2%, 29.4% and 26.7% in Stage I and 60.2%, 21.6% and 8.8% in Stage II). The statistics of functional genus were also conducted by searching the function of every genus in the literature. Bacterial populations involved in integrating denitrification with anaerobic digestion in the presence of reduced sulphur were classified as sulphur-oxidizing nitrate-reducing bacteria (soNRB), heterotrophic nitrate-reducing bacteria (hNRB) and fermentative bacteria (FB), as shown in Figure 5(b). The coexistence of soNRB, hNRB and FB further indicated that sulphur oxidation and carbon

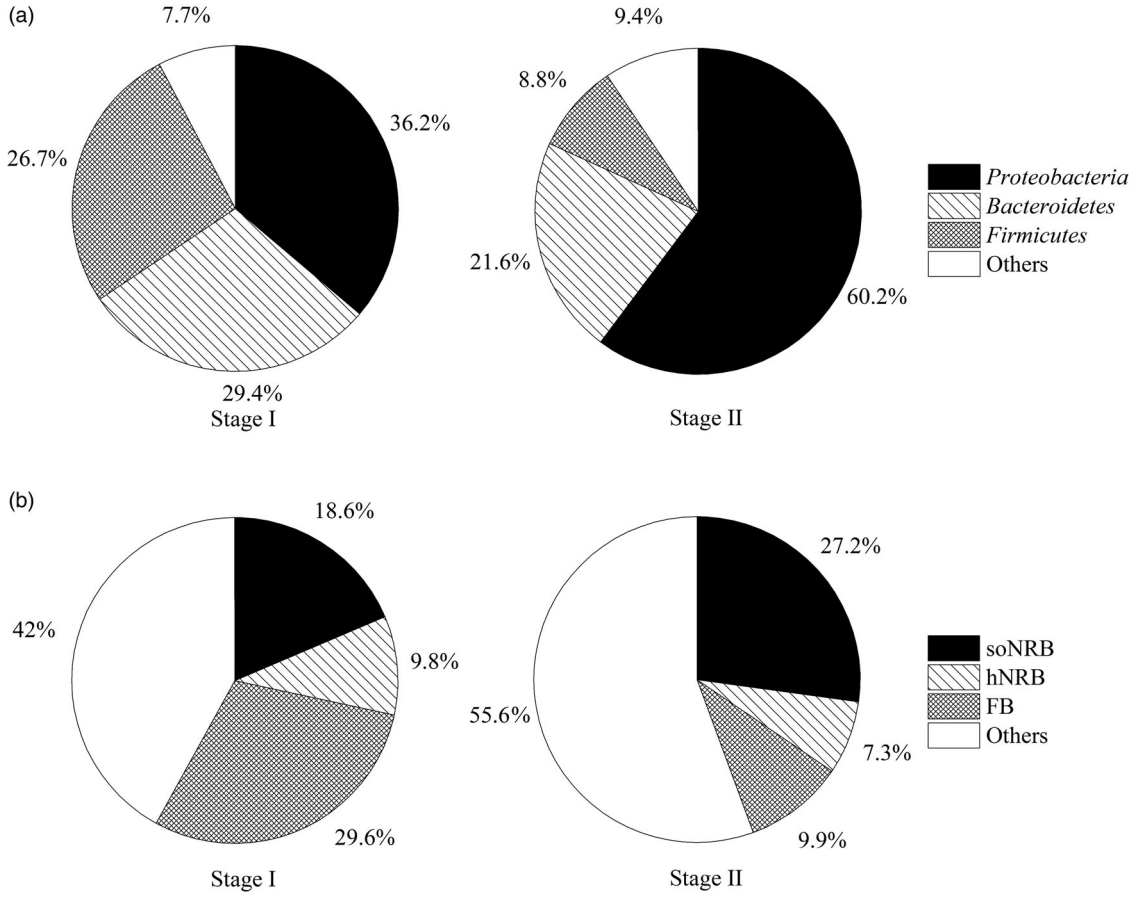

Figure 5. Phylum (a) and functional genus (b) level distributions of bacterial populations in compartment 3 of the ABR (Stage I, day 40; Stage II, day 60). FB, fermentation bacteria; hNRB, heterotrophic nitrate-reducing bacteria; soNRB, sulphur-oxidizing nitrate-reducing bacteria.

oxidation were coupled to denitrification and anaerobic fermentation.

