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Impacts of reduced sulfur components on active and resting ammonia oxidizers

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Abstract While there has been significant research on the nature and extent of the impact of inhibitory reduced sulfur with respect to anaerobic (e.g., methanogenic and sulfidogenic) microbial systems, only limited study has yet been conducted on the comparable effects of soluble sulfides which might occur within aerobic wastewater treatment systems. Admittedly, aerobic reactors would not normally be considered conducive to the presence of reduced sulfur constituents, but there do appear to be a number of processing scenarios under which related impacts could develop, particularly for sensitive reactions like nitrification. Indeed, the following scenarios might well involve elevated levels of reduced sulfur within an aerobic reactor environment: (1) mixed liquor recycle back through sulfide-generating anaerobic zones (e.g., in conjunction with biological nutrient removal processes, etc.), (2) high-level side-stream sulfide recycle via sludge digestion, etc., back to aerobic reactors, and (3) high-level influent sulfide inputs to wastewater treatment facilities via specific industrial, septage, etc., streams. The objective of this study was, therefore, to determine the subsequent metabolic impact of soluble sulfide under aerated and unaerated conditions, focusing in particular on ammonia-oxidizing bacteria due to their critical first-step role with nitrification. The obtained results indicated that, under catabolically active conditions, cultures of ammonia oxidizers were extremely sensitive to the presence of sulfide. At total soluble sulfide concentrations of 0.25 mg l^{-1} S, active ammonia oxidation was completely inhibited. However, immediately following the removal of this soluble sulfide presence, ammonia oxidation started to recover; and it continued to improve over the next 24 h. Similar sulfide impact tests conducted with inactive ammonia oxidizers exposed during anaerobic conditions, albeit at higher dosage levels, also revealed that their subsequent aerobic activity would correspondingly be retarded. These results indicated that, after sulfide exposure under unaerated conditions, subsequent aerobic oxidative activity rates rapidly decreased as the soluble sulfide exposure was increased from 0.5 gm l^{-1} S to 5 mg l^{-1} S and that further reductions in this activity progressively developed as the concentration was increased to 200 mg l^{-1} S. The recovery following unaerated exposure to sulfide was significantly higher at pH 7, as compared with pH 8, and although the specific nature of this variation was not established, a hypothetical explanation appeared warranted.

Keywords Nitrification · Activated sludge · Toxicity · Sulfide

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Introduction

Background

Reduced sulfur compounds are not considered likely constituents within aerobic wastewater systems such as nitrification, in that normally they would neither be expected as metabolic products nor retained as sustainable incoming components under the involved oxidizing conditions. Given these expectations, therefore, and no doubt also due to the inherent experimental challenge (i.e., trying to characterize aerobic activity in the fleeting presence of various sulfide forms), only nominal prior research interest [12] has been given to the issue of sulfide impacts on nitrification.

Contrasted against this situation, though, there do appear to be a number of process scenarios under which

nitrifier systems might become an important operational issue. On this basis, therefore, there is a conceptual basis for considering the potentially inhibitory impacts of reduced sulfur under both aerobic and anaerobic circumstances.

Soluble inorganic sulfur compounds represent a rather commonplace constituent in wastewaters, given their typical presence in raw groundwater sources, their additional input via human waste generation, and their frequent occurrence in industrial waste streams. For example, municipal wastewaters tied to a groundwater source, such as that generated in Midwestern United States communities, often have total inorganic sulfur levels ranging over 10–50 mg l^{-1} S. As for industrial contributions, sulfuric acid is widely used and then released, given that it is produced in larger quantities than any other industrially manufactured chemical substance [10]. Several industrial wastes have been summarized in the literature [7], including a cane molasses alcohol production plant bearing sulfate concentrations of 950 mg l^{-1} S, a distillery having an effluent sulfate concentration of 2,000 mg l^{-1} S, and a sugar beet molasses effluent having a sulfate concentration as high as 1,500 mg l^{-1} S; and these illustrate the potential occurrence of high sulfur concentrations in some industrial waste streams. Aside from the prevalence of inorganic sulfur compounds within these types of industrial waste, though, it must also be recognized that a commonplace practice to reduce treatment energy costs within industrial operations is to pre-treat wastewaters in an anaerobic fashion prior to downstream discharge into activated sludge facilities. In turn, sulfates entering these anaerobic pre-treatment steps would then be reduced to, and released as, sulfides.

Yet another circumstance which might also contribute to this sulfide generation and exposure circumstance for aerobic biomass would be that of biological nutrient removal (BNR) plants and their component reliance upon anaerobic, reducing zones with environments of low-level oxidation/reduction potential (ORP) to secure and manipulate the involved phosphate-storing bacteria. For sulfate reduction to occur, an ORP of approximately −220 mV is required [15]. While this may not be present in all BNR anaerobic zones, an ORP has been observed to drop to approximately −225 mV in the anaerobic zone of BNR batch reactors [23]. In studies that examined sulfate reduction in BNR batch tests, it was found that phosphate release and sulfate reduction occurred simultaneously during anaerobic exposure [22]. In other tests, it was found that sulfate concentrations in bench-scale BNR configurations could be reduced approximately ten-fold (i.e., from ≥35.0 mg l^{-1} to ca. 3.5 mg l^{-1} SO_4-S) within the BNR anaerobic zone [21]. More recently, the abundance of sulfate-reducing bacteria (SRB) was studied in a 1.6×10^6 population-equivalent BNR plant in Germany with an influent SO_4-S concentration of 51 mg l^{-1} [16]. It was determined that the SRB accounted for over 6% of the total cell count in the anaerobic zone of the activated sludge reactor.

