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# Ammonium-based bioleaching of toxic metals from sewage sludge in a continuous bioreactor

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## ABSTRACT

The broader reuse of sewage sludge as a soil fertilizer or conditioner is impeded by the presence of toxic metals. Bioleaching, a process that leverages microbial metabolisms and metabolites for metal extraction, is viewed as an economically and environmentally feasible approach for metal removal. This study presents an innovative bioleaching process based on microbial oxidation of ammonia released from sludge hydrolysis, mediated by a novel acid tolerant ammonia-oxidizing bacteria (AOB), *Ca. Nitrosoglobus*. Over a span of 1024 days, a laboratory-scale bioleaching reactor processing anaerobically digested (AD) sludge achieved an *in-situ* pH of  $2.5 \pm 0.3$ . This acidic environment facilitated efficient leaching of toxic metals from AD sludge, upgrading its quality from Grade C to Grade A (qualified for unrestricted use), according to both stabilization and contaminants criteria. The improved quality of AD sludge could potentially reduce sludge disposal expenses and enable a broader reuse of biosolids. Furthermore, this study revealed a pH-dependent total ammonia affinity of *Ca. Nitrosoglobus*, with a higher affinity constant at pH 3.5 ( $67.3 \pm 20.7$  mg N/L) compared to pH 4.5–7.5 (7.6–9.6 mg N/L). This finding indicates that by optimizing ammonium concentrations, the efficiency of this novel ammonium-based bioleaching process could be significantly increased.

## 1. Introduction

Sewage treatment plants (STPs) are known to generate substantial quantities of sludge, the safe disposal of which is a pressing concern. One of the most cost-effective methods of sludge disposal is land application, where sewage sludge is repurposed as a soil fertilizer or conditioner. This approach offers multiple benefits to soil (D'Imporzano and Adani, 2023), such as supplying essential nutrients like nitrogen, phosphorus, potassium for plant growth, enhancing soil organic matter, and improving the soil's water retention capacity, among others. However, the feasibility of land application is limited by the presence of toxic metals in sludge, like cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn) (Singh and Agrawal, 2008). These toxic metals, once introduced into the soil, can be ingested by living organisms and bioaccumulate up the food chain, thereby posing a significant risk to human health (Luo et al., 2024). Consequently, it is crucial to reduce the content of toxic metals to ensure a safe reuse of sewage sludge.

The most effective approach to managing toxic metals in sewage

sludge is source control by regulating the discharge of metal-rich industrial wastewaters (Babel and del Mundo Dacera, 2006). However, when sources identification is challenging, it becomes imperative to remove toxic metals from sludge. Current approaches include chemical leaching and bioleaching. Chemical leaching employs various chemicals like inorganic acids (Bayat and Sari, 2010), organic acids (Molaey et al., 2021a), chelating agents (Yao et al., 2021), to extract toxic metals from the solid phase. Nevertheless, its practical application is limited due to high operating costs associated with intensive chemical use. Bioleaching utilizes microbial metabolisms and metabolites for metal extraction. For instance, iron- and sulfur-oxidizing bacteria (SOB) can solubilize metals through direct oxidation of metallic sulfide (Suzuki, 2001), or indirect production of leaching agents such as sulfuric acids (H<sub>2</sub>SO<sub>4</sub>) or Fe<sup>3+</sup> (Seidel et al., 2004). More recently, anaerobic bioleaching was proposed, leveraging the self-sustaining volatile fatty acids (VFAs) produced during sludge fermentation as the leaching agent (Meulepas et al., 2015; Molaey et al., 2021b; Tonanzi et al., 2022; Yesil et al., 2021a). Compared to chemical leaching, bioleaching significantly reduces chemical consumption by up to 80 % (Tyagi et al., 1988), making it a

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more viable solution for toxic metal removal.

The recent discovery of an acid-tolerant ammonia-oxidizing bacterium (AOB), *Candidatus* (*Ca.*) Nitrosoglobus (Hayatsu et al., 2017; Wang et al., 2021d), has led to the proposal of a novel bioleaching approach based on microbial ammonia oxidation ( $\text{NH}_4^+ + 1.5\text{O}_2 \rightarrow \text{NO}_2^- + 2\text{H}^+$ ). Unlike sulfur- and iron-based bioleaching, which requires the dosage of sulfur or  $\text{Fe}^{2+}$ , ammonium-based bioleaching utilizes the inherent ammonium in sewage sludge (~ 1.5–6.0 % dry weight) (Metcalf et al., 1991), eliminating the need for external chemicals. A previous study clearly showed, via batch tests, that the anaerobically digested sludge could be acidified from pH 7.5 to pH 2 within 5 days following the inoculation of this novel AOB, accompanied by efficient solubilization of toxic metals such as Cu and Zn (Wang et al., 2021b).

