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Effect of Sulfide on the Processes of Transformation of Nitrogen Compounds and the Microbial Community Composition in the Anammox Bioreactor

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Abstract: Anammox is one of the most important processes in the global nitrogen cycle and the basis for an efficient technology of nitrogen removal from wastewater. The effect of the presence of sulfide in wastewater on the transformation of nitrogen compounds by the anammox community has been insufficiently studied. The present work dealt with the effect of sulfide on nitrogen removal efficiency and the dynamics of nitrogen species in a laboratory sequencing batch bioreactor modeling the functioning of the anammox community carrying out ammonium oxidation via nitritation and anammox and nitrite oxidation. The 16S rRNA gene profiling of the community of the anammox-activated sludge attached to the stationary carrier revealed members of the key physiological groups: ammonium oxidizers of the genus *Nitrosomonas*, nitrite oxidizers of the genus *Nitrosospira*, and anammox bacteria of the genera *Candidatus* Brocadia and *Ca*. Jettenia, as well as members of other bacterial genera. Nitrate removal was not sensitive to sulfide at concentrations up to 50 mg S/L and decreased by 17% at 100 mg/L. The threshold of sulfide sensitivity for group I nitrifiers was ~50 mg/L, while anammox bacteria were resistant to sulfide concentrations of up to 100 mg S/L in the incoming water. Group II nitrifiers were probably the most sulfide-sensitive components of the community. A drastic increase in the abundance of members of the family *Hydrogenophilaceae* at elevated sulfide concentrations, together with the precipitation of elemental sulfur, may indicate sulfide oxidation either by molecular oxygen or via nitrate reduction; this finding requires further investigation. This is the first report on the different effects of sulfide on the growth rate of members of the nitrifying genus *Nitrosomonas*, increasing/decreasing or not affecting it for different phylotypes at elevated sulfide concentrations.

Keywords: anammox process; anammox community; sulfide; nitrogen removal efficiency; sulfide sensitivity; metatranscriptome analysis

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1. Introduction

At wastewater treatment plants (WWTPs), sulfides enter the bioreactors with incoming wastewater, where their concentration can exceed 3 mg S/L [1]. They are usually associated either with sulfate reduction at the anaerobic stages of water treatment or in the sewage system or with sulfides of industrial wastewater [1,2]. The inhibitory effect of sulfide on the processes of biological water treatment, especially on nitrification, as well as their unpleasant odor and corrosive properties, resulted in elevated attention to sulfide in wastewater [3–5].

Ammonium and sulfide may be present simultaneously in such wastewater as reject water of anaerobic digesters, where both anaerobic degradation of amino acids and nucleic

acids (with ammonium release) and sulfate reduction (with sulfide release) occur. Anaerobic ammonium oxidation (anammox) is considered a mature technology for the treatment of such wastewater. In deammox, one of the varieties of anammox-based technologies, it was even suggested to use sulfide as an electron donor for nitrite production via partial denitritation [6]. Thus, investigation of the effect of sulfide on the anammox process and anammox bacteria is an urgent issue.

Although sulfide is considered a classical inorganic inhibitor of the anammox activity, its effect on this process has been rather poorly studied [7–9]. The inhibitory effect of sulfide is considered as depending, apart from its concentration, also on the nature of the anammox community (composition of the microbial consortium from activated sludge or environmental samples), conditions of the anammox process, concentration of the substrates (ammonium and nitrite), pH, and other factors. The inhibitory sulfide concentration determined in the experiments varies widely and depends significantly on the duration of the effect of the inhibitor on anammox bacteria. Thus, 50% inhibition of the anammox community from activated sludge involved in water treatment occurred at 264 mg S/L in the case of short-term sulfide application, while its long-term action resulted in a manifold decrease in activity already at 32 mg S/L [10]. The inhibitory sulfide concentrations cited in the literature usually vary within the range of 16–190 mg S/L [11–13]. In some works [14,15], inhibitory action against the anammox bacteria was especially pronounced for the unionized form of hydrogen sulfide, with activity decreasing twofold at concentrations as low as 1–5 mg H_2S-S/L. Since the ratio of sulfide forms in solution depends on pH, at the same total sulfide concentrations, the inhibitory effect increases under acidic conditions.

In the nitrification/anammox technological scheme (N/A, deammonification), the effect of sulfide on the balance of nitrogen species is determined by its action not only on the anammox bacteria but also on the nitrifying microorganisms responsible for stages I and II of nitrification. A number of works indicate that while sulfide inhibits both groups of nitrifiers, the activity of nitrite oxidizers is suppressed to a greater extent, making it possible to use hydrogen sulfide for optimization of the N/A process by suppressing stage II of nitrification and accumulating nitrite, the substrate for anammox bacteria [4,16–18]. The 50% inhibitory sulfide concentrations for group I nitrifiers are usually within the range of 0.73–14 mg S/L, although long-term exposure to sulfide results in the gradual adaptation of ammonium oxidizers to this inhibitor [19]. The effect of H_2S on the nitrogen removal efficiency of each anammox community is different. The goal of the present work was, therefore, to investigate the effect of sulfides on nitrogen removal efficiency in the previously described bioreactor [20,21] and to reveal the blocks of the nitrogen cycle most sensitive to this factor by monitoring the changes in bacterial abundance and levels of the key enzymes.