In addition to the above potential sources of sulfide toxicity, a number of wastewater treatment facilities control odors (i.e., generated by way of influent force-main off-gas, sludge-processing off-gas, etc.) by diffusing these malodorous gas streams, rich with reduced organic and inorganic sulfur species, into the bottom of their activated sludge reactors. It was recently estimated that at least 30 facilities in the United States use this method to control odors [5]; and this strategy does appear to be gaining popularity. While this review [5] listed the highest hydrogen sulfide concentration in these municipal wastewater treatment odorous gasses to be approximately 100 ppm, there are certainly industrial instances (e.g., such as pork-processing facilities), where percentile-level sulfide levels can be found in their off-gas streams [8].

Sulfide toxicity to nitrifying organisms

By whatever means these elevated soluble sulfide levels might be developed, therefore, the consequent concern would be that of potential inhibition of aerobic bacteria and particularly that of a group of organisms (i.e., *Nitrosomonas*) with recognized levels of environmental and chemical sensitivity. Indeed, the growth rate for this species is often considered a key rate-limiting step in the design of wastewater treatment facilities, whereby any reduction in their activity which might be incurred during sulfide exposure could be extremely significant. This growth rate dictates the ultimate size of the aeration basins and, therefore, any reduction in this rate may either lead to an increase in the effluent ammonia concentration or create the need for larger aeration basins. The available literature, however, does not offer definitive documentation as to whether the presence, or persistence, of soluble sulfides in unaerated, or only partially aerated, zones included in process configurations (e.g., sequencing batch reactors, BNRs, anoxic selectors, etc.) might lead to retardation of ammonia oxidation (AO) activity.

Conversely, the impact of soluble sulfides on other types of bacteria in anaerobic wastewater and biosolids systems has been studied extensively [11, 19]. The toxicity of soluble sulfides in these anaerobic studies suggests that pH affects the degree of toxicity due to partitioning between ionized [both mono- (HS^-) and divalent (S^{2-})] and non-ionized (H_2S) sulfide species. Reactor pH dictates that the fraction of total soluble sulfide present in the non-ionized form adheres to the following equations:

$$H_2S \leftrightarrow H^+ + HS^- \quad (1)$$

$$HS^- \leftrightarrow H^+ + S^{2-} \quad (2)$$

The dissociation constant (p *K*a) is 7.2 for Eq. 1 and 11.89 for Eq. 2 (both at 20°C [9]). The significance of this shift in free versus ionized-inhibitor presence with respect to anaerobe sulfide sensitivity has already been

demonstrated [11, 19]; and inhibition work with *Nitrosomonas* [2] to similar shifts in reduced-nitrogen forms (i.e., $NH_4^+ \rightarrow NH_3$) provides a compelling motivation to include this factor as a research objective regarding sulfide-induced impacts.

While limited research has been conducted on soluble sulfide inhibition of nitrification in activated sludge, a substantial amount of research has been performed relating to organic sulfur compounds. This research has demonstrated varying degrees of inhibition, depending on the form of organic sulfur compound and its concentration [6, 20]. More recently, a comprehensive review was published [17] detailing nitrifier inhibitor mechanisms and impacts, including 40 compounds that were listed as possible co-metabolic substrates of the ammonia monooxygenase (AMO) enzyme (i.e., responsible for the first two-electron oxidation from ammonia to hydroxylamine) which could also act to competitively inhibit NH_3 oxidation. This review also acknowledged that in some cases (e.g., inhibition of thioethers through oxidation to sulfoxides), a sulfur atom could bind at the primary site of oxidation by AMO, which would then effectively lead to competitive inhibition. Other forms of sulfur-induced inhibition have also been projected to involve the role of copper in the activity of AMO. One such mode of AMO inhibition or debilitation would then develop when sulfur-containing compounds bound onto, or formed, a chelating complex that effectively drew copper away from otherwise active enzymes.

Objectives

The objectives of this study were to determine the metabolic impact of soluble sulfide under aerated and unaerated conditions, focusing in particular on AO bacteria due to their critical first-step role with nitrification. Also, to establish whether sulfide speciation (i.e., ionized vs non-ionized) has an impact on this activity.

Materials and methods

General testing options

The impact of sulfide on nitrifying bacteria was evaluated under two basic exposure conditions, aerated and unaerated environments, along with additional variations in dosage level and pH. Table 1 provides a summary overview of these testing conditions.

Enriched nitrifier culture maintenance and preparation

All experiments were conducted with a nitrifying biomass cultured in a suspended-growth form within a bench-scale, continuous-flow 10-l reactor supplied with a high-strength ammonium bicarbonate (i.e., 1,000 mg l^{-1} N) feed stream. With the exception of a nominal phosphate supplement, which was added as dibasic sodium phosphate (20 mg l^{-1} P), all remaining minor and trace element requirements were supplied via this influent's groundwater diluent. The reactor was covered with an opaque hood in order to prevent light-induced inhibition of the culture [1, 12] and automated on-line pH control was provided, via sodium bicarbonate addition, to hold the reactor pH at 7.2. Routinely maintained with a hydraulic retention time of 1 day and a solids retention time (SRT) of ca. 25 days, the enhanced activity of this culture with ca. 2,000 mg l^{-1} volatile suspended solids (VSS) was confirmed by way of its typical maximum ammonium oxidation rates (i.e., with excess substrate addition) at 20–25 mg N mg^{-1} VSS h^{-1}. While this nitrifying culture contained no measurable heterotrophic respiration, there were sightings of colonial stalk ciliate within the flocs, which likely survived from the products released during cell lysis. Therefore, the terminology "enriched" nitrifier culture rather than "pure" culture was used to describe this suspension.