The soNRB (mainly *Paracoccus*) and hNRB (mainly *Pseudoxanthomonas* and *Pseudomonas*) mostly belonged to *Proteobacteria*. *Paracoccus* comprising autotrophic denitrifier can utilize inorganic sulphur compounds such as sulphide, sulphur and thiosulfate as electron donors and nitrate as an electron acceptor.[31]. Studies also reported that some *Paracoccus* species are able to use both inorganic and organic compounds as sole carbon source for cell growth and some species are able to grow only via a heterotrophic pathway.[32,33] Both *Pseudomonas* and *Pseudoxanthomonas* reportedly function as heterotrophic denitrifiers, utilizing various organic substrates and reducing nitrate to nitrogen gas.[34–37]

The change in the bacterial composition to cope with the new condition (Stage II) of high sulphide concentration also reflected the effects of sulphide on the integration of sulphur-based autotrophic denitrification and heterotrophic denitrification with anaerobic fermentation. Thus, the soNRB were stimulated by sulphide addition during Stage II, as seen in Figure 5(b) by their proportional increase in the bacterial population (from 18.6% to 27.2%). By contrast, FB were severely inhibited by the high sulphide concentration, with a 66.6% decrease in the size of the population. Additionally, sulphide addition suppressed hNRB, which resulted decline (25.5%) in their population size and thereby causing a shift from heterotrophic denitrification to heterotrophic DNRA (as discussed in Section 3.2).

3.4. Conceptual model

Based on this study, a possible pathway of electron-flow-coupled sulphur, carbon, and nitrogen cycles has been proposed as shown in Figure 6. The possible mechanism involving reductive sulphur in nitrate reduction at different environments (e.g. sediments, groundwater and wastewater) is also elucidated. According to this pathway, besides reduced sulphur (sulphide and elemental sulphur) contained in the influent, sulphide is produced via dissimilatory sulphate reduction (r_1) or hydrolysis of org-S(r_2) in an anaerobic environment. The generated

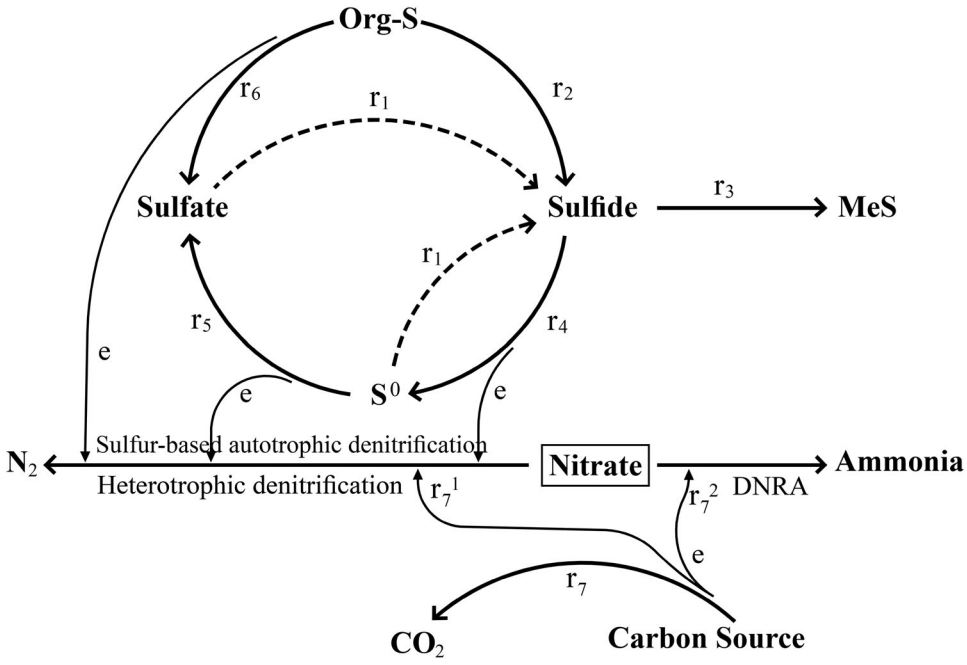

Figure 6. The proposed electron-flow-coupled sulphur, carbon and nitrogen cycles.

sulphide first precipitates with metals in compartment 1 (r_3). Both reduced sulphur and organic carbon are oxidized via autotrophic and heterotrophic denitrification pathways. The oxidation of sulphide to insoluble elemental sulphur (S^0) (r_4) and then to sulphate (r_5), together with the oxidation of reductive org-S to sulphate (r_6) and the oxidation of carbon source to carbon dioxide (r_7), generates electrons for use in nitrate reduction. In addition, in this study a high level of sulphide did not only stimulate autotrophic denitrification, but also inhibited heterotrophic denitrification(r_7^1), thus causing a shift from heterotrophic denitrification to heterotrophic DNRA (r_7^2).

