Biokinetics of microbial consortia using biogenic sulfur as a novel electron donor for sustainable denitrification

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ABSTRACT

In this study, the biokinetics of autotrophic denitrification with biogenic S⁰ (ADBIOS) for the treatment of nitrogen pollution in wastewaters were investigated. The used biogenic S⁰, a by-product of gas desulfurization, was an elemental microcrystalline orthorhombic sulfur with a median size of 4.69 µm and a specific surface area of 3.38 m²/g, which made S⁰ particularly reactive and bioavailable. During denitrification, the biomass enriched on nitrite (NO₂⁻) was capable of degrading up to 240 mg/l NO₂⁻-N with a denitrification activity of 339.5 mg NO₂⁻-N/g VSS·d. The use of biogenic S⁰ induced a low NO₂⁻-N accumulation, hindering the NO₂⁻-N negative impact on the denitrifying consortia and resulting in a specific denitrification activity of 223.0 mg NO₃⁻-N/g VSS·d. Besides Thiobacillus being the most abundant genus, Moheibacter and Thermomonas were predominantly selected for denitrification and denitritration, respectively.

1. Introduction

The increased depletion of resources, the rising water stress, the need of decreasing the carbon footprint and the stringent nutrient discharge limits encourage the development of new bioprocesses for nitrogen removal from wastewater. Conventionally, nitrate (NO₃⁻) and nitrite (NO₂⁻) reduction is coupled to the oxidation of organic matter by heterotrophic microorganisms in wastewater treatment plants.

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(WWTPs). However, heterotrophic denitrification and denitritation typically require the supply of organic matter, resulting in higher sludge production and operational costs (Sun and Nemati, 2012).

To overcome these disadvantages, chemically synthesized elemental sulfur (S⁰) has been used as a cheaper and effective electron donor for autotrophic denitrifying microorganisms treating wastewaters poor in organics (Wang et al., 2016). However, the low water solubility of chemically synthesized S⁰ limits its availability to microorganisms and makes denitritification and denitrification kinetics slower than that achieved with more soluble electron donors (Kiskira et al., 2017a; Mora et al., 2015; Park and Yoo, 2009; Zou et al., 2016). Additionally, elevated NO₂⁻ concentrations when using chemically synthesized S⁰ during autotrophic denitritation can decrease the overall process efficiency (Christianson et al., 2015; Kostrytsia et al., 2018). Specifically, the sulfur to nitrogen (S/N) molar ratio, the feed pH and the microbial community structure are known to be among the main factors controlling the NO₂⁻ accumulation. Therefore, it is crucial to investigate the potential of alternative electron donors for both NO₃⁻ and NO₂⁻ removal, such as biogenic S⁰.

Biogenic S⁰ (or biosulfur) is a biological product obtained from the incomplete oxidation of sulfide in gaseous streams under oxygen-limiting conditions by S-oxidizing microorganisms (Florentino et al., 2015). The Thiopaq⁰ technology (Paques BV, the Netherlands) is, for instance, a well-established process aimed at the biological gas desulfurization that integrates hydrogen sulfide (H₂S) removal and biogenic S⁰ recovery, with more than 200 installations worldwide (‘THIOPAQ Biogas desulphurization,’ 2018). Biogenic S⁰ globules, generated by different strains of bacteria, are considered to be hydrophilic, with a structure made of orthorhombic S⁰ crystals surrounded by a hydrated layer of long-chain polymers or polythionates (Kamyshny et al., 2009; Kleinjan et al., 2003). The chemical composition of biogenic S⁰ globules and their small particle size (2–40 µm) affect the S⁰ (bio)chemical reactivity and make it more bioavailable for microorganisms (Findlay et al., 2014).

These exclusive properties of biogenic S⁰ have promoted its application as a fertilizer (FERTIPAQ, the Netherlands) and in metal recovery technologies (Florentino et al., 2015). Only in the last two years, biogenic S⁰ has been suggested for denitrification applications (Di Capua et al., 2016) due to its bioavailable nature and the possibility to offer a more affordable and sustainable nutrient removal solution. In this line, a possible integrated solution combining desulfurization of biogas with nitrogen removal from wastewaters can become applicable in the future, enabling to upgrade the current wastewater treatment configurations on a novel water resource recovery facility, in agreement with the EU Action Plan for the Circular Economy. To do so, more research on the chemistry and microbiology behind the use of biogenic S⁰ for NO₃⁻ and NO₂⁻ removal is required.