The sulfide exposure tests were conducted with mixed liquor aliquots removed from this active suspension and provided with appropriate dilution in order to secure a consistent initial biomass concentration of ca. 1,000 mg l^{-1} VSS. In the case of the anaerobic exposure tests, a pre-washing step was also used with the stock culture prior to dilution in order to remove soluble sulfate.

Sulfide toxicity experiments

Test condition 1: nitrifier response during and after aerobic sulfide exposure

This initial series of tests was conducted to assess the impact of sulfide toxicity on metabolically active AO bacteria. Dissolved oxygen (DO) and pH were continuously measured throughout the tests. A pure oxygen feed via a diffuser stone was inserted in each nitrifier culture flask, with oxygen being introduced on an as-needed basis to maintain the mixed liquor bulk DO

Table 1 Summary of testing conditions for AO sulfide inhibition response

	Sulfide Exposure		Mixed Liquor pH		Sulfide Concentration Range (mgS/L)
Test Condition 1	Aerated		7	8	0 1 10 100 200
Test Condition 2		Unaerated	7	8	0 1 10 100 200

concentration at 2–6 mg l^{-1} O_2. To ensure that the nitrifiers were metabolically active, ammonia bicarbonate (plus additional sodium bicarbonate buffer) was added to achieve an initial concentration of approximately 35 mg l^{-1} N.

Following the addition of ammonium bicarbonate, a 60 mM solution of sodium sulfide (1.92 g l^{-1} S) was continuously pumped into the flask for a period of 2 h, with the objective of maintaining a relatively constant residual total sulfide concentration under aerobic conditions (i.e., rate of sulfide addition = rate of sulfide oxidation + rate of sulfide stripping + rate of sulfide precipitation). During this period, sub-samples were removed from the test beaker every 15 min and analyzed for soluble total sulfide and ammonia-N.

At least in theory, this reactor setup and sulfide delivery system was configured to secure a constant level of sulfide tension within the reactor for the exposed ammonia oxidizers. However, the pragmatic circumstance of delicately balancing the ''sinks'' for sulfide (i.e., oxidation, stripping, precipitation) with slight changes in the low-level sulfide delivery pump inevitably resulted in some degree of fluctuation in the targeted sulfide value (as can be visually seen in the resultant data plots). For example, when oxygen was introduced into the reactor, there were inevitable changes with sulfide stripping rates. The total volume of sulfide pumped into the flasks, though, represented less than 2% of the total liquid volume, such that dilution effects could effectively be ignored.

Following 2 h of continuous sulfide exposure under aerobic conditions, the chemical delivery pump was then stopped and the sulfide exposure phase of each test was concluded. Aeration was continued in each case (i.e., with DO above 2 mg l^{-1} O_2) and sub-sample testing for residual ammonia was then continued every 15–20 min for a period of 2 h.

In this manner of testing, therefore, nitrifier AO rates were determined both during and following their exposure to sulfide under aerobic conditions. As a means of confirming whether there was any degree of lingering inhibition extending beyond the initial 2-h post-exposure period, the AO rates were again reassessed 24 h following each test. All of these tests were also conducted at mixed liquor pH levels of 7 and 8 with the intent of securing further insights as to whether the inhibition appeared linked to total or non-ionized sulfide. The observed AO rates were compared with the AO rates obtained in a control reactor which was operated in an identical manner as the experimental reactors but without the addition of sodium sulfide. This allowed a direct comparison of the effect of sulfide on the AO rate.

Test condition 2: nitrifier response following anaerobic sulfide exposure

The pre-washed and diluted nitrifier suspension was first placed in 500-ml Erlenmeyer flasks and purged with nitrogen gas for 60 min to ensure that no residual DO was present. The mixed liquor pH was then adjusted to either 7 or 8. Varied aliquots of a 60 mM stock sodium sulfide solution (1.92 g l^{-1} S; pH adjusted to 7 or 8 with HCl) were then added to each sample to increase the sulfide concentration to the desired level. These 500-ml flasks were capped and allowed to gently mix on a magnetic mixer for 2 h. After 2 h, stirring was stopped and the mixed liquor was allowed to settle. The samples were then decanted and the concentrated mixed liquor washed with oxygen-free water to remove any remaining sulfide.

Following the second washing step and re-dilution to the original volume, ammonium bicarbonate and supplemental sodium bicarbonate buffer were added to the samples and aeration was initiated. Sub-samples were then removed every 15 min for a total of 2 h and analyzed for ammonia-N. After 24 h, the AO rates were again measured to quantify the remaining inhibition level. Similar to the previous test condition, AO rates were compared with the AO rates obtained in a control reactor which had been handled in an identical fashion as the experimental reactors but without the addition of sodium sulfide.