2. Materials and Methods

2.1. Experimental Setup

The experiments were carried out in two identical sequencing batch reactors operating in parallel. The working volume was 4.5 L; a Polivom cylindrical carrier of fibrous polyethylene and polypropylene (Etek, Kaluga, Russia) with a surface area of 11.6 dm^2 was used as the carrier for biofilm development. The synthetic medium contained the following (g/L): $(NH_4)_2SO_4$, 0.942 (200 mg N–NH_4/L); $NaCH_3COO{\cdot}3H_2O$, 0.04; KH_2PO_4, 0.044; and $NaHCO_3$, 2.1. The reactors were operated in the same mode at 32 ± 2 °C and pH 8.3 ± 0.1. At this pH, sulfide dissociates in the water solution and is present mainly as HS^- [22], without its massive release into the gas phase. pH was determined with an EXPERT-03 m ("Econix", Moscow, Russia). All chemicals and instruments were supplied by Dia-M, Moscow, Russia.

The duration of each cycle was 6 h. Each cycle consisted of three phases: alternated aeration and mechanical stirring (310 min); sedimentation (20 min); and simultaneous supply of 1 L of fresh medium and removal of 1 L of the processed one (30 min). Fresh medium

was supplied from the bottom, which resulted in simultaneous piston-like displacement of purified water from the top without mixing. The minimal and maximal concentrations of dissolved oxygen during the periods without and with aeration (2 min each) were 0.2 and 2 (±10%) mg/L, respectively. A detailed description of the bioreactors and the analytical techniques has been provided previously [20,21].

The reactor was inoculated with the anammox-activated sludge (AS) grown in the setup described above and collected by mechanical removal from the load after short-term storage at +4 °C. To launch the new cycle of operation, AS was suspended in fresh medium (1–1.5 L) and introduced into the working volume (2.25 g per reactor). Attachment of almost all AS to the load occurred within two weeks. Throughout the incubation, the amount of free-floating sludge in the reactor was minimal, not exceeding 2% of the introduced AS.

2.2. Experiments on the Effect of Sulfide on Nitrogen Removal Efficiency, Abundance of Bacteria, and Activity of the Specific Genes

Reactors no. 1 and no. 2 were the control and experimental ones, respectively. Sulfide was added to reactor no. 2 in stepwise-increasing concentrations, from 5 to 100 mg S per liter of the incoming solution. Sulfide concentrations were increased to 5, 15, 25, 50, and 100 mg S/L on days 44, 78, 83, 85, and 90 of the experiment, respectively. The experiment was carried out until a considerable biological effect was achieved, i.e., decreased nitrogen removal efficiency and complete suppression of group II nitrifiers. Moreover, massive precipitation of amorphous sulfur on the components of the reactor occurred on days 6–7 after the sulfide concentration was increased to 100 mg S/L; this was another reason to terminate the experiment. Its total duration was 100 days.

AS samples for DNA isolation and determination of the composition of the microbial community were collected at specified time intervals.

To determine the genes involved in the response of the anammox community to a drastic increase in sulfide concentration, a special experiment was carried out. After the end of the main experiment described above (on day 100), AS from the control reactor no. 1 was collected for RNA isolation, and sulfide (100 mg S/L) was added to the incoming water. AS for RNA isolation was then collected after 24 h, when the inhibitor concentration in the liquid phase was 68 mg S/L (at 100 mg S/L in the incoming medium).

Samples of outflowing water (for chemical analysis) and of the biofilm developing on the load (for molecular profiling of the community composition) were regularly collected.

The concentrations of ammonium, nitrate, and nitrite ions were determined using standard methods [23]. Sulfide concentration was measured on a Kapel 205 capillary electrophoresis system (Lumex, Saint Petersburg, Russia). Identification and quantitative determination were carried out by indirect detection measuring ultraviolet absorption at 254 nm. Electrophoresis was carried out in untreated fused quartz capillaries 60 mm long (effective length, 50 cm) and 75 μm in internal diameter, using the background electrolyte based on chromium (VI) oxide supplemented with diethanolamine and cetyltrimethylammonium hydroxide. The temperature in the capillary was maintained at 20 °C, and the applied voltage was −17 kV. The calibration curve for HS^- was obtained using dilutions of the Na_2S stock solution (5–100 mg S/L) in phosphate buffer (Na_2HPO_4, 0.4 g/L, pH 9 ± 0.2). The amount of removed nitrogen (per 1 L of the liquid phase) was calculated as the difference between the concentration of ammonium nitrogen (N–NH_4) in the incoming medium and the total concentration of the mineral nitrogen species (N–NH_4, N–NO_2, and N–NO_3) in processed water. The efficiency of nitrogen removal was calculated as the percentage of removed nitrogen from its concentration in the inflowing medium.

2.3. Analysis of the Composition of Microbial Communities

The composition of the activated sludge microbial communities was analyzed by high-throughput sequencing of the 16S rRNA gene fragments. Activated sludge was sampled from the reactor after 44, 72, 85, 92, and 99 days of operation.

DNA isolation and taxonomic analysis of the composition of microbial communities were carried out according to the method described in the work of Kallistova et al. [24].

2.4. Metatranscriptome Analysis

Total RNA was isolated from the activated sludge biomass with the RNA PowerSoil Total RNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA USA). Ribosomal RNA was removed using the QIAseq FastSelect -5S/16S/23S Kit (Qiagen, Hilden, Germany). Total RNA concentration was determined by the fluorescence method using the Qubit RNA HS Assay Kit (ThermoFisher, Waltham, MA, USA). RNA quality was determined by microcapillary electrophoresis using the RNA 6000 Pico Kit (Agilent, Waldbronn, Germany). The cDNA libraries were prepared with the NEBNext® mRNA Library Prep Reagent Set for Illumina (New England Biolabs, Inc, Ipswich, MA, USA) according to the manufacturer's protocols. Sequencing of cDNA was performed on the HiSeq-2500 platform (Illumina, San Diego, CA, USA) (in the 2 × 50 nt format). The experiments were carried out in triplicate.