The present research aims to investigate the fundamental aspects of autotrophic denitrification with biogenic S⁰ (ADBIOS) using high-strength NO₃⁻ and NO₂⁻ synthetic waters. The main objectives of this study were to: (i) perform a physico-chemical and elemental characterization of the biogenic S⁰ used; (ii) enrich a microbial consortium capable of NO₃⁻ and NO₂⁻ reduction and concomitant oxidation of biogenic S⁰ in batch; (iii) use the enriched microbial community to evaluate the kinetics of biogenic S⁰-based autotrophic denitrification, denitritation, and simultaneous denitritification-denitrification in batch bioassays; and (iv) investigate the structure of the bacterial communities in the presence of NO₃⁻, NO₂⁻ or both. The impact of this study for the design and scale-up of biogenic S⁰-driven denitrification and denitritation systems is discussed.

2. Materials and methods

2.1. Source of biogenic S⁰ and development of the biogenic S⁰-oxidizing microbial consortium

The biogenic S⁰ (FertipaQ BV, the Netherlands, purity > 99%, 11% moisture content) from the Thiopaq process (Paques BV, the Netherlands) was used as electron donor in the batch bioassays aimed at denitrification and denitritation. An activated sludge collected from the denitrifying tank of a municipal wastewater treatment plant (Cassino, Italy) was used as inoculum (10% v/v) in the batch bioassays. The biogenic S⁰-based denitrifying bacterial cultures were enriched for 3.5 months in 125 ml serum bottles with a working volume of 100 ml. The bottles were fed with the basal medium and trace elements as reported by Kostrytsia et al. (2018). NO₃⁻ or NO₂⁻ were individually added to the serum bottles with an initial concentration of 240 mg N/l. To ensure the presence of an adequate concentration of biogenic S⁰ for complete denitrification or denitritation, an excess S⁰ was used to maintain an S:N (g/g) ratio of 3.76 (1.5 times higher than the stoichiometric value). NaHCO₃ was added as buffer and carbon source with a concentration of 2.0 g/l.

Each bottle was purged with helium gas for 3 min to exclude oxygen, prior to sealing the bottle with a rubber stopper and an aluminum crimp. Subsequently, the bottles were placed on a gyratory shaker at 300 rpm and temperature was maintained at 20 °C by means of a water bath. The enrichment was subcultured when NO₃⁻ or NO₂⁻ concentrations approached zero. An enrichment was treated as ‘stable’ when the achieved denitrification or denitritation rates of the subcultures alternated by less than 5%.

2.2. Kinetic experiments

To knowledge of the authors, for the first time biogenic S⁰-oxidizing microbial consortia capable of reducing NO₃⁻ or NO₂⁻ were developed. Three sets of batch bioassays were set up using the enriched biomass to investigate the kinetics of ADBIOS, i.e. denitrification (A: NO₃⁻ and S⁰), denitritation (B: NO₂⁻ and S⁰) and simultaneous denitritation-denitrification (C: NO₂⁻, NO₃⁻ and S⁰). The initial NO₃⁻ or NO₂⁻ concentrations were similar (i.e. 5% difference) to those used in a previous batch study on autotrophic denitrification with chemically synthesized S⁰ (Kostrytsia et al., 2018). In the first experiment (A), batch bioassays were conducted to investigate the denitrification characteristics (NO₃⁻-N reduction rate, NO₂⁻-N accumulation and NO₃⁻-N reduction rates). During the second set of the experiments (B), NO₃⁻-N was used as the sole electron acceptor in order to evaluate the denitrification kinetics (NO₃⁻-N reduction rate) and assess the impact of biomass acclimation to NO₃⁻-N degradation. The simultaneous denitritation-denitrification experiment (C) was performed to study the effect of NO₂⁻-N on NO₃⁻-N degradation.

