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 (ABD BIOS) 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 denitrification, 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 denitritation 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 NO3\textsuperscript{−} concentrations when using chemically synthesized S0 during autotrophic denitrification 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 NO3\textsuperscript{−} accumulation. Therefore, it is crucial to investigate the potential of alternative electron donors for both NO3\textsuperscript{−} and NO2\textsuperscript{−} 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 Thiopaq® 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\textsuperscript{−} and NO2\textsuperscript{−} removal is required.

The present research aims to investigate the fundamental aspects of autotrophic denitrification with biogenic S0 (ADBIOS) using high-strength NO3\textsuperscript{−} and NO2\textsuperscript{−} 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\textsuperscript{−} and NO2\textsuperscript{−} 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 denitrification-denitritation in batch bioassays; and (iv) investigate the structure of the bacterial communities in the presence of NO3\textsuperscript{−}, NO2\textsuperscript{−} 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\textsuperscript{−} or NO2\textsuperscript{−} 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 clip. Subsequently, the bottles were placed on a gyratory shaker at 300 rpm and temperature was maintained at 30 ± 2 °C by means of a water bath. The enrichment was subcultured when NO3\textsuperscript{−}-N or NO2\textsuperscript{−}-N 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\textsuperscript{−} or NO2\textsuperscript{−} 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\textsuperscript{−} and S0), denitritation (B: NO2\textsuperscript{−} and S0) and simultaneous denitrification-denitritation (C: NO3\textsuperscript{−}, NO2\textsuperscript{−} and S0). The initial NO3\textsuperscript{−} or NO2\textsuperscript{−} 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). For the first experiment (A), batch bioassays were conducted to investigate the denitrification characteristics (NO3\textsuperscript{−}-N reduction rate, NO2\textsuperscript{−}-N accumulation and NO3\textsuperscript{−}-N reduction rates). During the second set of the experiments (B), NO3\textsuperscript{−}-N was used as the sole electron acceptor in order to evaluate the denitritation kinetics (NO2\textsuperscript{−}-N reduction rate) and assess the impact of biomass acclimation to NO3\textsuperscript{−}-N degradation. The simultaneous denitritation-denitrification experiment (C) was performed to study the effect of NO3\textsuperscript{−}-N on NO3\textsuperscript{−}-N degradation.

At the beginning of each experiment, the required amount of NO3\textsuperscript{−}-N and NO2\textsuperscript{−}-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\textsuperscript{−} or NO2\textsuperscript{−}) 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\textsuperscript{−}-N and NO2\textsuperscript{−}-N degradations, respectively, versus time and expressed as mg NOx\textsuperscript{−}-N/l/d. The biomass specific denitritation and denitrification activities (mg NOx\textsuperscript{−}-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 °C prior to analysis. NO3\textsuperscript{−}, NO2\textsuperscript{−} and sulfate (SO4\textsuperscript{2−}) concentrations were determined by ion chromatography, as reported elsewhere (Kiskira et al., 2017a; Mora et al., 2015; Park and Yoo, 2009; Zou et al., 2016). The liquid samples were filtered through 0.2 μm glass fiber filters (Whatman, Molsheim, France) prior to analysis. The samples were filtered and the filtrate was acidified with 0.5% (v/v) concentrated hydrochloric acid to avoid bacterial growth during analysis. The samples were stored at −20 °C until analysis. The pH of all samples was monitored during the experiments and it was maintained between 6.6 and 7.4.
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.

### Table 1

| Experiment | Initial concentration (mg/l) | pH |
|------------|-------------------------------|----|
| A: Denitrification (NO₂⁻ and S⁰) | 15 225 240 850 220^a | 8.7 ± 0.1 |
| B: Denitrification (NO₂⁻ and S⁰) | 240 – 240 850 215^b | 8.9 ± 0.1 |
| C: Denitrification and denitrification (NO₂⁻, NO₃⁻ and S⁰) | 110 70 180 680 220^c | 8.8 ± 0.1 |
| NO₂⁻ and NO₃⁻ controls | – – 850 220 | 8.8 ± 0.1 |
| S⁰-free controls | 240 – 240 – 220 8.9 ± 0.1 |
| Abiotic controls | 240 240 850 – 8.9 ± 0.1 |

|^a| Microbial source: biomass enriched on NO₂⁻-N and S⁰. |
|^b| Microbial source: biomass enriched on NO₃⁻-N and S⁰. |

2017b). Elemental S⁰ was determined by reversed-phase chromatography as originally described by Kamysny et al. (2009). In this study, a high-performance liquid chromatography (HPLC) system (Prominence LC-20A Series, Shimadzu, Japan) equipped with a Kinetex 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 HydroG sample dispersion wet unit. The measurement range of the instrument was from 0.02 to 2000 µm. Size parameters of the diameters dₐ, dₘ, and dₕ 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 (Freeczone 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 67–69% HNO₃ (ROMIL–Spa™, USA) and 3 ml of 30% H₂O₂ (TraceSelect® Ultra ≥ 30%, Sigma Aldrich, Germany) (Healy et al., 2016). The digested samples were transferred into trace metal-free centrifuge tubes (Labcon, Pelatuna, USA), and elemental concentrations were determined using a PerkinElmer ELAN DRCe ICP-MS (Perkin Elmer, USA) using both standard and dynamic reaction cell (DRC) mode with methane as the carrier gas (Ratcliffe et al., 2016) in a class1000 (ISO class 6) clean room.