The translation of this novel concept into a practical process remains challenging. Among others, the ammonium concentration achievable in a continuous bioleaching reactor is expected to be much lower than that in a batch test (Cassidy et al., 2000). This is a critical issue considering *Ca.* Nitrosoglobus's high affinity constant with total ammonia ( $\text{NH}_3 + \text{NH}_4^+$ ) ( $K_{s,\text{total}}$ ), reported to be  $13.5 \pm 3.3$  mg N/L at pH 5.0 (Wang et al., 2021a) and anticipated to be even higher at pH <3 as required by efficient bioleaching. This is because a low pH shifts the equilibrium  $\text{NH}_3 + \text{H}^+ \rightleftharpoons \text{NH}_4^+$  towards  $\text{NH}_4^+$ , thus reducing the fraction of ammonia ( $\text{NH}_3$ )—the true substrate of AOB. However, the  $K_{s,\text{total}}$  of *Ca.* Nitrosoglobus at different pH remains unknown. Therefore, it is crucial to investigate the pH dependence of  $K_{s,\text{total}}$  for *Ca.* Nitrosoglobus and its effects on the feasibility of a continuous ammonium-based bioleaching process.

This study aims to identify and address the key challenges in turning the novel ammonium-based bioleaching concept into a practical process in a continuous bioreactor. A laboratory-scale bioleaching reactor receiving anaerobically digested (AD) sludge was established through the inoculation of an enriched *Ca.* Nitrosoglobus culture. The reactor was continuously operated for ~ 1000 days to attain a pH conducive to bioleaching (pH < 3). The concentrations of heavy metals and their distributions between the liquid and solid phases of the treated sludge were monitored throughout the experiments, along with the pathogen levels and the specific oxygen uptake rate. The pH and concentrations of nitrogenous compounds were also closely monitored. The  $K_{s,\text{total}}$  value of *Ca.* Nitrosoglobus was assessed at pH levels of 3.5, 4.5, 7.5, to identify potential ammonia limitation.

## 2. Materials and methods

### 2.1. Sources of AD sludge

The feeding AD sludge was collected from a full-scale anaerobic digester in a local STP (Brisbane, Australia), which receives a mixture of primary and secondary sludge, at a ratio of ~ 1:1 based on volatile solids (VS) concentrations. The main characteristics of the AD sludge are listed in Table S1. The AD sludge was collected weekly, transported to the lab within an hour, and stored in a 4 °C fridge prior to use.

### 2.2. Set-up and operation of the bioleaching reactor

A laboratory-scale bioleaching reactor was established in an air-conditioned laboratory at a room temperature of  $22 \pm 1$  °C. The reactor has a total capacity of 1 L and operates at a working volume of 650 mL. Initially, the bioleaching reactor was inoculated with 650 mL aerobic sludge, enriched with *Ca.* Nitrosoglobus from a parent reactor. This inoculum, which contained *Ca.* Nitrosoglobus as the only detectable AOB ( $55.1 \pm 0.5$  % relative abundance based on 16S rRNA gene sequencing), along with the operation and performance of the parent reactor, was detailed in a previous study (Wang et al., 2021a).

A predetermined amount of sludge was manually removed from the reactor daily and replaced with an equivalent amount of new AD sludge. The quantity of sludge fed and discharged was determined by the

adopted hydraulic retention time (HRT), as detailed below. The reactor was constantly aerated with compressed air at a flowrate of 2 L/min through an air stone, ensuring a dissolved oxygen (DO) concentration exceeding  $4 \text{ mg L}^{-1}$  (METTLER TOLEDO, Switzerland). The reactor was equipped with a pH probe and a transmitter (both from TPS, Australia) for pH monitoring. The pH was not controlled but recorded prior to each sludge discharge/feeding event. The reactor was mixed using a magnetic stirrer at 250 rpm.

The bioleaching reactor was operated continuously for 1024 days, divided into three phases. In Phase 1 (Day 0–500), the reactor was operated with an HRT of 13 days. The HRT was extended to 26 days in Phase 2 (Day 501–900), to further reduce the pH. In Phase 3 (Day 901–1024), 2.6 mL ammonium chloride ( $\text{NH}_4\text{Cl}$ ) stock solution (62.5 g  $\text{NH}_4^+$ -N/L) was added to the reactor daily along with the sludge, aiming to elevate the  $\text{NH}_4^+$  concentration by 250 mg N/L.

### 2.3. Monitoring protocol

The monitoring protocol is briefly summarized here, with a detailed description of each analysis provided below. The dissolved nitrogen (ammonium, nitrite, and nitrate) concentrations were assayed three times per week. The total inorganic nitrogen (TIN) was calculated as the sum of ammonium, nitrite, and nitrate concentrations.