At the beginning of each experiment, the required amount of NO₃⁻-N and NO₂⁻-N from stock solutions was added into the serum bottles to achieve the desired initial concentration as reported in Table 1. Biogenic S⁰, NaHCO₃, the basal medium and trace elements solution were supplied at the same concentrations as in the enrichment phase. Each serum bottle was inoculated with an enriched culture with an amount of approximately 217.5 (± 2.5) mg/l of volatile suspended solids (VSS). Abiotic controls were used to monitor the possible chemical reactions involving the electron donor and electron acceptor. Controls without electron donor (biogenic S⁰) or electron acceptor (NO₃⁻ or NO₂⁻) were carried out to estimate their possible degradation not associated with S⁰-driven denitrification or denitritation. In each experiment, the denitrification and denitritation rates were calculated from the slope of the curve describing NO₃⁻-N and NO₂⁻-N degradations, respectively, versus time and expressed as mg NO₃⁻-N/l/d. The biomass specific denitrification and denitritation activities (mg NO₃⁻-N/g VSS d) were calculated by normalizing the denitrification and denitritation rate data with the initial biomass concentration (g VSS/l).

2.3. Sampling and analytical techniques

The liquid samples were taken twice a day and stored at −20 ℃ prior to analysis. NO₃⁻, NO₂⁻ and sulfate (SO₄²⁻) concentrations were determined by ion chromatography, as reported elsewhere (Kiskira et al., 2017a).
2.4.1. DNA extraction and high-throughput sequencing

The total genomic DNA was extracted from the inoculum (Section 2.1) and the biomass at the beginning and at the end of the experiments (Table 1) in triplicate, following the protocol described by Griffiths et al. (2000). A high-throughput sequencing of partial 16S rRNA gene on DNA samples was conducted by the Illumina MiSeq sequencing service (FISABIO, Spain). The primers 515F and 806R were applied to target the 16S rRNA gene. The raw sequence files supporting the results of this article are available in the European Nucleotide Archive under the project accession number PRJEB27906.

2.4.2. Bioinformatics

The abundance table was generated by constructing operational taxonomic units (OTUs). Initially, the paired-end reads were pre-processed as described by Schirmer et al. (2015). Briefly, the paired-end reads were trimmed and filtered using Sickle v1.200. Then, PANDAseq v2.4 was used to assemble the forward and reverse reads into a single sequence spanning the entire V4 region. This resulted in consensus sequences for each sample on which VSEARCH v2.3.4 was used for OTU construction. The preprocessed reads from each sample were pooled together while barcodes were added. The reads were then dereplicated and sorted in order of decreasing abundance (Schirmer et al., 2015). Subsequently, the reads were clustered based on 97% similarity, followed by a removal of clusters (vssearch). Finally, the OTU table was generated by matching the original barcoded reads against clean OTUs (a total of 1104 OTUs for n = 19 samples) at 97% similarity. The representative OTUs were taxonomically classified against the SILVA SSU Ref NR v123 database. Multisequence aligned the OTUs and used them with FastTree v2.1.7 to generate the phylogenetic tree in NEWICK format. The biom file for the OTUs was then generated by combining the abundance table with the taxonomy information using Qime workflow.

2.4.3. Statistical analysis

Statistical analyses were performed in R v3.4.4 using the combined data generated from the bioinformatics as well as meta data associated with the study. The vegan package was used for alpha and beta diversity analyses. For alpha diversity measures, the following indexes were calculated: rarefied richness – the estimated number of species after rarefying the abundance table to minimum library size; Shannon entropy – a commonly used index to measure balance within a community. The ordination of the OTU table in a reduced space was done using Principal Coordinate Analysis (PCoA) plots of OTUs using two different distance measures: Bray-Curtis distance metric which considered OTU abundance counts and; Weighted UniFrac distance that combined the phylogenetic distances weighted with relative abundances. Phylogenetic distances within each sample were further characterized by calculating the nearest taxa index (NTI) and net relatedness index (NRI) (Kembel et al., 2010). This analysis helped to determine whether the community structure was stochastic (driven by competition among taxa) or deterministic (driven by environmental pressure).

Sparse Projection to Latent Structure – Discriminant Analysis (sPLS-DA) was performed with the R’s mixOmics package (Rohart et al., 2017). The procedure constructed artificial latent components of the predicted variables (OTU table collated at genus level) and the response variables by factorizing these matrices into scores and loading vectors in a new space such that the covariance between the scores of these two matrices in this space was maximized. The loading vector was constructed with the coefficients indicating the importance of each variable to define the component, i.e. non-zero coefficients in the loading vectors indicated the genera that vary significantly between the categories and were thus deemed as discriminants (Rohart et al., 2017). Fine tuning of the algorithm was applied by splitting the data into training and testing sets and then finding the classification error rates, employing two metrics, i.e. overall error rates and balanced error rates (BER).