Low-quality ends were trimmed with sickle v1.33. All reads were assembled into transcripts with v2.13.2. The protein-coding regions were predicted using prodigal v2.6.3 with the parameters for the metagenome. On assembled transcripts, transcriptome reads were superimposed using bowtie2 v2.3.5.1, and the read number per each protein-encoding gene was determined using htseq-count v2.0.2. Differential gene expression analysis was carried out using the R library of Deseq2: normalization of read number, calculation of the variation in expression level, estimation of *p*-value statistical significance, and adjusted *p*-value for expression variation. Functions of the genes were determined using the KEGG database with KofamScan v1.3.0 and using the Uniref database with diamond v2.0.6. KEGG annotation was used to analyze enrichment by the KEGG metabolic pathways using the R library of clusterProfiler v4.4.4. Taxonomic classification of the set of protein-coding genes was obtained using kaiju v1.8.2.

2.5. Statistical Treatment of the Results

Concentrations of nitrogen species and oxygen were determined in triplicate. Average values and average absolute deviations were determined for each point. This value was within the experimental error and did not exceed 4%. The differences exceeding 8% were therefore considered significant.

3. Results

3.1. Bioreactor Operation

The data on nitrogen removal (a) and the efficiency of bioreactor operation (b) are presented in Figure 1.

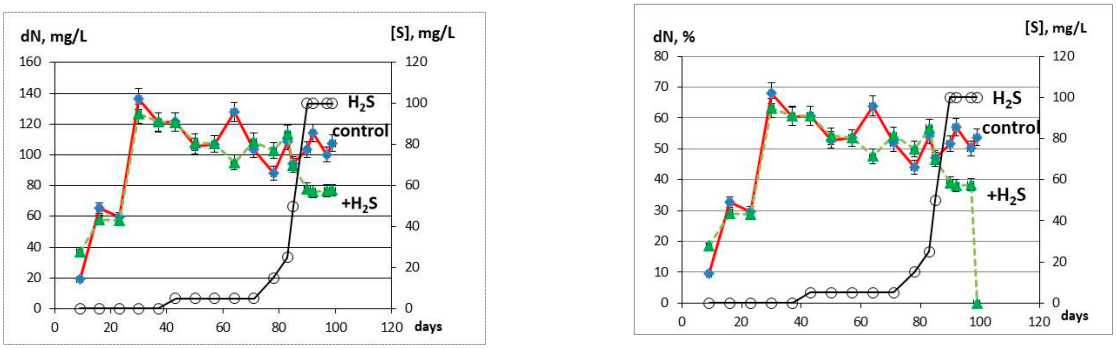

Figure 1. Operational parameters of the control (solid red line) and experimental (broken green line) bioreactors: nitrogen removal, dN, mg/L, (**a**) and its efficiency, % (**b**). The black broken line shows sulfide concentration in the incoming water.

The reactors achieved the stable operation mode after the first 43 days; by the end of this period, the removal of 120–140 mg/L nitrogen out of 200 mg/L in the incoming water was observed, and the nitrogen removal efficiency was 60–70%. This parameter decreased to 50% by day 100 of the experiment due to the accumulation of the activated sludge biomass on the carrier, which resulted in hindered oxygen diffusion into the biofilm's inner layers. An increase in oxygen supply to maintain the high nitrogen removal efficiency was not used in order to avoid adding a second variable (aeration) to the studied one (sulfide concentration).

Sulfide addition was commenced on day 44 of operation, when stable nitrogen removal was achieved in both reactors.

It was shown in a special preliminary experiment that under the conditions of the bioreactors used (aeration, active mixing of the liquid phase, open surfaces, and pH 8.0–8.4), sulfide was not blown out or oxidized abiotically by oxygen to S^0, with its concentration remaining constant throughout the 6 h cycle after the addition of the fresh medium; the sulfide concentration in the reactor agreed with the calculated values. This observation is supported by the literature data [25].

Unexpectedly, no decrease in the amount of removed nitrogen was observed at sulfide concentrations in the incoming solution from 5 to 50 mg S/L. The amount of removed nitrogen decreased from 93 to 78 mg/L (by 17%) only at a sulfide concentration of 100 mg S/L. Sulfide concentrations above 100 mg S/L were not tested due to the massive precipitation of elemental sulfur both on the activated sludge biomass and on the walls and tubing of the bioreactor.

Analysis of dependence between the concentrations of ammonium, nitrite, and nitrate (the substrates and intermediates of nitrogen transformation in the bioreactor) and sulfide concentration (Figure 2) was used to assess sulfide sensitivity of the individual bacterial groups: anammox bacteria (AB) and ammonium-oxidizing and nitrite-oxidizing bacteria (AOB and NOB, respectively).

Ammonium concentration in processed water increased significantly at a sulfide concentration of 50 mg S/L. At the same sulfate concentration, nitrite concentration decreased by 18% compared to the control. A significant decrease in nitrate concentration was observed already at 15 mg S/L sulfide, while at 100 mg S/L, it decreased tenfold. The threshold of sulfide sensitivity for group I nitrifiers, which were responsible for ammonium oxidation, was 50 mg S/L. Anammox bacteria were probably more resistant, since nitrite concentration decreased slightly, while inhibition of anammox bacteria with retained activity of group I nitrifiers was expected to result in its same or elevated level. The observed drastic decrease in nitrate concentration indicated that group II nitrifiers, responsible for nitrate accumulation, were the most sulfide-sensitive group.

3.2. Taxonomic Composition of the Microbial Community in the Presence of Sulfide

To assess the effect of sulfide on the composition of the microbial community, the taxonomic composition of microorganisms in the activated sludge of the control bioreactor (C), to which sulfide was not added, was compared to that of the experimental reactor (S), to which sulfide was added up to 100 mg S/L.