The concentrations of total kjeldahl nitrogen (TKN) and total phosphorus (TP) in the feed/discharged sludges were assayed weekly. The total nitrogen (TN) was computed as the sum of TKN, nitrite, and nitrate. The total solids (TS) and volatile solids (VS) concentrations in the feed/discharged sludges were measured biweekly. The solids destruction efficiencies of TS and VS were calculated as, taking TS as an example,  $(\text{TS}_{\text{in}} - \text{TS}_{\text{out}}) / \text{TS}_{\text{in}}$ , where  $\text{TS}_{\text{in}}$  and  $\text{TS}_{\text{out}}$  represent the TS concentration in the feed and the discharged sludges, respectively.

Sludge stabilization levels and metal distribution were evaluated every 1–2 weeks, at the steady state of each phase. The sludge stabilization levels are indicated by specific oxygen uptake rate (SOUR) and *Faecal Coliforms* abundances. The SOUR was calculated as the oxygen uptake rate (OUR) standardized by the VS concentration. The OUR was measured in an *ex situ* respirometer as described in a previous study (Wang et al., 2021c). The metal solubilization efficiency was calculated using the formula  $(M_s / M_{\text{total}}) * 100\%$ , where  $M_s$  and  $M_{\text{total}}$  represent the soluble and total metal concentrations, respectively.

### 2.4. Assessment of $K_{s,\text{total}}$ value at different pH levels

The  $K_{s,\text{total}}$  value of enriched *Ca.* Nitrosoglobus culture (collected from the digester on Day 800) was evaluated at three distinct pH levels 3.5, 4.5, and 7.5. For each pH level,  $K_{s,\text{total}}$  value was estimated based on the Monod equation.

$$\mu = \mu_m \frac{S_{\text{total ammonia}}}{K_{s,\text{total}} + S_{\text{total ammonia}}} \quad (1)$$

where  $\mu$  denotes the specific growth rate of *Ca.* Nitrosoglobus ( $\text{h}^{-1}$ ),  $\mu_m$  is the maximum growth rate ( $\text{h}^{-1}$ ), and  $S_{\text{total ammonia}}$  is the sum of  $\text{NH}_3$  and  $\text{NH}_4^+$  (mg N/L). The substrate utilization rate ( $r_{\text{O}_2}$ ) is related to the specific growth rate as follows:

$$r_{\text{O}_2} = \frac{\mu X}{Y} = \frac{\mu_m X}{Y} \frac{S_{\text{total ammonia}}}{K_{s,\text{total}} + S_{\text{total ammonia}}} \quad (2)$$

Where  $X$  is the *Ca.* Nitrosoglobus biomass concentration (mg/L), and  $Y$  represents the yield of *Ca.* Nitrosoglobus (mg biomass/mg  $\text{O}_2$ ).  $X$  can be seen as a constant over the batch duration (< 1 hour) due to the slow growth of autotrophic *Ca.* Nitrosoglobus (Wang et al., 2021a). As such,  $\frac{\mu_m X}{Y}$  is a constant and lumped as  $r_{\text{O}_2,m}$ , i.e., the maximum OUR derived from ammonia oxidation.

$$r_{O_2} = r_{O_2,m} \frac{S_{total\ ammonia}}{K_{s,total} + S_{total\ ammonia}} \quad (3)$$

Here, OUR derived from ammonia oxidation ( $r_{O_2}$ ) was measured across a gradient of total ammonia concentrations ranging from 0 to 500 mg N/L, under an unlimited DO concentration ( $> 8$  mg  $O_2$ /L), with an *ex-situ* respirometer. Detailed operational procedures of the respirometer are presented in *Supplementary Materials*. The  $K_{s,total}$  and the  $r_{O_2,m}$  value were determined through non-linear least-square regression. The 95 % confidence interval was obtained using the in-built model in Prism GraphPad (V 8.00) (San Diego, California).

## 2.5. Analytical methods

### 2.5.1. Chemical analysis

To determine the concentrations of dissolved nitrogen compounds, liquid samples were taken with syringes and filtered immediately through 0.45  $\mu$ m sterile millipore filters (Merck SLLHR04NL, U.S.). Concentrations of  $NO_2^-$ -N,  $NO_3^-$ -N, and  $NH_4^+$ -N were measured with a Flow Injection Analyzer (Lachat QuickChem8000, Milwaukee, WI). The TS, VS were measured according to standard methods (American Public Health Association, 2005).