### Table 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Initial concentration (mg/l)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: Denitrification (NO₃⁻ and S⁰)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO₂⁻-N</td>
<td>NO₃⁻-N</td>
<td>Total N</td>
</tr>
<tr>
<td>15</td>
<td>225</td>
<td>240</td>
</tr>
<tr>
<td>B: Denitrification (NO₂⁻ and S⁰)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO₂⁻-N</td>
<td>NO₃⁻-N</td>
<td>Total N</td>
</tr>
<tr>
<td>240</td>
<td>240</td>
<td>240</td>
</tr>
<tr>
<td>C: Denitrification and denitrification (NO₂⁻, NO₃⁻, and S⁰)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO₂⁻- and NO₃⁻-free</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO₂⁻-N</td>
<td>NO₃⁻-N</td>
<td>Total N</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S⁰-free controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO₂⁻-N</td>
<td>NO₃⁻-N</td>
<td>Total N</td>
</tr>
<tr>
<td>240</td>
<td>240</td>
<td>240</td>
</tr>
<tr>
<td>Abiotic controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO₂⁻-N</td>
<td>NO₃⁻-N</td>
<td>Total N</td>
</tr>
<tr>
<td>240</td>
<td>240</td>
<td>240</td>
</tr>
</tbody>
</table>

* Microbial source: biomass enriched on NO₃⁻-N and S⁰.
* Microbial source: biomass enriched on NO₂⁻-N and S⁰.

2017b). Elemental S⁰ was determined by reversed-phase chromatography as originally described by Kamyshny et al. (2009). In this study, a high-performance liquid chromatography (HPLC) system (Prominence LC-20A Series, Shimadzu, Japan) equipped with a Kinexet LC column (C18, 5100 Å) and a UV/Vis detector (SPD-20A, Shimadzu, Japan) at 230 nm was used to quantify elemental S⁰. Prior to and at the end of the batch kinetic experiments, total suspended solids (TSS) and VSS of the liquid samples were determined according to the Standard Methods (APHA, 2011).

Laser size particle analysis (LSPA) was performed to determine the particle size distribution (PSD) of the raw and freeze-dried biogenic S⁰ in a deionized water by a Mastersizer 2000 (Malvern Instruments, UK) laser diffraction particle size equipped with a HydroQ sample dispersion wet unit. The measurement range of the instrument was from 0.02 to 2000 µm. Size parameters of the diameters d₀.1, d₀.5 and d₀.9 were presented with 10%, 50% and 90%, respectively, of the volume of the particles below the given number.

To investigate the chemical and structural origin of biogenic S⁰, Raman spectra were obtained at random positions on the biogenic S⁰ material using a Horiba LabRAM II Raman spectrometer (Horiba Jobin-Yvon, France). The instrument was equipped with a 600 groove-mm⁻¹ grating, a confocal optical system, a Peltier-cooled CCD detector and an Olympus BX41 microscope arranged in 180° back-scatter geometry. The measurements were performed using a 532 nm laser channeled through a Leica L100X/0.75 objective, providing a laser spot diameter of ~1.5 µm.

Inductively coupled plasma mass spectrometry (ICP-MS) was used to determine quantifiable trace metals in biogenic S⁰. The biogenic S⁰ samples were freeze-dried at −52 °C (Freezone 12, Labconco, USA), and approximately 0.1 g of sample was digested with an optimized microwave digestion procedure (Anton Paar Multiwave 3000, Austria) using 3 ml of trace metal grade 67% HNO₃ (Anton Paar Multiwave 3000, Austria) and approximately 0.1 g of sample was digested with an optimized microwave digestion procedure (Anton Paar Multiwave 3000, Austria) using 3 ml of trace metal grade 67% HNO₃ (Anton Paar Multiwave 3000, Austria) and 3 ml of 30% H₂O₂ (Anton Paar Multiwave 3000, Austria) at 250 °C with biogenic S⁰ as an electron donor and an S:N (g/g) ratio of 3.76.
3. Results and discussion