Analytical methods

Prior to analysis, all samples were filtered through 0.45-μm filters. Sulfide samples were immediately analyzed following filtration, using the methylene blue method ([3], method 4500-S^{2-}D) and a Shimadzu UV 160 U spectrophotometer. Ammonia analyses were conducted within 3 h of sampling using the Nesslerization method ([4], method 4500-NH_3C), while total suspended solids and VSS were analyzed according to [3], methods 2540D and 2540E . ORP was measured according [3], method 2580, using an Ag/AgCl, 4 M KCl electrode and corrected to the standard hydrogen scale [14]. DO and pH analyses were performed according to [3], methods 4500-O G and 4500-H^+ B . Total and soluble metal analysis was performed using a Thermo Jarrell Ash Atom Scan 16 sequential inductively coupled plasma spectrophotometer. Sample preparation followed [3], methods 3030B and 3030E, for soluble metals and total metals, respectively. Digestion was achieved by adding concentrated nitric acid to the activated sludge sample, followed by heating to a slow boil. Nitric acid was added to the sample as needed to compensate for the evaporative losses. This was continued until the digestion was complete as indicated by a light-colored clear solution [3].

Results and discussion

Test condition 1: nitrifier response during and after aerobic sulfide exposure

To investigate the impact of soluble sulfide under aerobic conditions, three sulfide concentration ranges at two

mixed liquor pHs were studied. These three sulfide concentration ranges were selected to represent a low, medium, and high set of values which might occur in the initial stages of an aeration basin treating an influent sulfide-laden wastewater. While elevated levels of soluble sulfide may enter an aeration basin, chemical, biological, and absorption processes could normally be expected to incur an expedient reduction with this incoming bulk liquid sulfide level, such that the low, medium, and high concentration ranges selected for this period of study were respectively ≤ 0.5, 0.5–1.0, and 1.0–3.5 mg l^{-1} S. Furthermore, in order to observe the potential impact of sulfide speciation, these tests were completed at mixed liquor pH levels of both 7 and 8. Based on the dissociation constants, at a mixed liquor pH of 7, 61% of the total sulfide would have been non-ionized (39% ionized), while at a pH of 8, only 14% of the total sulfide would have been non-ionized (86% ionized).

These tests demonstrated that AO bacteria are extremely sensitive to the presence of soluble sulfide under aerobic conditions. The trend in all cases was similar: when sulfide was present, little or no AO occurred. Table 2 summarizes the results from all aerobic exposure tests, while Figs. 1, 2 illustrate the results from a representative test conducted at mixed liquor pH 7.0 and pH 8.0. These figures were constructed to illustrate the AO rate while sulfide was present (represented in the gray-shaded area) and immediately following sulfide removal (non-shaded area). Ammonia was plotted on the top portion of each figure (i.e., Figs. 1a, 2a) and soluble sulfide on the bottom (i.e., Figs. 1b, 2b). For comparison, the AO rate of the ''control'' is also provided in each figure. Under predominately non-ionized sulfide conditions (i.e., mixed liquor pH 7.0), concentrations of less than 0.50 mg l^{-1} S of total soluble sulfide (i.e., 0.2 mg l^{-1} S ionized, 0.30 mg l^{-1} S non-ionized) reduced AO by 93%, while under predominately ionized sulfide conditions (i.e., 0.43 mg l^{-1} S ionized, 0.07 mg l^{-1} S unionized, at pH 8.0) the AO rate was reduced by 68%. At exposures higher than 0.5 mg l^{-1} S of total soluble sulfide, the oxidation rate was reduced by over 95% at either mixed liquor pH. For those tests where some initial measure of AO might have been retained, inhibition escalated as exposure time progressed. After 105 min, AO had effectively stopped in all of these tests.

After sulfide addition had been terminated, the residual soluble sulfide was depleted to below detection within 15 min in all cases (i.e., via oxidation, stripping, or precipitation; see Figs. 1, 2). Following this depletion, AO promptly recovered, indicating that a degree of recovery was possible. However, since the depletion rate immediately following sulfide exposure ranged over only 51–74% of the maximum control rate, some measure of a lingering effect was still evident. Ammonia depletion rates measured 24 h after sulfide exposure indicated that additional recovery was possible, but a full recovery to the ''pre-sulfide-exposure'' AO rates was not achieved within this time interval. To confirm that sodium from the sodium sulfide did not contribute toward the inhibition, a series of tests were completed using sodium chloride as the feed instead of sodium sulfide. The results of these tests indicated that the impact of sodium on the AO rate was less than 1% when compared with the controls.