### 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 (vsearch). 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. Multisquence 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 Qiime 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).
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 small biogenic S⁰ particle size of the biogenic S⁰ was of the same order of magnitude of the chemically produced S⁰ (Kleinjan et al., 2003). In this study, the small biogenic S⁰ with a smaller particle size compared to that of the chemically produced S⁰ (Kleinjan et al., 2003). In contrast, chemically produced S⁰ is more hydrophobic 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 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 SSA of 3.38 m²/g, compared to that of raw biogenic sulfur that had a SSA of 3.38 m²/g, compared to that of raw biogenic sulfur that had a SSA of 3.38 m²/g, compared to that of raw biogenic sulfur that had a SSA of 3.38 m²/g, compared to that of raw biogenic sulfur that had a SSA of 3.38 m²/g, compared to that of raw biogenic sulfur that had a SSA of 3.38 m²/g.

A higher accumulation of NO₂⁻/NO₃⁻ detected using ICP-MS (averages ± standard deviation; n = 3).

| Element | Value (± SD) (µg g⁻¹) | Element | Value (± SD) (µg g⁻¹) | Element | Value (± SD) (µg g⁻¹) |
|---------|-----------------------|---------|-----------------------|---------|-----------------------|
| Al      | 5.11 ± 0.97           | Ni      | 1.04 ± 0.10           | Cr      | 9.63 ± 0.23           |
| Ti      | 0.89 ± 0.13           | Cu      | 6.79 ± 0.32           | Fe      | 129.05 ± 5.21         |
| Mn      | 4.19 ± 0.20           | Mo      | 2.11 ± 0.06           | Zn      | 19.69 ± 1.34          |
| Co      | 2.78 ± 0.11           | Ba      | 0.61 ± 0.04           |

3. Results and discussion

3.1. Physico-chemical and elemental characterization of biogenic S⁰

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| Co      | 2.78 ± 0.11           | Ba      | 0.61 ± 0.04           |

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 NO₂⁻-N/l·d allowed the complete NO₂⁻-N degradation in 14 days with a final biomass concentration of 450 mg VSS/l. The NO₂⁻-N removal efficiency reached up to 84% after the first 5 days, with NO₂⁻-N accumulating up to 135 mg/l. The NO₂⁻-N 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 Nemat, 2012). The high NO₂⁻-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 NO₂⁻-N concentration above 60 mg/l on the activity of denitrifying biomass (Guerrero et al., 2016).

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

A higher accumulation of NO₂⁻-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 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.
substrate (Sahinkaya et al., 2015; Simard et al., 2015). As the microcrystallinity and hydrophilic properties (Section 3.1) provided a higher bioavailability of biogenic S\textsuperscript{0}, a faster degradation of the accumulated NO\textsubscript{3}–N (20.9 mg NO\textsubscript{3}–N/l·d\textsuperscript{-1}) during denitrification was achieved (Fig. 2a and c). Even a higher NO\textsubscript{3}–N degradation rate (73.0 mg NO\textsubscript{3}–N/l·d\textsuperscript{-1}) could be obtained by using the biomass enriched on NO\textsubscript{2}–N (Fig. 2b). Therefore, the use of NO\textsubscript{2}–N-acclimated biomass is recommended for biogenic S\textsuperscript{0}-driven denitrification treating high-strength NO\textsubscript{3}–N wastewaters to control the high NO\textsubscript{2}–N accumulation.

SO\textsubscript{2}–S was the only sulfur product of the biogenic S\textsuperscript{0} oxidation (Fig. 2f). This observation was confirmed by the stoichiometric consumption of the biogenic S\textsuperscript{0} with NO\textsubscript{3}–N or NO\textsubscript{2}–N (Sun and Nemati, 2012). No denitrification and denitritation were observed in the abiotic and electron donor-free controls (data not shown). In this study, specific denitrification and denitritation activities of 223.0 mg NO\textsubscript{3}–N–N/g VSSd and 339.5 mg NO\textsubscript{3}–N–N/g VSSd, respectively, were achieved by the biogenic S\textsuperscript{0}-oxidizing microbial consortium (Table 3). The high solubility of biogenic S\textsuperscript{0}, which was likely attributed to the hydrophilic properties and the lower particle size of the biogenic S\textsuperscript{0} particles (Section 3.1), induced a significantly higher NO\textsubscript{3}–N degradation (Fig. 2a). The kinetics of AD-BIOS (including both denitrification and denitritation) was characterized by 10-time higher rates compared to those obtained with chemically synthesized S\textsuperscript{0} (Kostrytsia et al., 2018), with both studies being performed at similar initial NO\textsubscript{3}–N and NO\textsubscript{2}–N concentrations.