3.1. Physico-chemical and elemental characterization of biogenic S0

The PSD of biogenic S0 is shown in Fig. 1. The raw biogenic S0 sample consisted of particles with a median diameter of 241.16 µm and the 10% (6.88 µm) and 90% (508.89 µm) as measures of variability (Fig. 1). A surface weighted mean of 24.78 µm was quantified, which estimated the average diameter based on the surface area. In contrast, in previous studies on chemically synthesized S0-based denitrification, the S0 particle size was between 500 and 16000 µm (Christianson and Sahinkaya, 2014; Sahinkaya et al., 2014; Sahinkaya and Kilic, 2014), which is approximately two orders of magnitude higher than that of the S0 used in the current study.

To evaluate the degree of agglomeration of the biogenic S0 and its behavior in suspension, the size distribution of the S0 particles was also determined after mixing them in water (5 min at 300 rpm and 30 °C). The measured particles were with the median size of 4.69 µm and variability of the 10% (1.37 µm) and 90% (12.8 µm). This shows that the particle size of the biogenic S0 was of the same order of magnitude of the S0 particles (up to 1 µm) produced by sulfide-oxidizing bacteria in aqueous environment (Findlay et al., 2014). The contact of biogenic S0 with water under mixing (i.e. 300 rpm) was likely to break sulfur agglomerations (Fig. 1). This could be explained by the hydrophilic surface of biogenic S0, which hinders the particle-aggregation in water (Kleinjan et al., 2003). In contrast, chemically produced S0 is more hydrophobic and aggregates quickly in aqueous solutions (Findlay et al., 2014).

The specific surface area (SSA) of elemental S0 particles is a main driver for its biooxidation rate, including oxidation coupled to denitrification and denitritation (Kostrytsia et al., 2018). In previous studies, the higher reactivity of biogenic S0 was attributed not only to its unique surface characterization, but also to a higher SSA associated with a smaller particle size compared to that of the chemically produced S0 (Kleinjan et al., 2003). In this study, the small biogenic S0 grain size of 4.69 µm obtained after mixing provided a high SSA of 3.38 m²/g, compared to that of raw biogenic sulfur that had a 0.242 m²/g SSA corresponding to a 241.16 µm grain size. In addition, the results of LSPA (Fig. 1) and Raman spectroscopy (data not shown) confirmed that the biogenic S0 used in this study is an elemental microcrystalline orthorhombic sulfur. These findings are in line with a previously proposed biogenic model of a microcrystalline solid elemental sulfur covered by biopolymers (Janssen et al., 1999). Therefore, the microcrystallinity of biogenic S0 particles results in its higher reactivity and solubility, as suggested by Pasteris et al. (2001).


during denitrification, the reactions of NO3− or NO2− reduction to nitrogenous oxides are catalyzed by metalloenzymes (Shao et al., 2010). Among the quantifiable trace metals detected in biogenic S0 (Table 2), copper (Cu), molybdenum (Mo) and iron (Fe) are co-factors of metalloenzymes (Shao et al., 2010). Nitrite reductase (NIR) and nitrous oxide reductase (N2OR) enzymes contain Cu. Mo is covalently attached to the protein in nitrate reductase (NaR), and Fe is cofactor for nitric oxide reductase (NOR). Additionally, Fe-S proteins, so-called ferredoxins, mediate electron transfer during NO3− and nitric oxide (NO) reduction (Shao et al., 2010). Thus, the supply of trace metals is essential for the high performance of denitrification and denitritation, and biogenic S0 can effectively provide the necessary trace metals during these processes (Table 2). The possible inhibitory effect of heavy metals released by biosulfur on the activity of denitrifying biomass can be taken into consideration in a future study.

3.2. Effect of electron acceptor on ADBIOS kinetics

The evolution of pH, NO3−-N, NO2−-N and SO42−-S concentrations as well as the remaining S0 throughout the 2-week batch experiments is shown in Fig. 2. In the denitrification experiment (Fig. 2a and d), the achieved degradation rate of 49.4 mg NO3−-N/l·d allowed the complete NO3− degradation in 14 days with a final biomass concentration of 450 mg VSS/l. The NO3− removal efficiency reached up to 84% after the first 5 days, with NO3−-N accumulating up to 135 mg/l. The NO3− accumulation was most likely ascribed to a higher enzyme activity of NaR compared to NIR, as also reported elsewhere (Du et al., 2016; Sun and Nemati, 2012). The high NO3−-N build-up was followed by a drop of the NO3−-N degradation rate to 7.8 mg/l·d from day 5 onwards. This was likely due to the inhibition of a NO3−-N concentration above 60 mg/l on the activity of the denitrifying biomass (Guerrero et al., 2016).