A total of 153,698 sequences of the V3–V4 variable fragments of the 16S rRNA gene were analyzed. The Chao1 and Shannon biodiversity indices did not change significantly during the operation of the reactor, which indicated the stability of microbial diversity in the studied samples.

Clusterization resulted in 192 OTUs belonging to 22 bacterial phyla according to the GTDB system [26]. The four predominant phyla were *Chloroflexi* (12.91–42.59%), *Proteobacteria* (17.19–26.34%), *Bacteroidota* (17.5–48.42%), and *Planctomycetota* (2.64–16.68%). Their overall abundance in each sample exceeded 90%. Minor phyla, constituting not more than 3% in one of the samples, were represented by *Verrucomicrobiota*, *Spirochaetota*, *Bdellovibrionota*, WPS-2, *Actinobacteriota*, *Armatimonadota*, *Nitrospirota*, *Acidobacteriota*, *Myxococcota*, *Sumerlaeota*, *Desulfobacterota*, *SAR324_clade (Marine_group_B)*, *Dependentiae*, *Dadbacteria*, *Fir-*

micutes, *Patescibacteria*, and *Hydrogenedentes*. Archaea were not revealed in the communities of the studied bioreactors.

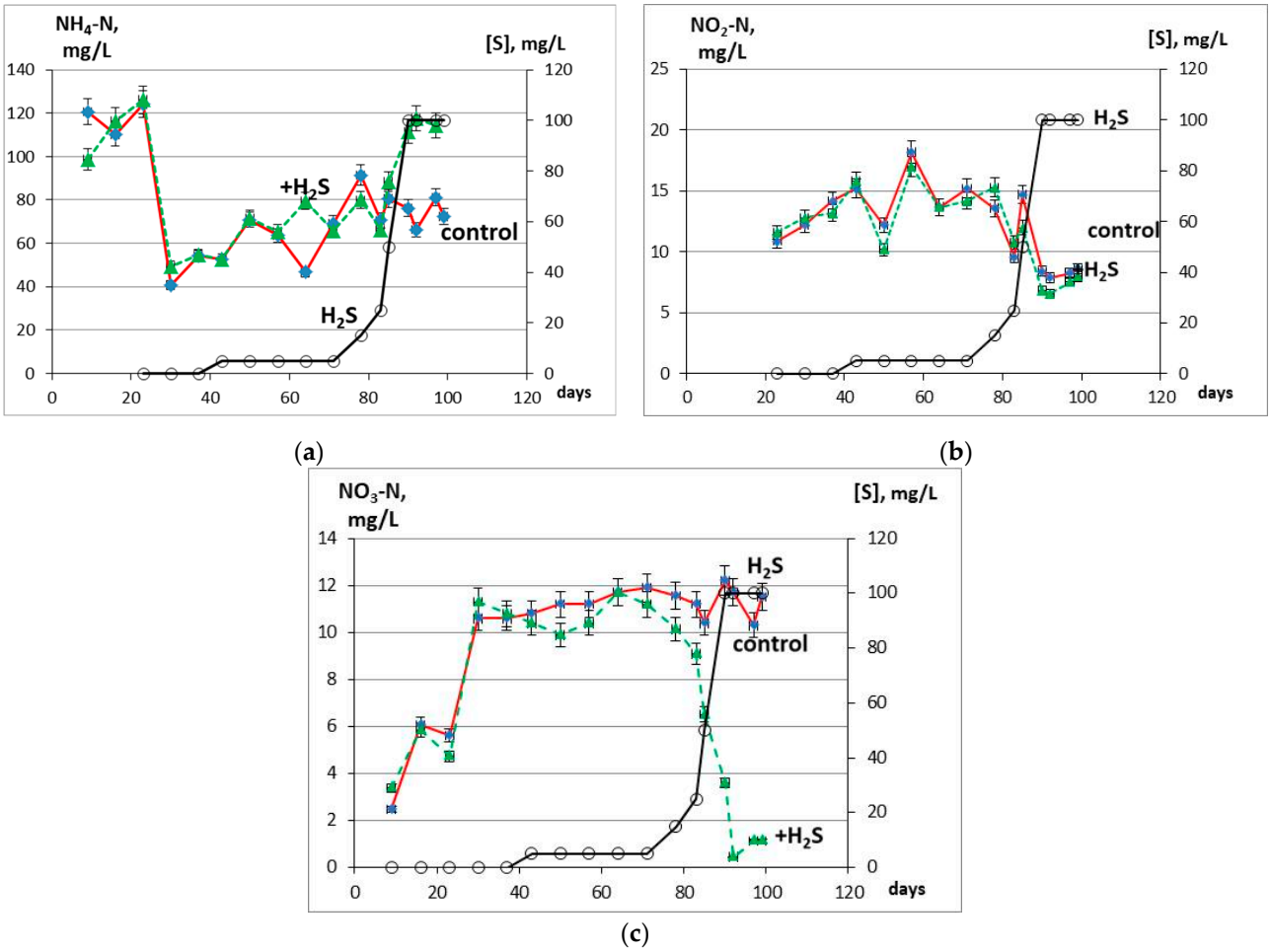

Figure 2. Dynamics of nitrogen species in the water treated in the anammox reactor: concentrations of ammonium (**a**), nitrite (**b**), and nitrate (**c**) in the control (red solid line) and experimental (green broken line) reactors. The black line shows sulfide concentration in the incoming water.

In general, the microbial taxonomic composition changed synchronously in the course of operation of two bioreactors (Figure 3).