The immediate return of AO after the sulfide was depleted indicates that the mode of inhibition is not mechanism-based. Mechanism-based inhibition is the result of the normal cycle of the AMO enzyme producing an inhibitory product from the original compound [17]. This type of irreversible inactivation (also referred to as ''suicide inhibition'') is dependent on the catalytic cycle of the enzyme [17]. There are three principal forms of reversible inhibition: competitive, non-competitive, and uncompetitive. In the case of competitive inhibition, sulfide would compete for the active site on the AMO enzyme and would eliminate any hydroxylamine production, thereby interrupting the flow of electron output during its subsequent oxidation. The importance of this latter ''interruption'' is that hydroxylamine oxidation plays a key role with the preceding AMO-catalyzed step, in that it recycles two electrons back to the initial AO reaction, effectively ''priming the pump'' as it were, to maintain AMO activity [12]. Since the structure on the active site of the AMO enzyme remains unchanged, the enzyme is still able to oxidize any ammonia that locates an active site. The actual level of inhibition is dependent on the

Table 2 Summary of AO rates under metabolically active conditions

	Fraction of Maximum Ammonia Depletion Rate Relative to Control					
	pH 7			pH 8		
Total Sulfide Presence (mgS/L)	S Present	Post S Removal (Immediate)	Post S Removal (24 hr)	S Present	Post S Removal (Immediate)	Post S Removal (24 hr)
Low (0-0.5 mg/L)	0.07	0.74	0.84	0.32	0.73	0.92
Medium (0.5-1.0 mg/L)	0.00	0.55	0.83	0.04	0.65	0.91
High (1-3.5 mg/L)	0.00	0.56	0.89	0.00	0.51	0.88

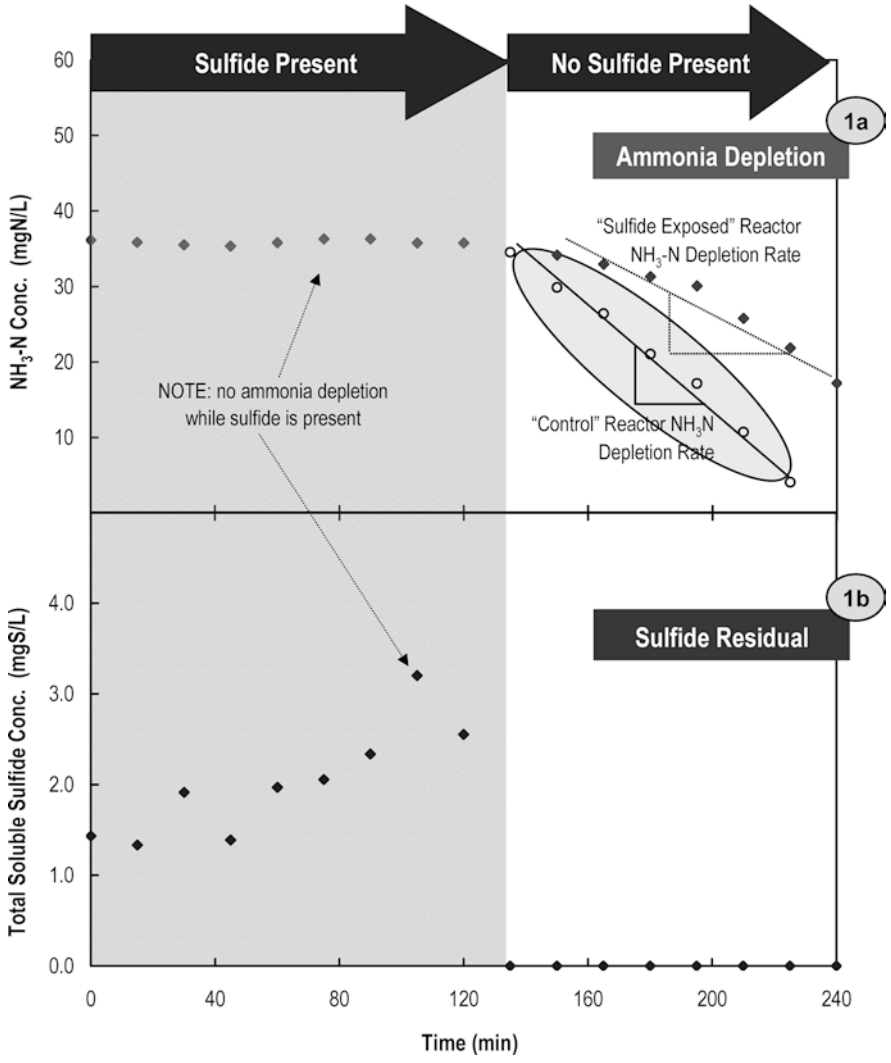

Fig. 1 Ammonia oxidation in the presence and absence of soluble sulfide at mixed liquor pH 7.0. . **a** Ammonia-N concentration when sulfide is present (*gray-shaded area*) and absent (*non-shaded area*). **b** Sulfide concentration. Control rate signifies AO with no previous sulfide exposure

concentration of the inhibitor and the substrate. For this reason, competitive inhibition can normally be overcome by simply increasing the substrate concentration. Since our experiments were conducted at high substrate concentrations far above the expected half-saturation concentration (K_s ca. 1 mg l^{-1} NH_3-N) and substrate utilization was only measured on one occasion in the presence of sulfide (e.g. at pH 8 with the low-sulfide exposure concentration), the mode of inhibition does not appear to be competitive in nature. In non-competitive inhibition, the sulfide molecule would be expected to attach to the AMO enzyme at a location other than the active site and change its structure such that it inactivates the enzyme. In this situation, as long as the sulfide molecule is attached to the enzyme, AO is either reduced or completely stopped. With uncompetitive inhibition, the inhibitor binds to the active site only after ammonia has attached to the AMO enzyme. Therefore, even though the enzyme is saturated with substrate, the attachment of the inhibitor creates an inactive complex. To verify whether the inhibition was uncompetitive or non-competitive, it would be necessary to conduct kinetic studies to establish the corresponding half saturation and maximum velocity constants. However, completing these tests would be extremely difficult, if not altogether impossible, due to the following complications: (a) the substrate concentrations at which AO ceases are so low (i.e., < 0.5 mg l^{-1} S at pH 7), and (b) the difficulty of maintaining constant residual sulfide concentrations within a reactor under aeration.