In the denitritation experiments, the potential of the biogenic S0-oxidizing biomass enriched on NO3− to reduce high NO2− concentrations was investigated (Fig. 2b and e). The bacteria were capable of completing NO3−-N removal with the rate of 73.0 mg NO3−-N/l·d, which resulted in a biomass growth up to 430 mg VSS/l. NO detrimental effects were observed on denitrification at a NO3−-N concentration as high as 240 mg/l. Additionally, the kinetics of simultaneous denitrification-denitritation were investigated in the presence of high NO3−-N concentrations and microbial community enriched on NO3− (Fig. 2c). During the experiment, the highest NO3−-N and NO2−-N removal rates were 31.3 and 21.8 mg/l·d, respectively. The final biomass concentration in simultaneous denitrification-denitritation experiments was 320 mg VSS/l. Thus, the presence of NO3−-N did not result in an inhibition of the NiR activity.

A higher accumulation of NO3−-N and its slow degradation are generally observed when using chemically synthesized S0 as electron donor for denitrification due to the low solubility of the S0-based
substrate (Sahinkaya et al., 2015; Simard et al., 2015). As the micro-crystallinity and hydrophilic properties (Section 3.1) provided a higher bioavailability of biogenic S0\(^2\), a faster degradation of the accumulated NO\(^3\)-N (20.9 mg NO\(^3\)-N/l·d\(^{-1}\)) during denitrification was achieved (Fig. 2a and c). Even a higher NO\(^3\)-N degradation rate (73.0 mg NO\(^3\)-N/l·d\(^{-1}\)) could be obtained by using the biomass enriched on NO\(^2\)-N (Fig. 2b). Therefore, the use of NO\(^2\)-N-acclimated biomass is recommended for biogenic S0-driven denitrification treating high-strength NO\(^3\)-N wastewaters to control the high NO\(^2\)-N accumulation.

SO\(^2\)-S was the only sulfur product of the biogenic S0 \(^2\) oxidation (Fig. 2d and f). This observation was confirmed by the stochiometric consumption of the biogenic S0 \(^2\) with NO\(^3\)-N or NO\(^2\)-N (Sun and Nemati, 2012). No denitrification and denitrification were observed in the abiotic and electron donor-free controls (data not shown). In this study, specific denitrification and denitrification activities of 223.0 mg NO\(^3\)-N/g VSS·d and 339.5 mg NO\(^3\)-N/g VSS·d, respectively, were achieved by the biogenic S0-oxidizing microbial consortium (Table 3). The high solubility of biogenic S0, which was likely attributed to the hydrophilic properties and the lower particle size of the biogenic S0 particles (Section 3.1), induced a significantly higher NO\(^3\)-N degradation (Fig. 2a). The kinetics of ADBIOS (including both denitrification and denitrification) was characterized by 10-time higher rates compared to those obtained with chemically synthesized S0 (Kostrytsia et al., 2018), with both studies being performed at similar initial NO\(^3\)-N and NO\(^2\)-N concentrations.

### 3.3. Effect of different electron acceptors on microbial communities performing ADBIOS

The efficiency of biological NO\(^3\)-N and NO\(^2\)-N reduction depends on the community composition of microorganisms (Shao et al., 2010). Thus, the genera prevailing under each experimental condition (Fig. 3a), i.e. denitrification (A), denitrification (B) and simultaneous denitrification-denitrification (C) at the beginning (T\(_0\)) and at the end (T\(_e\)) of the experiments, as well as the microbial community of the raw activated sludge (AS) used as inoculum, were analyzed in this study (Figs. 3 and 4).