The nitrifiers revealed belonged to the genera *Nitrosomonas* (OTU2, OTU11, OTU26, and OTU102) and *Nitrospira* (OTU94 and OTU162), belonging to the phyla *Proteobacteria* and *Nitrospirota*, respectively. In the control reactor, the abundance of nitrifiers did not change significantly during its operation and varied from 13.65 to 20.22% (Figure 4). Increased sulfide concentration in the medium resulted in various changes in the relative abundance of stage I nitrifiers of the genus *Nitrosomonas* (Figure 4). The share of *Nitrosomonas* OTU26 increased from 0.004 to 6.6%, while that of OTU2 decreased from 10 to 2%. The shares of OTU11 and OTU102 did not change in the presence of sulfide. The relative abundance of stage II nitrifiers of the genus *Nitrospira* did not change significantly during the operation of both the experimental and control reactors. At all sulfide concentrations, the overall abundance of *Nitrosomonas* in the activated sludge community was relatively constant (13–20%), with no significant differences between the experimental and control variants.

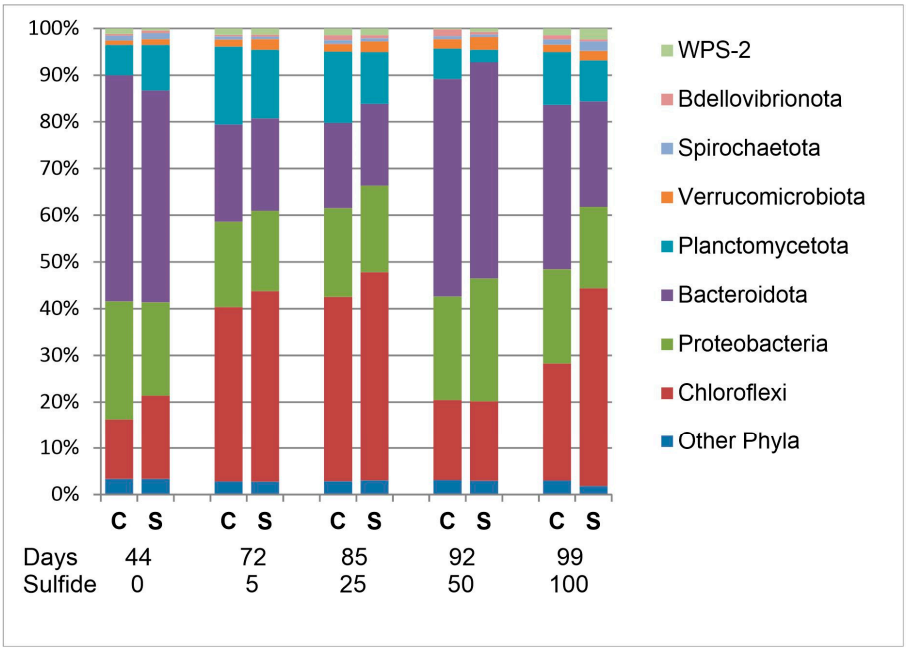

Figure 3. Relative abundance of bacterial phyla in the activated sludge communities of the control (C) and experimental with sulfides (S) bioreactors. The numerals indicate time of the bioreactor operation (days, upper row) and sulfide concentration in the incoming water (mg S/L, lower row).

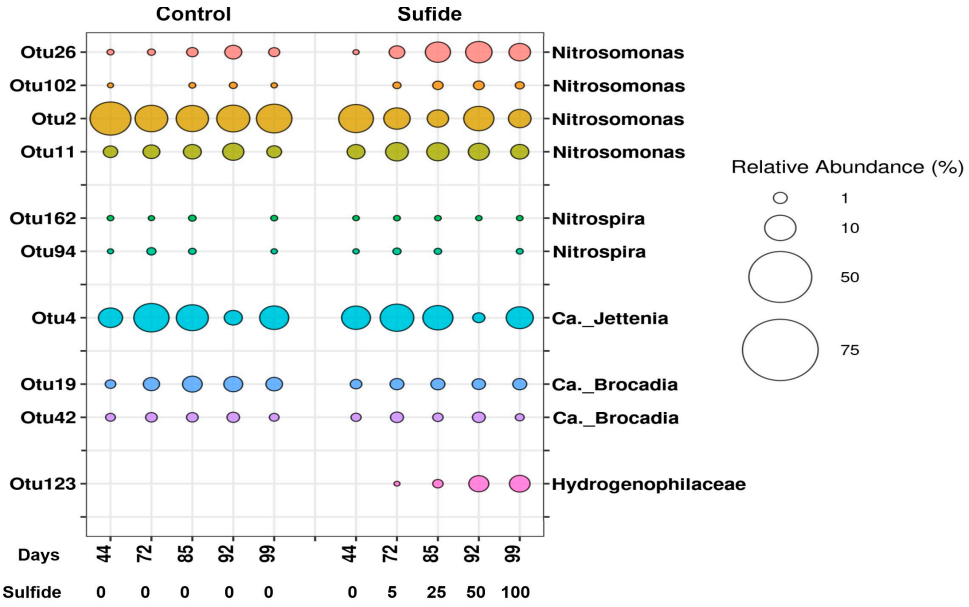

Figure 4. Relative abundance of bacterial genera involved in nitrogen metabolism.

Anammox bacteria were represented by two genera, *Ca*. Jettenia (Otu4) and *Ca*. Brocadia (Otu19 and Otu42). Members of the genus *Ca*. Jettenia were predominant (5–13%), while the share of *Ca*. Brocadia varied from 0.5 to 3.5%. The addition of sulfide had no effect on the relative abundance of anammox bacteria in the bioreactor. During the experiment, the shares of anammox bacteria in the control and experimental bioreactor changed similarly (Figure 4).