These results as they are, though, can be extrapolated to illustrate the importance of designing processes that dilute high-strength sulfide wastes as they enter an aeration basin. While it is recognized that several factors (e.g., type of aeration system, mixed liquor character, composition, pH, etc.) affect the rate at which sulfides are stripped, oxidized, or precipitated, the results of these experiments indicate that residual soluble sulfide can reduce the observed nitrification rates. Even after the soluble sulfide is completely oxidized, an adverse effect may linger, resulting in a reduction in the nitrification rate and indicating the need for a longer SRT. On a positive note, however, the results do indicate that time-dependent recovery is possible when ammonia

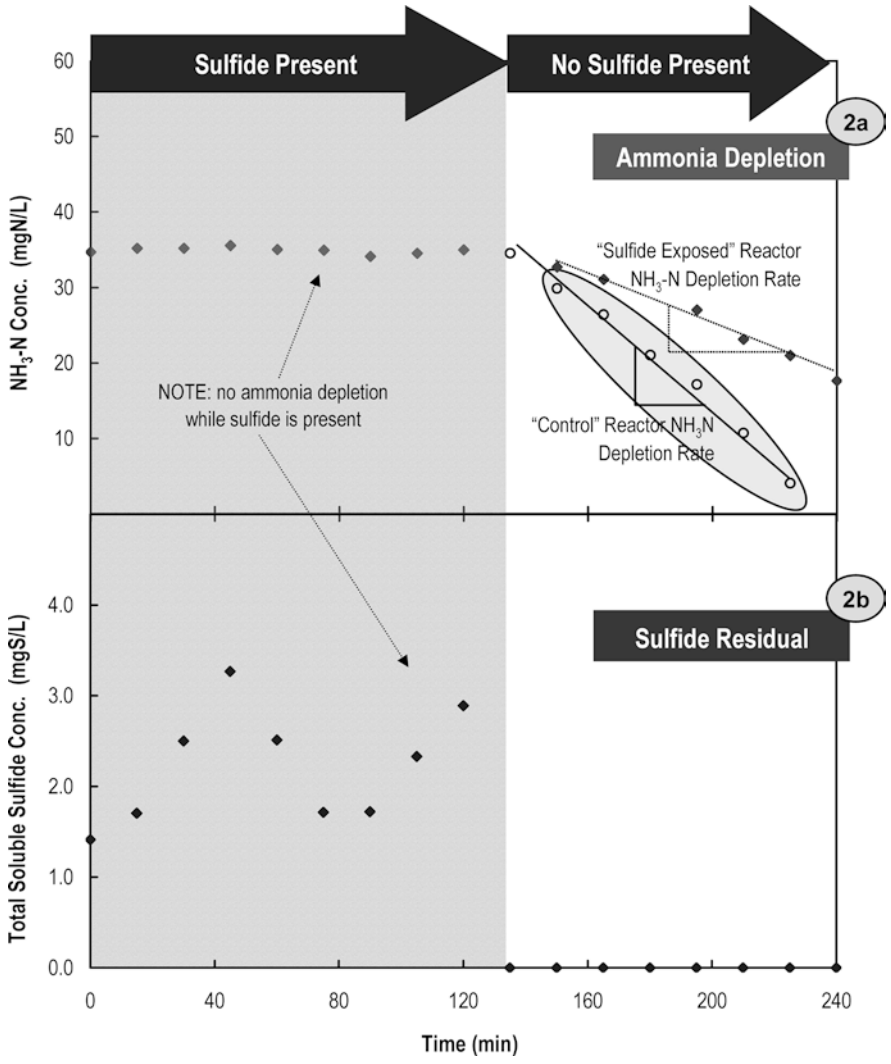

Fig. 2 Ammonia oxidation in the presence and absence of soluble sulfide at mixed liquor pH 8.0. **a** Ammonia-N concentration when sulfide is present (*gray shaded area*) and absent (*non-shaded area*). **b** Sulfide concentration. Control rate signifies AO with no previous sulfide exposure

oxidizers are exposed to soluble sulfide, at least under the concentration ranges tested in this study.

Test condition 2: nitrifier response following anaerobic sulfide exposure

For this test condition, the effect of sulfide added to AO bacteria under unaerated environments was evaluated. Higher residual sulfide concentrations were tested due to the ability of sulfides to accumulate under unaerated conditions. The concentrations investigated with these tests were 0.5, 1.0, 2.5, 5, 10, 50, and 200 mg l^{-1} S. Similar to test condition 1, the effect of sulfide speciation was observed by repeating each test at mixed liquor pH 7 and pH 8. At each concentration and pH, sulfide was added under unaerated conditions and allowed to persist for 2 h. The suspension was then washed with oxygen-free water and the ammonia depletion rate immediately measured. The rate was again measured 24 h later to determine whether any recovery occurred. Although plots of the depletion tests are not shown, each rate was determined by linear regression of at least seven data points. Each regression analysis had a coefficient of determination (R^2) greater than 0.97. The values representing the slope of each depletion test were plotted with respect to a maximum control rate and are shown in Fig. 3.