4. Conclusions

A lab-scale study was conducted to demonstrate the interaction of organic carbon, reduced sulphur and nitrate for leachate treatment using an ABR. Denitrification was the main nitrate reduction pathway (>80%) during the test period. Various reduced sulphur (e.g. sulphide, elemental sulphur and organic sulphur) could be involved in the nitrate reduction process via sulphur-based autotrophic denitrification with the DOC/nitrate ratio decreased below 1.6. The addition of sulphide (Stages II and IV) not only enhanced autotrophic denitrification but also suppressed heterotrophic denitrification (with decreases of 43.2% and 52.8% in Stages II and IV, respectively) thereby causing a small shift in the nitrate reduction pathway from denitrification to DNRA (19.6% and 11.1%, respectively). High-throughput 16S rRNA gene sequencing and subsequent analysis also support the role of reduced sulphur in nitrate reduction.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplemental data

Supplemental data for this article can be accessed at 10.1080/09593330.2015.1102331

References

[1] Nie Y. Development and prospects of municipal solid waste (MSW) incineration in China. Front Environ Sci Eng China. 2008;2:1–7.
[2] Chen D, Christensen TH. Life-cycle assessment (EASEWASTE) of two municipal solid waste incineration technologies in China. Waste Manag Res. 2010;28:508–519.
[3] Rodriguez J, Castrillón L, Marañón E, et al. Removal of non-biodegradable organic matter from landfill leachates by adsorption. Water Res. 2004;38:3297–3303.
[4] Liu J, Zhong J, Wang Y, et al. Effective bio-treatment of fresh leachate from pretreated municipal solid waste in