Among sulfate reducers, members of the phylum *Desulfobacteriota* were found (not more than 0.2%); their relative abundance was not affected by the presence of sulfide.

Interestingly, during the operation of the experimental bioreactor, the relative abundance of members of the family *Hydrogenophilaceae* (OTU123) increased from 0 to 3.4%,

while these organisms have never been detected in the control bioreactor (Figure 4). The sequences most closely related to OTU123 belong to uncultured microorganisms retrieved from wastewater and the activated sludge of a wastewater-processing laboratory MBR reactor (JX040326—99%, MH761772—100%).

3.3. Metatranscriptomic Analysis of the Microbial Community

The profiles of gene transcription in the microbial community were analyzed prior to sulfide addition and 24 h after it. Analysis was performed in three replicates, and a total of 1072.51 × 10^6 reads were obtained, with at least 163.6 × 10^6 reads for each sample.

Metatranscriptome assembly resulted in the identification of 84,342 protein-coding genes, among which the expression of 13,985 increased more than twice and 16,370 decreased more than twice. Transcription levels of the remaining 53,987 genes did not change.

Analysis of transcription profiles of the key genes of nitrogen and sulfur metabolism (Figure 5) revealed significant changes in the expression of some of them upon sulfide addition. Thus, the expression of transcription of the key genes of the anammox reaction, nitrite reductases *nirK* and *nirS* (2.8 and 3.9 times, respectively), and hydrazine synthase *hzs* (2.6 times) was observed for anammox bacteria of the order Ca. *Brocadiales*. The transcription level of the *hdh* hydrazine dehydrogenase did not change. This gene encodes the enzyme responsible for the last stage of the anammox process: hydrazine oxidation with the formation of dinitrogen.

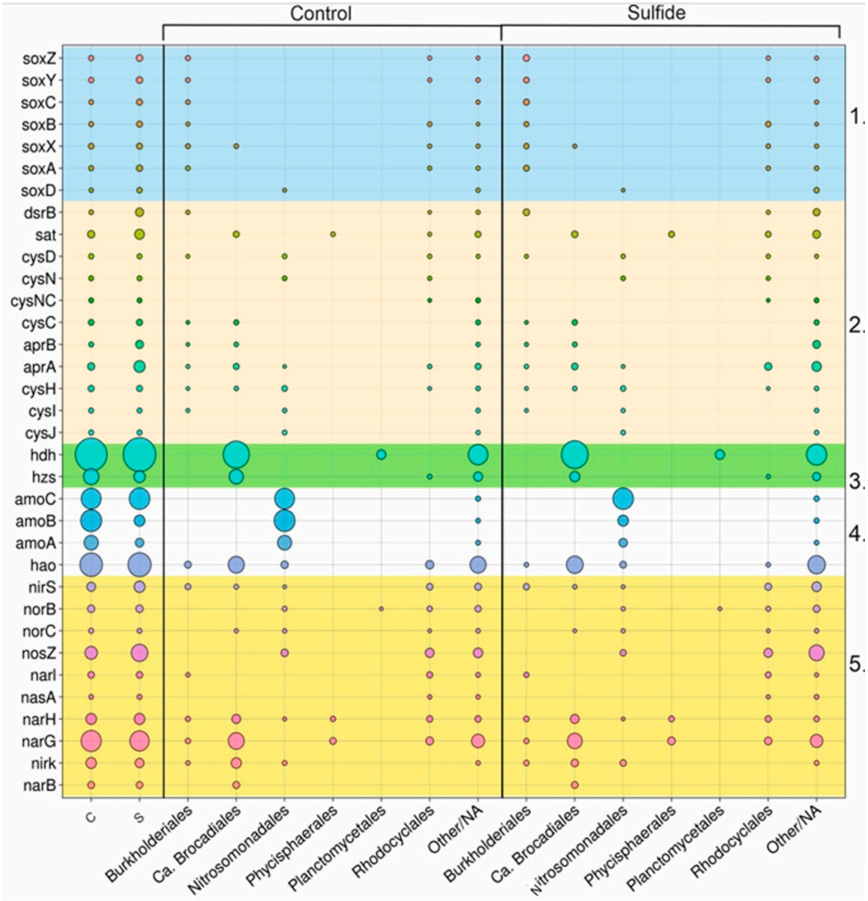

Figure 5. Profiles of expression of the genes of sulfur and nitrogen metabolism in various taxonomic groups. The functional genes were identified according to the KEGG database. Columns C and S are summarized profiles before and after sulfide addition, respectively. The numerals at the right and colored shading indicate the metabolic pathways: sulfur oxidation (1), sulfate reduction (2), anammox (3), nitrification (4), and denitrification (5).

The addition of sulfide resulted in a significantly decreased transcription of the ammonium monooxygenase genes *amoB* and *amoC* (six and four times, respectively) in the nitrifiers of the order *Nitrosomonodales*. Members of this order also exhibited decreased transcription of the gene for a *norB* subunit of nitric oxide reductase (3-fold), which is involved in N_2O formation out of NO.

The transcription of most genes of the sulfur cycle increased twofold and more upon the addition of sulfide. These were the genes associated with sulfur oxidation (*soxA*, *soxB*, *soxC*, *soxD*, *soxZ*, *soxX*, and *soxY*), as well as those associated with dissimilatory sulfate reduction (*aprA*, *aprB*, and *dsrB).* Most of these genes belonged to members of the orders *Burkholderiales* and *Rhodocyclales*.

4. Discussion

Analysis of the activity of the individual bacterial groups assumed that NOB are mainly responsible for nitrate production, while AB are responsible for nitrogen removal; the NO_2^- level depends on the activity of both AB and AOB: AB cause a decrease in nitrite content, while AOB cause an increase in it. The direction in which NO_2^- and NO_3^- concentrations change was used as an indicator of activities of AB, NOB, and AOB.