### 2.5.2. Metal concentrations analysis

Metal concentrations were measured using an inductively coupled plasma-optical emission spectrometry (ICP-OES) (Thermo Scientific iCAP 7000 plus Series, U.S.). For total metal concentrations, 5 mL of mixed sludge liquor was digested with 5 mL of 70 %  $HNO_3$  at 200  $^\circ$ C for 30 min in a microwave (MARS 6, U.S.), and then analysed by ICP-OES. Solubilized metal concentrations were determined by centrifuging 10 mL of mixed sludge at 13,000 rpm for 15 min, filtering the supernatant (3.6 mL) through 0.45  $\mu$ m sterile millipore filters (Merck SLLHR04NL, U.S.), and digesting the filtered supernatant (3.6 mL) with 70 %  $HNO_3$  (0.4 mL) at 160  $^\circ$ C for 20 min. The digested supernatant was then analysed to determine the solubilized metal concentrations.

### 2.5.3. Faecal Coliform analysis

The abundance of *Faecal Coliforms* in the feed and discharged sludges were quantified using the Colilert®–18 Test kit (IDEXX laboratories, Australia) as per the manufacture's instructions. Sludge samples were initially diluted with sterilized reverse osmosis water to ensure pathogen concentrations within the detection limit of the test kit (1–2420 cells per 100 mL). The diluted sludge samples were mixed with Colilert®–18 reagents and transferred into a Quanti-Tray® 2000, which was sealed in

the Quanti-Tray sealer (IDEXX laboratories, Australia). The sealed tray was incubated at  $45 \pm 1$   $^\circ$ C in an incubator with a shaking speed of 120 rpm. After 18-hour incubation, wells indicating positive *Faecal Coliforms* signal turn yellow. The number of positive wells (yellow) was counted and recorded. Based on the number of positive wells, the most probable number (MPN) of *Faecal Coliforms* was estimated using the standard table provided by the supplier (IDEXX laboratories, Australia).

## 3. Results

### 3.1. Attaining a low pH conducive to bioleaching

The pH level is a crucial determinant of metal leaching efficiency. Over the course of 1024 days, the pH profiles of both the feed and discharged sludge were closely monitored, as depicted in Fig. 1. The feed sludge maintained a relatively stable pH of around 8 throughout the entire experimental duration. During the initial 20-day start-up period, the pH of the discharged sludge decreased from 7 to roughly 5, indicating successful acidification. Subsequently, for a period of 500 days (Phase 1), the reactor's pH consistently maintained at  $4.7 \pm 0.5$  at an HRT of 13 days. Despite the presence of residual ammonium ( $166 \pm 71$  mg N/L) in the reactor (Table 1), there was no further decrease in pH. This is likely due to the short sludge retention time (SRT) of *Ca. Nitrosoglobus*, which was equivalent to HRT (13 days), thereby limiting the extent of ammonium oxidation.

To achieve a pH level conducive to bioleaching, the SRT was extended to 26 days in Phase 2. This extension resulted in a further decrease in pH to  $3.5 \pm 0.4$ . In the steady state of this phase, the reactor contained ammonium at  $44.3 \pm 11.2$  mg N/L, a level likely limited the activity of the acid tolerant AOB (Wang et al., 2021a).

We evaluated the total ammonia affinity constant of *Ca. Nitrosoglobus* at pH 3.5, to test the hypothesis that ammonium was a limiting substrate in Phase 2, preventing the pH from decreasing further. The  $K_{s,total}$  value was estimated to be  $67.25 \pm 20.69$  mgN/L at pH 3.5 (Fig. 2). Such a high  $K_{s,total}$  value could be due to that low pH of 3.5 reduced the availability of ammonia—the true substrate of AOB. Given a total ammonium concentration of  $44 \pm 11$  mg N/L in Phase 2 (Table 1), the *in situ* AOB rate was only 40 % of the maximum rate according to the Monod equation. This result suggests that the *Ca. Nitrosoglobus* was indeed subject to ammonia limitation. To overcome this substrate limitation,  $NH_4Cl$  of 250 mg  $NH_4$ -N  $L^{-1}$  was introduced to the feed sludge from Day 901 onwards. The addition of  $NH_4^+$  led to a further decrease in pH to  $2.5 \pm 0.3$  in Phase 3 (Fig. 1). This supported our hypothesis.