an expanded granular sludge bed bioreactor. Bioresour Technol. 2010;101:1447–1452.
[5] Osman NA, Della TS. Anaerobic/aerobic treatment of municipal landfill leachate in sequential two-stage upflow anaerobic sludge blanket reactor (UASB)/ completely stirred tank reactor (CSTR) system. Process Biochem. 2005;40:895–902.
[6] Wang CC, Lee PH, Kumar M, et al. Simultaneous partial nitrification, anaerobic ammonium oxidation and denitrification (SNAD) in a full-scale landfill-leachate treatment plant. J Hazard Mater. 2010;175:622–628.
[7] Ruscalleda M, Lopez H, Ganigue R, et al. Heterotrophic denitrification on granular anammox SBR treating urban landfill leachate. Water Sci Technol. 2008;58:1749–1755.
[8] He P, Li M, Xu S, et al. Anaerobic treatment of fresh leachate from a municipal solid waste incinerator by upflow blanket filter reactor. Front Environ Sci Eng China. 2009;3:404–411.
[9] Fang F, Abbas AA, Chen YP, et al. Anaerobic/aerobic/coagulation treatment of leachate from a municipal solid wastes incineration plant. Environ Technol. 2012;33:927–935.
[10] Dang Y, Ye J, Mu Y, et al. Effective anaerobic treatment of fresh leachate from MSW incineration plant and dynamic characteristics of microbial community in granular sludge. Appl Microbiol Biotechnol. 2013;97:10563–10574.
[11] Xie L, Chen JR, Wang R, et al. Effect of carbon source and COD/NO_3^--N ratio on anaerobic simultaneous denitrification and methanogenesis for high-strength wastewater treatment. J Biosci Bioeng. 2012;113:759–764.
[12] Reyes-Avila JS, Razo-Flores E, Gomez J. Simultaneous biological removal of nitrogen, carbon and sulfur by denitrification. Water Res. 2004;38:3313–3321.
[13] Wong BT, Lee DJ. Sulfide enhances methanogenesis in nitrate-containing methanogenic cultures. Bioresour Technol. 2011;102:2427–2432.
[14] Wong BT, Lee DJ. Denitrifying sulfide removal and carbon methanogenesis in a mesophilic, methanogenic culture. Bioresour Technol. 2011;102:6673–6679.
[15] Tugtas AE, Pavlostathis SG. Effect of sulfide on nitrate reduction in mixed methanogenic cultures. Biotechnol Bioeng. 2007;97:1448–1459.
[16] Truper HG, Schlegel HG. Sulphur metabolism in Thiorhodaceae 1.Quantitative measurements on growing cells of *Chromatium okenii*. Antonie Van Leeuwenhoek J. Microbiol Serol. 1964;30:225–238.
[17] APHA (American Public Health Association). Standard methods for the examination of water and wastewater. 20th ed. Washington, DC: American Public Health Association; 1998.
[18] Zhang TC, Lampe DG. Sulfur: limestone autotrophic denitrification processes for treatment of nitrate-contaminated water: Batch experiments. Water Res. 1999;33:599–608.
[19] Kim SY, Jung HJ, Kim KS, et al. Treatment of high nitrate-containing wastewaters by sequential heterotrophic and autotrophic denitrification. J Environ Eng. 2004;130:1475–1480.
[20] Moon HS, Ahn KH, Lee S, et al. Use of autotrophic sulphur-oxidizers to remove nitrate from bank filtrate in a permeable reactive barrier system. Environ Pollut. 2004;129:499–507.
[21] Brunet RC, Garcia-Gil LJ. Sulfide-induced dissimilatory nitrate reduction to ammonia in anaerobic freshwater sediments. FEMS Microbiol Ecol. 1996;21:131–138.
[22] Flere JM, Zhang TC. Nitrate removal with sulfur-limestone autotrophic denitrification process. J Environ Eng. 1999;125:721–729.
[23] Koenig A, Liu LH. Autotrophic denitrification of landfill leachate using elemental sulfur. Water Sci Technol. 1996;34:533–540.
[24] Trouve C, Chazal PM, Gueroux B, et al. Denitrification by new strains of Thiobacillus denitrificans under non-standard physicochemical conditions. Effect of temperature, pH, and sulphur source. Environ Technol. 1998;19:601–610.
[25] Li W, Zhao QL, Liu H. Sulfide removal by simultaneous autotrophic and heterotrophic desulfurization-denitrification process. J Hazard Mater. 2009;162:848–853.
[26] Chen C, Ren NQ, Wang AJ, et al. Simultaneous biological removal of sulfur, nitrogen and carbon using EGSB reactor. Appl Microbiol Biotechnol. 2008;78:1057–1063.
[27] Furumai H, Tagui H, Fujita K. Effects of pH and alkalinity on sulfur-denitrification in a biological granular filter. Wat Sci Tech. 1996;34:355–362.
[28] Chao A, Lee S. Estimating the number of classes via sample coverage. J Am Stat Assoc. 1992;87:210–217.
[29] Chao A. Nonparametric estimation of the number of classes in a population. Scan J Stat. 1984;11:265–270.
[30] Krebs C. Ecological methodology. Menlo Park, CA: Benjamin/Cummings; 1999.
[31] Vaiopoulou E, Melidis P, Aivasidis A. Sulfide removal in wastewater from petrochemical industries by autotrophic denitrification. Water Res. 2005;39:4101–4109.
[32] Lipski A, Reichert K, Reuter B, et al. Identification of bacterial isolates from biofilters as Paracoccus alkenifer sp. nov. and Paracoccuss olventivorans with emended description of Paracoccuss olventivorans. Int J Syst Bacteriol. 1998;48:529–536.
[33] Siller H, Rainey FA, Stackebrandt E, et al. Isolation and characterization of a new gram- negative acetone-degrading, nitrate-reducing bacterium from soil, Paracoccuss olventivoran ssp. nov. Int J Syst Bacteriol. 1996;46:1125–1130.
[34] Mechichi T, Stackebrandt E, Gadon N, et al. Phylogenetic and metabolic diversity of bacteria degrading aromatic compounds under denitrifying conditions, and description of Thauera phenylacetica sp. nov., Thauera aminoaromatica sp. nov., and Azoarcus buckelii sp. Nov. Arch Microbiol. 2002;178:26–35.
[35] Sorokin DY. Oxidation of inorganic sulfur compounds by obligately organotrophic bacteria. Microbiology. 2003;72:641–653.
[36] Park S, Seon J, Byun I, et al. Comparison of nitrogen removal and microbial distribution in wastewater treatment process under different electron donor conditions. Bioresour Technol. 2010;101:2988–2995.
[37] Trois C, Coulon F, de Combret CP, et al. Effect of pine bark and compost on the biological denitrification process of non-hazardous landfill leachate: focus on the microbiology. J Hazard Mater. 2010;181:1163–1169.