Based on our data, it may be concluded that NOB were sensitive to sulfide: since a decrease in nitrate concentration was observed at a sulfide concentration of 15 mg S/L, and it decreased tenfold at 100 mg/L, NOB were the group most sensitive to sulfide, and AB were the most resistant group, which retained activity at up to 50 mg S/L and showed only an insignificant decrease at 100 mg S/L. For group I nitrifiers, the threshold of sulfide sensitivity was 50 mg S/L.

The high resistance of AB to sulfide may be explained by their location within aggregates and in the deeper layers of the carrier-immobilized biocenosis.

Aggregation of the anammox bacterial biomass is known to alleviate the inhibitory effect of sulfide. Inhibition of the anammox process is most pronounced in diluted suspensions and is less efficient in the case of aggregated dense cultures. Thus, Jensen et al. [27] found that for short-term treatment of communities of the Black Sea water samples, the anammox-inhibiting sulfide concentration was as low as 4 μmol/L (128 μg S/L). Most works in this area used granular biomass enriched with anammox bacteria, for which the inhibitory sulfide concentrations reached tens of milligrams per liter. A comparative assessment of resistance of aggregated and non-aggregated biomass to inhibitors was presented by Carvajal-Arroyo et al. [14]. Sulfide concentration causing 50% inhibition of anammox activity in granular biomass (with predominance of *Candidatus* Brocadia) was 3.3 times higher than in the case of a cell suspension. Apart from sulfide, higher resistance of granular activated sludge to other toxic compounds (heavy metals, organic toxicants, and antibiotics), as well as to shock situations, has been reported in a number of works [28–30].

The retention of high anammox activity is also explained by the protective properties of the extracellular polymers, which bind the inhibitors and prevent their contact with the cells [31,32].

Most studies have been made using the cultures enriched with *Candidatus* Kuenenia stuttgartiensis [9] and *Candidatus* Brocadia sp. [15]. In the present work, anammox bacteria of the genera *Ca*. Brocadia and *Ca*. Jettenia were present in the activated sludge.

The literature data on the inhibitory sulfide concentrations are usually within the range of 16–190 mg S/L [11–13]. In our case, 100 mg S/L caused an activity loss of not more than 17%.

The undissociated form of H_2S, rather than the total sulfide concentration, was shown to have the most pronounced effect on the anammox process. Carvajal-Arroyo et al. [14] showed that for undissociated H_2S, the concentrations causing 50% inhibition were 0.03 and 0.1 mM for suspensions and aggregates, respectively. Since in our bioreactor, the pH of the medium was 8.3, such that only ~4% of the introduced sulfide was present in the non-ionized form (H_2S), 0.1 mM H_2S concentration (0.32 mg/L) was achieved at the total sulfide concentration of at least 1 mM (32 mg S/L). The resistance of the studied anammox

association was, in fact, considerably higher. Cases of AB resistant to sulfide concentrations of up to 190 mg S/L have been reported previously [11–13].

Preadaptation of AB to sulfide, an additional factor explaining their high sulfide resistance, was due to the fact that this microbial community was initially obtained from the sludge of a sludge-processing bioreactor in which sulfide was always present.

The higher sensitivity of nitrifying bacteria to sulfides, compared to the anammox bacteria, may also be explained by stratification in the activated sludge attached to the carrier. Aerobic AOB and NOB are localized in the surface layers of the sludge aggregates with higher sulfide availability.

Ortiz et al. [4] reported the half-inhibitory sulfide concentrations of 2.6 and 1.2 mg S/L for AOB and NOB, respectively, while Joye & Hollibaugh [3] observed 100% inhibition of nitrification (NOB) at 32 mg S/L, which was comparable to our results. In our study, however, AOB were much more resistant than in other works, which reported a 50% decrease in their activity already at 0.73–14 mg S/L [19].

An investigation of the taxonomic composition of the microbial community in the presence of sulfide revealed that no significant changes occurred during long-term cultivation. Observed variations in the relative abundance of the major phyla were probably associated with the conditions of bioreactor operation (temperature, etc.), rather than with the addition of sulfide to the medium. In the studied bioreactor, anammox bacteria were represented by two genera, *Ca*. Jettenia and *Ca*. Brocadia, probably occupying different ecological niches [33]. Among the nitrifiers, *Nitrosomonas* and *Nitrospira* were present, which have also been revealed in other wastewater-treating bioreactors. Interestingly, 4 *Nitrosomonas* phylotypes were found in the studied microbial community. In the course of cultivation, the relative abundance of OTU2 decreased and that of OTU26 increased, while the overall share of this microbial group did not change significantly. It may be hypothesized that the addition of sulfide acted as a selective factor for OTU26.

Interestingly, the addition of sulfide favored the development of members of the family *Hydrogenophilaceae*. It is known from the literature that some *Hydrogenophilaceae* members are able to grow in the presence of sulfide. The presence of sulfide as an electron donor and of molecular oxygen or nitrogen oxides as electron acceptors are crucially important for growth [34]. Many colorless sulfur bacteria of the family *Hydrogenophilaceae* grow in narrow zones and gradients, where sulfide and oxygen occur simultaneously [35], e.g., in stratified lakes and at the boundaries between oxic and anoxic zones, sediments, and wet soils. In our experiments, a massive deposition of amorphous sulfur on the walls and components of the bioreactor was observed, which confirmed the occurrence of sulfide oxidation with oxygen, nitrite, and/or nitrate with the production of elemental sulfur. The role of sulfate reduction to elemental sulfur was negligible due to the absence of suitable electron donors and the presence of both oxygen and nitrate in the medium. It may be hypothesized that the decrease in nitrate concentration at the highest sulfide concentration in the reactor was partially associated with the activity of *Hydrogenophilaceae* members. According to the series of preferable electron acceptors, oxygen, rather than nitrate, should have been primarily used for sulfide oxidation. At the same time, some studies revealed that filamentous colorless sulfur oxidizers are capable of using nitrate instead of oxygen for sulfide oxidation. They migrate between the surface, where they acquire nitrates and store them in their vacuoles, and the sulfide zone, where they access sulfides [36]. Thus, the issue of the preferable electron acceptor for sulfide oxidation to elemental sulfur in anammox reactors is of interest and requires further investigation.