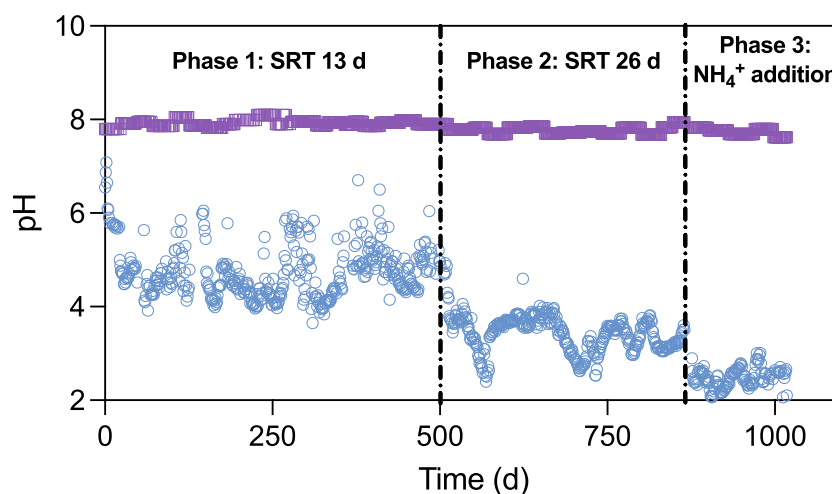


Fig. 1. The pH profiles of feed (purple squares) and discharged sludge (blue circles) across three phases. Phase 1 and Phase 2 have different SRTs of 13 days and 26 days, respectively. An SRT of 26 days was maintained in Phase 3, but ammonium chloride was added to the feed sludge, increasing its ammonium concentration by 250 mg N/L.

**Table 1**

Nitrogenous compounds concentrations in feed and discharged sludge across the three phases.

Parameters	Feed sludge	Discharged sludge		
		Phase 1	Phase 2	Phase 3
Total Kjeldahl nitrogen (TKN) (mg N/L)	3278.5 ± 437.2	1310.7 ± 161.8	788.0 ± 116.2	776.5 ± 98.3
Soluble Kjeldahl nitrogen (SKN) (mg N/L)	1309.8 ± 8.3	219.2 ± 101.4	94.4 ± 11.8	78.7 ± 7.0
Ammonium (mg N/L)	1206.2 ± 120.1	165.5 ± 70.9	44.3 ± 11.2	65.4 ± 22.6
Nitrite (mg N/L)	0.0 ± 0.0	165.4 ± 71.2	11.7 ± 5.6	0.6 ± 0.0
Nitrate (mg N/L)	0.0 ± 0.0	83.3 ± 51.2	200.4 ± 66.5	213.2 ± 52.5
Total inorganic nitrogen (TIN) <sup>1</sup> (mg N/L)	1206.2 ± 120.1	414.2 ± 158.7	256.4 ± 68.2	279.2 ± 38.3
Total nitrogen (TN) <sup>2</sup> (mg N/L)	3278.5 ± 437.2	1559.4 ± 106.2	1000.1 ± 77.4	990.3 ± 50.8
Organic nitrogen <sup>3</sup> (mg N/L) <sup>4</sup>	2072.3 ± 329.3	1145.2 ± 134.9	743.7 ± 73.0	711.1 ± 65.2

<sup>1</sup> TIN was calculated as the sum of ammonium, nitrite, and nitrate.

<sup>2</sup> TN was calculated as the sum of TKN, nitrite, and nitrate.

<sup>3</sup> Organic nitrogen was calculated as the difference between TN and TIN.

<sup>4</sup> N/A means not applicable.