The enrichment in both denitrification (A) and simultaneous denitrification-denitrification (C) (i.e. NO\(^3\)-N and NO\(^2\)-N both involved) led to similar microbial communities with samples A \(_T_\_\_\_\) and C \(_T_\_\_\_\) clustering closer to each other on the PCoA plots (Fig. 3d). In contrast, when denitrification was performed alone (B) (i.e. with the sole NO\(^2\)-N involved), a distinct community was formed (B \(_T_\_\_\_\)) (Fig. 3d). Thus,

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**Table 3**

<table>
<thead>
<tr>
<th>Electron acceptor</th>
<th>Biogenic S0</th>
<th>Chemically synthesized S0 (Kostrytsia et al., 2018)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO(^3)-N</td>
<td>Denitrification rate (mg NO(^3)-N/l·d)(^{a})</td>
<td>49.4</td>
</tr>
<tr>
<td></td>
<td>Specific denitrification activity (mg NO(^3)-N/g VSS·d(^{a}))</td>
<td>223.0</td>
</tr>
<tr>
<td>NO(^2)-N</td>
<td>Denitrification rate (mg NO(^2)-N/l·d)(^{a})</td>
<td>73.0</td>
</tr>
<tr>
<td></td>
<td>Specific denitrification activity (mg NO(^2)-N/g VSS·d(^{a}))</td>
<td>339.5</td>
</tr>
</tbody>
</table>

a Biomass enriched for 3.5 months on NO\(^3\)-N and S0.

b Biomass enriched for 3.5 months on NO\(^2\)-N and S0.
different key representative genera are selected when both (A and C) or only one electron acceptor (B) are used, with the former enriching for *Petrimonas*, *Bacillus*, *Truepera*, *Ferruginibacter*, *Castellaniella*, *Aminobacter* and the latter comprising of *Comamonas*, *Truepera*, *Bacillus* and *Clostridium sensu stricto 13*, based on taxa differential analysis (Fig. 3b).

Some members of the genera *Comamonas* and *Bacillus* have been shown to be involved in NO$_3^-$ reduction (Park and Yoo, 2009; Zhang et al., 2015), while in this study these were also abundant in the denitrification (B) experiment.

Additionally, in the top 25 most abundant genera *Thiobacillus* and *Moheibacter* were predominantly selected for the conditions with two electron acceptors (A and C) (Fig. 3b). In contrast, the condition with one electron acceptor (B) selected for different communities predominantly (~75%) comprising of *Thiobacillus* and *Thermomonas* (Fig. 3b). *Thiobacillus* has been reported as ubiquitous in denitrification applications with reduced sulfur compounds, e.g. particulate chemically synthesized S$^0$ and soluble S$_2$O$_3^{2-}$ (Di Capua et al., 2016; Kostrytsia et al., 2018) and is capable of withstanding high NO$_2^-$ concentrations (Chen et al., 2018; Gao et al., 2017; Zhang et al., 2015), as also observed in this study with hydrophilic biosulfur (Fig. 2b). The recently isolated species within the *Moheibacter* genus were not yet reported to perform NO$_2^-$ reduction (Schauss et al., 2016).

Fig. 3. a) The sampling points used for microbial community analysis: activated sludge (AS) used as inoculum, and samples at the beginning (T0) and at the end (TF) of the denitrification (A), denitrification (B) and simultaneous denitrification-denitrification (C) experiments. b) Taxa plots with the relative abundances of the 25 most abundant genera and their correlation with the environmental data; c) Alpha diversity metrics; d) PCoA of community data using Bray-Curtis distance and Weighted Unifrac dissimilarity; e) Stochastic vs deterministic nature of communities using NRI and NTI.
Following the sPLS-DA algorithm, only 40 genera were varying between the conditions in the kinetic experiments (Fig. 4a-c). The communities, when two (A and C) or one electron acceptor (B) were used, mainly differed in terms of genera represented by *Stenotrophomonas*, *Kaistia*, *Moheibacter*, *Brevundimonas*, *Thauera*, *Propionibacteria*, *Seculamonas ecuadoriensis* (block b1); and *Bryobacter*, *Diaphorobacter*, *Actinobacteria*, *Rhodanobacter*, *Microbacterium*, *Pseudaminobacter*, *Dokdonella*, *Intrasporangium*, *Halothiobacillus*, *Thermomonas*, and *Sphingopyxis* (block b4). Block 1 was under expressed in denitrification (B), whereas block b4 was over expressed in denitrification (B), and vice versa for denitrification (A) and simultaneous denitrification-denitrification (C). Similarly, *Stenotrophomonas*, *Thauera*, *Diaphorobacter* and *Halothiobacillus* were also reported in denitrifying reactors with chemically synthesized $S^0$ (Xu et al., 2015; Zhang et al., 2015). *Rhodanobacter*, *Dokdonella* and *Thermomonas* genera within the *Xanthomonadaceae* family are capable of using organic products from cell lysis to fuel denitrification (Xu et al., 2015). *Pseudaminobacter* is capable to oxidize reduced sulfur compounds directly to $SO_4^{2-}$ (Ghosh and Dam, 2009).