### 3.3. Effect of different electron acceptors on microbial communities performing AD-BIOS

The efficiency of biological NO\textsubscript{3}–N and NO\textsubscript{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), denitritation (B) and simultaneous denitrification-denitrification (C) at the beginning (T\textsubscript{0}) and at the end (T\textsubscript{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\textsubscript{3}–N and NO\textsubscript{2}–N both involved) led to similar microbial communities with samples A T\textsubscript{E} and C T\textsubscript{E} 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\textsubscript{2}–N involved), a distinct community was formed (B T\textsubscript{E}) (Fig. 3d). Thus,

### Table 3

| Electron acceptor | Biogenic S\textsuperscript{0} | Chemically synthesized S\textsuperscript{0} (Kostrytsia et al., 2018) |
|------------------|-----------------------------|---------------------------------------------------------------------|
| NO\textsubscript{3}–N | Denitrification rate (mg NO\textsubscript{3}–N/l·d\textsuperscript{-1}) | 49.4 | 20.9 |
| NO\textsubscript{2}–N | Specific denitrification activity (mg NO\textsubscript{3}–N/g VSSd\textsuperscript{-1}) | 223.0 | 20.9 |
| NO\textsubscript{2}–N | Denitrification rate (mg NO\textsubscript{2}–N/l·d\textsuperscript{-1}) | 73.0 | 10.7 |
| NO\textsubscript{2}–N | Specific denitrification activity (mg NO\textsubscript{2}–N/g VSSd\textsuperscript{-1}) | 339.5 | 10.7 |

\( ^a \) Biomass enriched for 3.5 months on NO\textsubscript{3}–N and S\textsuperscript{0}.

\( ^b \) Biomass enriched for 3.5 months on NO\textsubscript{2}–N and S\textsuperscript{0}.
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 NO3⁻ 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 only 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 S⁰ and soluble S₂O₃²⁻ (Di Capua et al., 2016; Kostrytsia et al., 2018) and is capable of withstanding high NO₂⁻ 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 NO₂⁻ reduction (Schauss et al., 2016).
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, Propionibac- cella, Seculamonas ecuadoriensis (block b1); and Bryobacter, Diaphorobacter, Actinotalea, 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 $\text{S}^0$ (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, Pleomomomas, Simplicispira, Limnobacter (block b2); and Nakamurella, Truepera, Cellulomonas, Petrimonas, Clostridium sensu stricto 13, Bacillus, Ferruginibacter, Aminobacter, Castellaniella, 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.
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\textsuperscript{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\textsuperscript{0}, make it technologically attractive and economically feasible. In addition, the high NO\textsubscript{2}\textsuperscript{–} degradation rate in the presence of a NO\textsubscript{2}\textsuperscript{–}-enriched biomass suggests that ADBIOS can also be applied for NO\textsubscript{2}\textsuperscript{–} removal from wastewaters (Fig. 5 [1]). Generally, ADBIOS implements the reuse of a waste resource (S\textsuperscript{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\textsuperscript{0} supply, and biosulfur transportation might be required, which needs to be considered within an economic balance.

4. Conclusions

The biogenic S\textsuperscript{0}-oxidizing microbial consortia capable of reducing NO\textsubscript{3}\textsuperscript{–} or NO\textsubscript{2}\textsuperscript{–} mostly included *Thiobacillus*, *Moheibacter* and *Thermomonas*. The biogenic S\textsuperscript{0} showed an orthorhombic crystalline structure, having a 4.69 μm median particle size and a 3.38 m\textsuperscript{2}/g SSA, which made it particularly reactive and bioavailable. The specific denitrification and denitritation activities as high as 223.0 mg NO\textsubscript{3}\textsuperscript{–}/N/g VSS·d and 339.5 mg NO\textsubscript{2}\textsuperscript{–}/N/g VSS·d, respectively, resulted in enhanced denitrification and denitritation rates compared to those of chemically synthesized S\textsuperscript{0}. Moreover, the use of biogenic S\textsuperscript{0} induced a lower accumulation of NO\textsubscript{2}, alleviating the activity of the denitrifying consortia.

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Notes

The authors declare no competing financial interest.

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Fig. 5. Integrated ADBIOS (1) with NO\textsubscript{3}– and NO\textsubscript{2}– used as electron acceptors and biological gas desulfurization Thiopaq* (2).
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