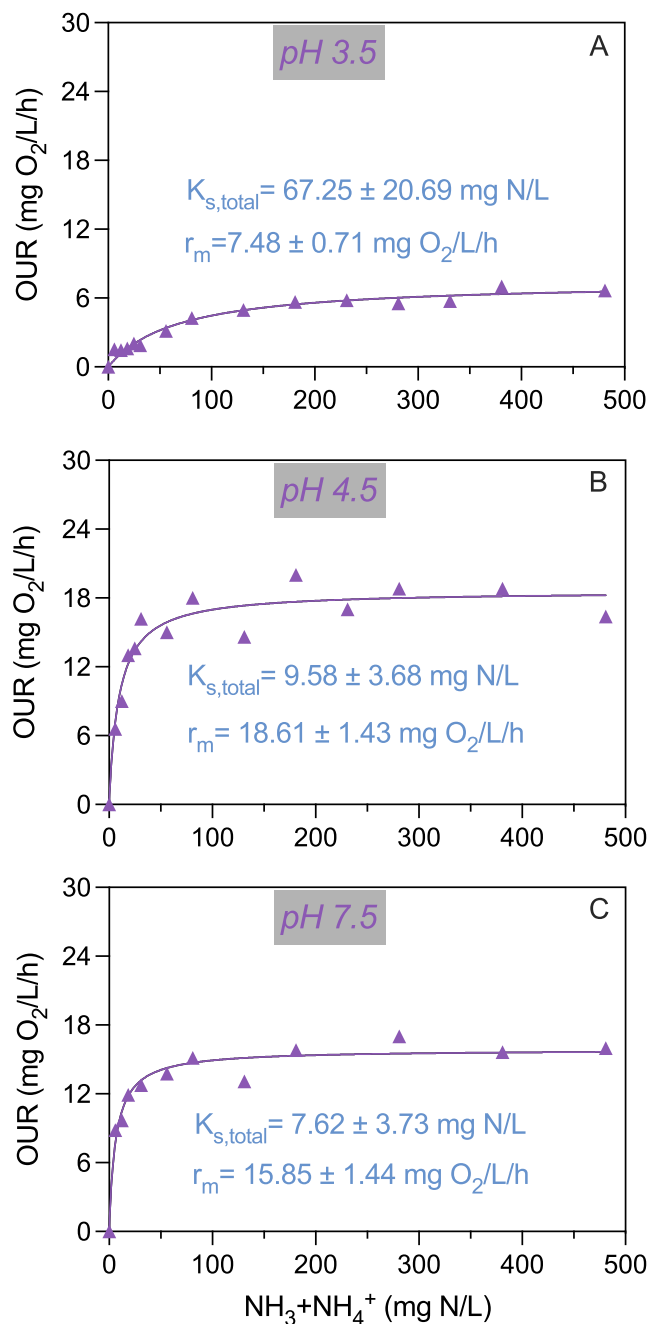
We also evaluated the total ammonia affinity constant of *Ca. Nitrosoglobus* at pH 4.5 and 7.5 (Fig. 2). Interestingly, we found comparable  $K_{s,total}$  values at these two pH levels, with values of  $9.58 \pm 3.68$  and  $7.62 \pm 3.73$  mg N/L, respectively. These values align with previous studies which reported  $K_{s,total}$  values of  $13.5 \pm 3.3$  and  $9.6 \pm 4.6$  mg N/L at pH 5.0 and 5.5, respectively (Wang et al., 2021b,2021a). Contrary to our speculation that  $K_{s,total}$  of *Ca. Nitrosoglobus* is pH-dependent, the  $K_{s,total}$  value remains relatively stable within a pH range of 4.5–7.5. It is unclear why  $K_{s,total}$  value increases at pH 3.5, which requires further investigation.

Around 50–70 % of influent N was lost during bioleaching (Table 1). Part of the TN loss could result from the emissions of NO and N<sub>2</sub>O, both of which are gaseous intermediates of ammonium oxidation. However, the emission of NO and N<sub>2</sub>O only accounted for 1.3 % and 5.8 % of TN loss Phase 3 (Table S3). The remaining N loss is likely due to denitrification to N<sub>2</sub>, which may occur in the micro-anoxic zones created by high solids concentrations (approximately 20 g/L) and insufficient mixing/aeration. The N loss could also be mediated by aerobic denitrifiers which respire oxygen and nitrogen oxides concurrently (Hu et al., 2023; Lu et al., 2023).

### 3.2. Solubilisation of toxic metals

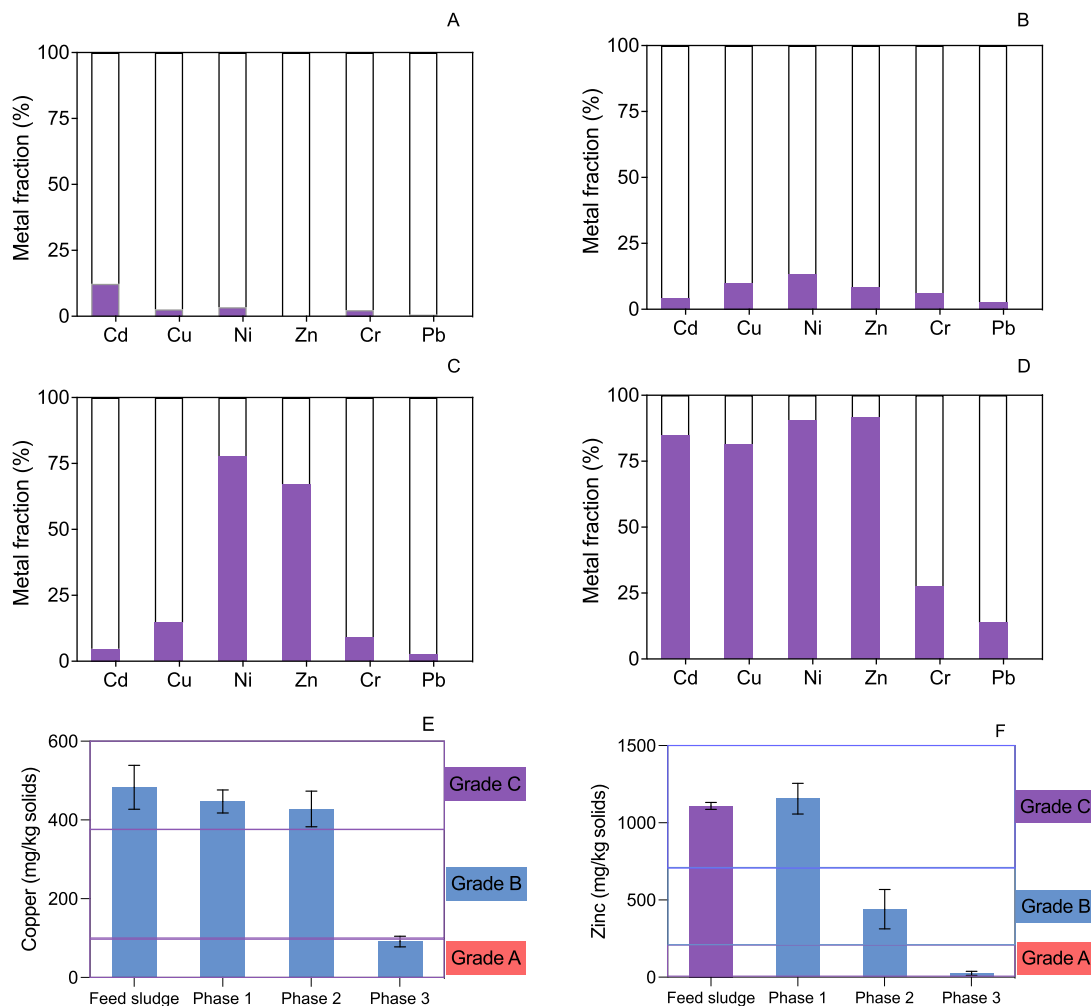
We monitored six toxic metals, including Cd, Cu, Ni, Zn, Cr, and Pb, as these are regulated by NSW-EPA biosolids standards (New South Wales Environment Protection Authority: Sydney, 2000), across all three phases.