The results indicate that under metabolically inactive conditions, AO bacteria are negatively affected by the presence of sulfides. Post-exposure ammonia depletion rates decreased rapidly as the level of sulfide which these cells had previously experienced under unaerated conditions was increased from 0.5 mg l^{-1} S to 5.0 mg l^{-1} S. At 10 mg l^{-1} S sulfide exposure, the AO rates were only 72% and 64% of the control rate at pH 7 and pH 8, respectively. At sulfide concentrations above 5 mg l^{-1} S, the AO rates further decreased, although not as rapidly.

While the immediate effect of sulfide at each pH was similar, there was a significant difference in the recovery after 24 h. At mixed liquor pH 7, a much higher degree of recovery was measured, as compared with exposure at pH 8 (see Fig. 3). Visual observations of the mixed liquor at pH 7 revealed a much darker mixed liquor

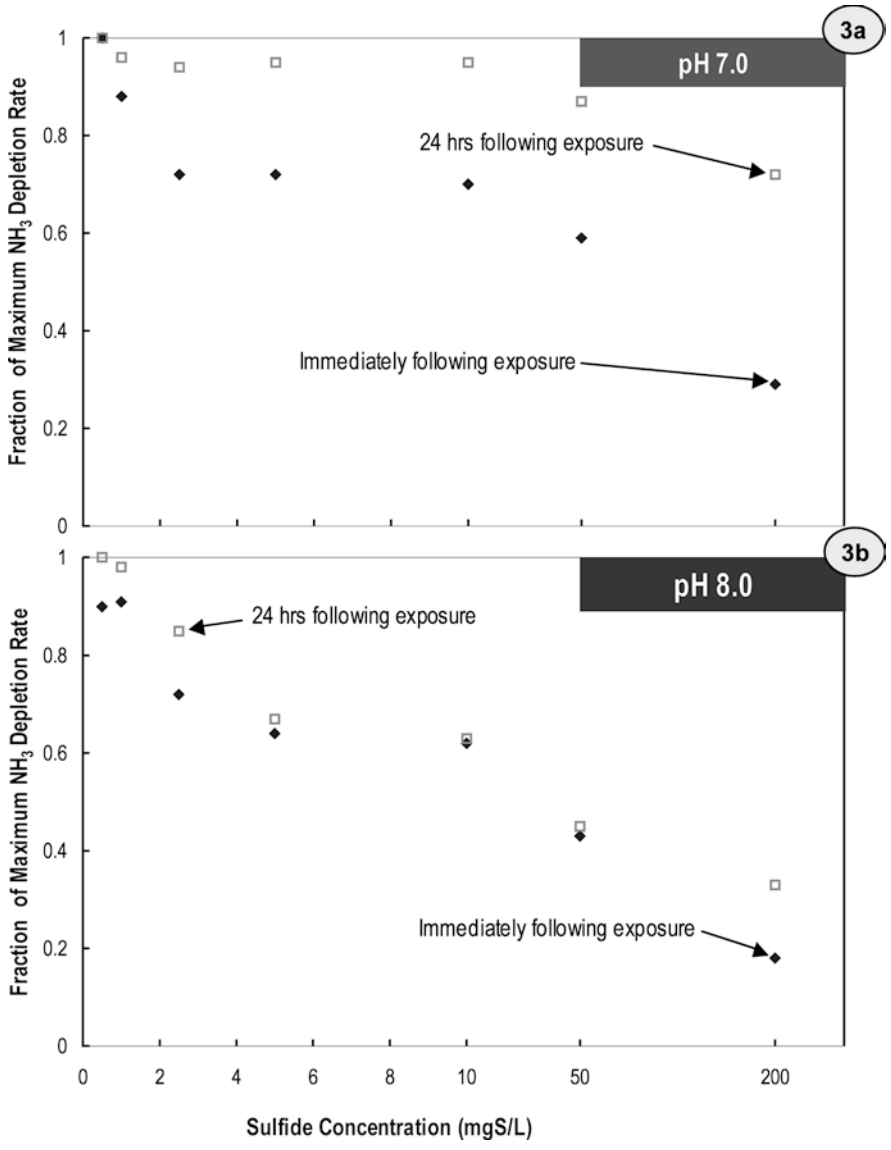

Fig. 3 Reduction in ammonia oxidizer activity following unaerated exposure to sulfide, at pH 7 (**a**) and pH 8 (**b**). AO rates measured immediately after exposure and 24 h following exposure

suspended solids, with 200 mg l^{-1} S, than that observed at pH 8. To determine whether metals precipitation could be the cause of the reduced oxidation rate and contributed to the difference in inhibition at the two mixed liquor pHs, total and soluble metals analysis were performed for the following: calcium, cadmium, cobalt, copper, iron, magnesium, manganese, nickel, and zinc. To help identify metal speciation, ORP was measured during each unaerated experiment.