The exact mechanisms of action of sulfide on anammox bacteria are presently unknown [9]. In the present work, metatranscriptome analysis was used to investigate the effect of sulfide on the transcription of the key genes of nitrogen and sulfur metabolism. Since the metatranscriptome experiment was carried out in one day, no significant changes in the composition of the microbial community were observed. The addition of sulfide resulted in a noticeable decrease in the activity of the genes of the initial stage of the anammox metabolism, *nirK*, *nirS*, and *hzs*, in members of the genus *Brocadiales*. At the

same time, transcription of the hydrazine dehydrogenase gene, responsible for hydrazine decomposition to nitrogen and hydrogen in the annamoxosome, did not change. The anammox process requires nitrite, which was produced in the bioreactor via ammonium oxidation by stage I nitrifiers of the genus *Nitrosomonas*. Our metatranscriptome data indicate decreased transcription of ammonium monooxygenase genes in the nitrifiers of the order *Nitrosomonodales* upon the addition of sulfide, which is in agreement with the previous data [4,5]. This resulted in the decreased production of nitrite, which the anammox bacteria require for ammonium oxidation. At the same time, nitrite was not consumed completely (Figure 2b), i.e., the anammox bacteria did not face serious nitrite limitation.

Enhanced transcription of the genes of sulfur metabolism indicated the activity of sulfur-oxidizing bacteria and of the pathway of assimilatory sulfate reduction, which occurs in various microbial groups.

The observed contradiction between an insignificant effect of sulfide on the functional activity of the anammox bacteria and stage I nitrifiers (a decrease not exceeding 17%) and a significant (several times) decrease in transcription of the genes responsible for the synthesis of crucially important enzymes in the presence of sulfide may be explained by a considerable safety margin of the biological systems. Moreover, the experiment on the effect of sulfide was carried out with its concentrations increasing gradually so that preadaptation to this toxic agent was possible.

The observed suppression of nitrification, especially of its first stage, while the abundance of the relevant microorganisms did not decrease, may be explained by the fact that attached activated sludge prevents dynamic selection, i.e., washing out of the non-growing or slowly growing species.

Our results suggest an unexpected and technologically important conclusion that sulfides may be used in the anammox process as useful components of the medium, which probably may inhibit the NOB, competitors of the anammox bacteria, thus stabilizing the anammox process and decreasing nitrate concentrations in the processed water.

Our results may also explain the high activity of anammox in the natural ecotopes with high sulfide concentrations, e.g., in the Black Sea. At the depth of 100–150 m, where oxygen concentration does not exceed several milligrams per liter [37], ammonium may be oxidized by AOB to nitrite, which is utilized by AB. Since both bacterial groups may be active at H_2S concentrations up to 70 mg S/L, while the actual sulfide concentration at this depth is up to 1 mg S/L [38], nitrogen removal via anammox is observed [39,40].

The fact that the nitrifying bacteria may either decrease their growth rate at increasing sulfide concentrations (Otu2), increase it (Otu26 and Otu102), or be insensitive to this compound (Otu11) is both interesting and unexpected. Only inhibition of nitrifying bacteria in the presence of sulfide was reported in all previous studies [4,5,41].

Inhibition of the nitrifiers in the presence of sulfide is probably enhanced by the competition of the substrate, nitrite and/or nitrate, with members of the family *Hydrogenophilaceae*, which use the oxidized nitrogen species for sulfide oxidation.

5. Conclusions

In this study, the effect of sulfide on the transformation of nitrogen compounds by the anammox community has been studied through the use of a laboratory sequencing batch bioreactor with a stationary biomass carrier. The 16S rRNA gene profiling of the community of the anammox-activated sludge revealed members of the key physiological groups: ammonium oxidizers of the genus *Nitrosomonas*, nitrite oxidizers of the genus *Nitrosospira*, and anammox bacteria of the genera *Candidatus* Brocadia and *Ca*. Jettenia, as well as members of other bacterial genera. Nitrate removal was not sensitive to sulfide at concentrations up to 50 mg S/L and decreased by 17% at 100 mg/L. The threshold of sulfide sensitivity for group I nitrifiers was ~50 mg/L, while anammox bacteria were resistant to sulfide concentrations of up to 100 mg S/L in the incoming water. Group II nitrifiers were obviously the most sulfide-sensitive components of the community. A practice-wise consequence of this research is that Anammox technologies can be applied

towards wastewaters with a high sulfide load. A drastic increase in the abundance of members of the family *Hydrogenophilaceae* at elevated sulfide concentrations, together with the precipitation of elemental sulfur, was detected. This may indicate sulfide oxidation either by molecular oxygen or by nitrate; this finding requires further investigation. This is the first report on the different effects of sulfide on the growth rate of members of the nitrifying genus Nitrosomonas, increasing/decreasing or not affecting it for different phylotypes at elevated sulfide concentrations. Further investigations are necessary to cultivate and to isolate unique sulfide-tolerant and "sulfide-loving" *Nitrosomonas*.

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