The distribution of these toxic metals between liquid and solid phases is illustrated in Fig. 3A–D (the results of other metals were presented in Fig. S1). In the feed AD sludge, these metals predominantly exist in solid phase, with less than 10 % in the aqueous phase (Fig. 3A). The soluble metal level only marginally increased in Phase 1 (pH  $4.7 \pm 0.5$ ) (Fig. 3B). However, in Phase 2 with an *in-situ* pH of  $3.5 \pm 0.4$ , Ni and Zn were substantially solubilized (Fig. 3C), with a liquid fraction of 77.6 % and 67.1 %, respectively. The solubilization efficiencies for the other four metals remain below 10 %. As the pH further decreased to  $2.5 \pm 0.3$  in Phase 3, more than 75 % of Cd, Cu, Ni, Zn effectively solubilized, whereas the liquid fractions of Cr and Pb remain at relatively low levels of 28 % and 14 %, respectively. (Fig. 3D).



**Fig. 2.** Estimation of the affinity constant with respect to total ammonia at pH 3.5 (A), 4.5 (B), and 7.5 (C). The activity was represented by oxygen uptake rate (OUR) derived from ammonia oxidation. The half saturation constants ( $K_s$ ) and maximum rates ( $r_m$ ) were estimated based on the Monod equation.

The toxic metal concentrations in the feed and discharged sludges are compared against the biosolids grading thresholds in Fig. 3E and F, and Table S2. Due to the effective sources control, Cd, Ni, Cr, and Pb already complied with the Grade A standard in the feed sludge. However, the feed AD sludge contained  $483.1 \pm 55.8$  mg Cu per kg solids and  $1109.6 \pm 22.5$  mg Zn per kg solids, which only met Grade C standards (New South Wales Environment Protection Authority: Sydney, 2000). In Phase 1, the soluble Cu and Zn concentrations increased slightly but the metal contents in the treated sludge elevated due to solids destruction (as will be further discussed). In Phase 2, bioleaching significantly decreased the Zn concentration to  $440.5 \pm 127.0$  mg/kg solid (Grade B standards), but the Cu concentration remained at Grade C biosolids level with a concentration of  $427.9 \pm 45.4$  mg Cu/kg solids. The further



**Fig. 3.** The distribution of six toxic metals between liquid (filled) and solids (empty) phases of the feed (A), and the discharged sludge in Phase 1 (B), Phase 2 (C), and Phase 3 (D). Concentrations of copper (E) and zinc (F) in the feed and discharged sludges are compared against the biosolids grading criteria. The reuse of Grade B & C biosolids need to be strictly managed while Grade A biosolids qualify for unrestricted use.

acidification in Phase 3 successfully decreased both Zn and Cu contents to levels that complies to the Grade A standards. The above results indicates that the ammonium-based bioleaching can upgrade AD sludge from Grade C to Grade A when the pH is decreased to  $\sim 2.5$ ; however, additional ammonium is required to attain such a low pH.