As expected, the total metal concentration for each condition was comparable. However, soluble metal concentrations were generally slightly higher at mixed liquor pH 7 versus pH 8 (Table 3). While cadmium, cobalt, copper, iron, manganese, nickel, and zinc all have relatively low solubility products when combined with sulfide (ranging from 3×10^{-14} for MnS to 2×10^{-25} for ZnS), a reduction in soluble species can also be created by redox conditions. For example, the redox potential at the 200 mg l^{-1} S condition was −182 mV at pH 7, but −279 mV at pH 8. This leads to a condition that is favorable for Fe^{2+} to dominate at pH 7, while at pH 8 the equilibrium would be shifted towards $Fe(OH)_2$. The Fe^{2+} would in turn react with sulfide at pH 7 to form a dark precipitate, while at mixed liquor pH 8, $Fe(OH)_2$ would be present, which is a colorless solid. This may be one explanation of the observed difference in the appearance of the mixed liquor between the two pH conditions. Although soluble metal concentrations were generally higher at pH 7, there was only one condition where a measured metal was present at pH 7 and not present at pH 8 (e.g., cobalt at 50 mg l^{-1} S). When a higher concentration of sulfide was added (e.g., 200 mg l^{-1} S), cobalt concentrations at both pH conditions were reduced to below detection limits. However, even with the soluble cobalt concentration below the detection limit at each pH condition, recovery was significantly lower at mixed liquor pH 8. Based on these observations, it is not likely that the

Table 3 Soluble metal concentrations (mg l^{-1}) under unaerated conditions following sulfide addition

	10 mgS/L		50 mgS/L		200 mgS/L	
Soluble Element	pH 7 (mg/L)	pH 8 (mg/L)	pH 7 (mg/L)	pH 8 (mg/L)	pH 7 (mg/L)	pH 8 (mg/L)
Cadmium	0.005	0.002	0.006	0.002	0.006	0.002
Cobalt	0.025	0.015	0.011	0.000	0.000	0.000
Copper	0.004	0.003	0.004	0.004	0.000	0.000
Iron	0.331	0.055	0.314	0.057	0.194	0.053
Manganese	1.624	0.308	1.502	0.264	1.341	0.274
Nickel	0.044	0.032	0.044	0.030	0.042	0.028
Zinc	0.022	0.019	0.023	0.015	0.023	0.012
Calcium	94.6	76.7	91.6	72.6	92.2	64.8
Magnesium	32.2	31.2	33.5	30.7	29.3	26.9

limited availability of soluble metals for growth was the primary cause of inhibition created by the addition of sulfide under unaerated conditions. This is further supported by the fact that the yield of ammonia oxidizers is very low (e.g., 0.1–0.15 g VSS g^{-1} NH_3-N) and as such the growth of new cells over the oxidation period of 2 h is minimal (i.e., $<0.5\%$ new cell growth, based on existing biomass concentration). However, this is not to construe that metals did not play a role in the reduction of AO activity. In fact, it is possible that sulfide addition created a condition for metals to be extracted from the AMO enzyme, leading to a reduction in its activity. For example, evidence has shown that metals such as copper play an important role in AMO activity [17] and that certain sulfur compounds could form complexes which can chelate copper and inhibit AMO activity. In this set of experiments, the AMO enzyme was inactive when exposed to sulfide due to the unaerated conditions. This eliminated the need to consider competitive inhibition as the cause of the reduced AO rate. Of the remaining modes of action of nitrification inhibitors, only non-competitive inhibition or the binding of metals such as copper on the AMO enzyme seem appropriate. While it cannot be confirmed that copper extraction was the primary reason for the decline in AO activity, the results in Table 3 indicate that only minimal amounts were present at either pH after sulfide addition. The increased extraction of copper ions from the AMO enzyme at the higher mixed liquor pH (i.e., due to a lower hydrogen ion concentration) would support the observation of a higher level of inhibition at mixed liquor pH 8, as compared with pH 7. Also, the remaining soluble metal concentrations were generally lower at the higher pH value (pH 8.0), which would also create a higher driving force from enzyme-associated metals to soluble ions in order to maintain equilibrium. These soluble metal ions would then in turn be precipitated by residual sulfides, leading to the possibility of increased inhibition at the higher pH.

Finally, to confirm that AO bacteria were not lost from the flocs during the washing step described above, a series of similar tests were conducted. In these tests, the AO bacteria were exposed to anaerobic conditions and immediately aerated to remove the sulfide, thereby eliminating the washing step. AO rates measured following sulfide oxidation illustrated the same differential trend at pH 7 vs pH 8, as shown in Fig. 3.

Overall, these results illustrate the importance of mixed liquor pH when exposing ammonia oxidizers to sulfide under unaerated conditions, as might be realized in many activated sludge or sequencing batch reactor configurations. While the immediate effect of sulfide is similar at both pHs, significantly less recovery was observed at the higher exposure pH.

Conclusions

1. Ammonia oxidizers are extremely sensitive to the presence of soluble sulfide under metabolically active conditions. The presence of 0.5 mg l^{-1} S as total soluble sulfide within nitrifying cultures can completely inhibit the oxidation of ammonia.
2. Enhanced cultures of ammonia oxidizers are sensitive to the presence of soluble sulfide under metabolically inactive conditions. Following unaerated exposure to a soluble sulfide concentration of 5.0 mg l^{-1} S, the post-exposure aerobic rate of AO decreased to 72% and 64% of the maximum control rate at mixed liquor pH 7 and pH 8, respectively.
3. There was a distinct difference in the post-exposure recovery patterns for ammonia oxidizers previously exposed to soluble sulfide under unaerated conditions, in that measurably higher rates were observed for mixed liquor pH 7 vs pH 8. While a hypothesis was developed to explain this phenomenon, no clear basis for this difference could be ascertained.

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