The co-presence of the two electron acceptors mainly selected for (Fig. 4d): *Shinella*, *Rhizobium*, *Pleomorphomonas*, *Simplicispira*, *Limnobacter* (block b2); and *Nakamurella*, *Truiperia*, *Cellulomonas*, *Petrimonas*, *Clostridium sensu stricto 13*, *Bacillus*, *Ferruginibacter*, *Aminobacter*, *Cassellaniella*, *Isosphaera* (block b3). *Shinella*, *Rhizobium*, *Simplicispira* and *Limnobacter* were detected in reactors with reduced sulfur compounds treating NO$_3^-$ pollution (Christianson et al., 2015; Zhang et al., 2015).

Fig. 4. a) The number of latent components for genera table after evaluating the performance of the PLS-DA algorithm. b) The number of discriminating features in each of 3 components with minimum classification error rates. c) Color-coded clustered image map of the discriminating genera with the hierarchical agglomeration clustering on rows and columns shown as dendrograms.
The Nakamuraella, Truepera, Petromonas, Clostridium sensu stricto 13, Bacillus, Ferruginibacter Aminobacter, Castellaniella, Isophaera genera are known to comprise of some denitrifying bacteria (He et al., 2018; Zhao et al., 2016). In addition, looking at block b3, the right tree comprising Clostridium sensu stricto 13, Bacillus, Ferruginibacter, Aminobacter, Castellaniella, and Isophaera is overexpressed for both denitrification (B) and denitrification (A), indicating that those genera can degrade NO$_3^-$ (Park and Yoo, 2009; Spain and Krumholz, 2012; Xu et al., 2017).

3.4. Opportunities for ADBIOS as a part of a sustainable and integrated wastewater treatment system

ADBIOS (Fig. 5 [1]) provides a sustainable technological solution for biological nitrogen removal fueled by biogenic S$^0$, as a by-product of biogas desulfurization (Fig. 5 [2]). The benefits of the process, such as a 10-time faster kinetics (Section 3.2) compared to that of autotrophic denitrification with chemically synthesized S$^0$, make it technologically attractive and economically feasible. In addition, the high NO$_2^-$ degradation rate in the presence of a NO$_2^-$-enriched biomass suggests that ADBIOS can also be applied for NO$_2^-$ removal from wastewaters (Fig. 5 [1]). Generally, ADBIOS implements the reuse of a waste resource (S$^0$) into conventional nitrogen removal systems and creates a potential for an integrated process combining wastewater and flue gas treatment. Therefore, the scale-up of ADBIOS is of a great interest, and the current study can serve as the basis of the necessary fundamental information on the process. However, not each site may have readily-available biogenic S$^0$ supply, and biosulfur transportation might be required, which needs to be considered within an economic balance.

4. Conclusions

The biogenic S$^0$-oxidizing microbial consortia capable of reducing NO$_3^-$ or NO$_2^-$ mostly included Thiobacillus, Moebiabacter and Thermomonas. The biogenic S$^0$ showed an orthorhombic crystalline structure, having a 4.69 μm median particle size and a 3.38 m$^2$/g SSA, which made it particularly reactive and bioavailable. The specific denitrification and denitrification activities as high as 223.0 mg NO$_3^-$-N/g VSS/d and 339.5 mg NO$_2^-$-N/g VSS/d, respectively, resulted in enhanced denitrification and denitrification rates compared to those of chemically synthesized S$^0$. Moreover, the use of biogenic S$^0$ induced a lower accumulation of NO$_2^-$, alleviating the activity of the denitrifying consortia.

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Notes

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References