### 3.3. Simultaneous solids reduction and stabilization

Apart from efficient metal solubilization, the bioleaching reactor concurrently achieved solids reduction and stabilization. In Phase 1, the TS was reduced by approximately 30 %, from  $31.6 \pm 1.0$  in the feed sludge to  $21.8 \pm 0.6$  g/L in the treated sludge (Fig. 3A). The extended HRT in Phase 2 led to an additional TS reduction of 17 %, bringing the concentration down to  $18.0 \pm 0.5$  g/L. Despite a further lowered pH in Phase 3, there was no significant further TS reduction compared to Phase 2, possibly due to most biodegradable solids already being destroyed. Likewise, the VS concentration decreased from an initial  $22.6 \pm 0.5$  g/L in the feed sludge to  $15.5 \pm 0.2$  g/L in Phase 1, further to  $12.2 \pm 0.4$  g/L in Phase 2, and finally to  $11.6 \pm 0.4$  g/L in Phase 3 (Fig. 3B). By Phase 3, the bioleaching reactor had achieved an overall reduction efficiency of 43 % for TS and 46 % for VS.

The level of sludge stabilization, as indicated by *Faecal Coliform* abundances and SOUR, was also evaluated. The feed sludge was characterised with a *Faecal Coliform* count of  $4.22 \pm 0.05$  log (MPN/gTS) and an SOUR of  $2.09 \pm 0.12$  mg O<sub>2</sub>/gVS/h (Fig. 3C and 3D), both meeting

only the standards for Class B biosolids according to U.S EPA 40 CFR Part 503 Regulation and New South Wales Environment Protection Authority's standard. The bioleaching process effectively decreased the *Faecal Coliform* abundance to levels that met the standards of Grade A biosolids: down to log count of  $2.35 \pm 0.11$  (Phase 1), then to  $2.07 \pm 0.13$  (Phase 2), and finally to  $1.90 \pm 0.2$  (Phase 3) log (MPN/gTS) (Fig. 3C). The SOUR levels of the discharged sludge were also significantly decreased across all three phases, falling into Grade A category (Fig. 3D). In conclusion, the ammonium-based continuous bioleaching process successfully upgraded AD sludge from Grade B to Grade A in terms of stabilization levels.

## 4. Discussion

This study presents, for the first time, the application of ammonium-based bioleaching for sewage sludge treatment in a continuous reactor. Over a period of 1024 days, the reactor pH was successfully reduced to  $2.5 \pm 0.3$  (Day 901–1024). This low pH environment facilitated efficient leaching of heavy metals from AD sludge, thereby elevating sludge contaminants quality to meet Grade A biosolids standards. Concurrently, the stabilization level achieved during bioleaching also satisfied Grade A criteria. In sum, the ammonium-based leaching process successfully upgraded AD sludge, an important by-product of STPs, from Grade C to Grade A biosolids. These upgraded biosolids are suitable for unrestricted reuse, promoting the principles of a circular economy.

Furthermore, the enhanced quality of the sludge could potentially reduce sludge disposal costs, as it allows for local disposal, eliminating the need for transportation to distant disposal sites.

This study presents an interesting comparison of the affinity constant of the novel AOB, *Ca. Nitrosoglobus*, across a range of pH levels (3.5–7.5). The  $K_{s,\text{total}}$  value increases at pH 3.5, while remaining relatively stable within the pH range of 4.5–7.5 (Fig. 4). Since  $\text{NH}_3$  is generally regarded as the true substrate of AOB, the  $K_{s,\text{NH}_3}$  value should be relatively constant, irrespective of pH levels. Contrary to this expectation,  $K_{s,\text{NH}_3}$  decreases as pH declines (Fig. 5A), indicating a higher affinity for ammonia at lower pH values. This observation could be potentially attributed to cellular adaptations to acidic environment, such as an increased expression of  $\text{NH}_3/\text{NH}_4^+$  permease (Herbold et al., 2017). However, this hypothesis needs further investigation through transcriptomic studies.

As shown in Fig. 5, among known acid-tolerant ammonia oxidizers, the *Ca. Nitrosoglobus* used in this study exhibits the highest affinity for ammonia under extremely acidic (pH < 4) conditions (Hayatsu et al., 2017; Picone et al., 2021; Wang et al., 2021a,2021c). Therefore, *Ca. Nitrosoglobus* is the best candidate for ammonium-based bioleaching among known ammonia oxidizers, notwithstanding the identified ammonia limitation issue. This limitation can be mitigated by applying this process to ammonia-rich sludge treatment, such as AD digestate with thermal hydrolysis pretreatment (Abelleira et al., 2012), high-solids anaerobic digestate (Fagbohngbe et al., 2015), or digestate from the co-digestion of sewage sludge and food wastes (Breunig et al., 2017). These opportunities will be explored in further studies.

Apart from using different sludge sources, the ammonia limitation issue could also be alleviated through process optimization. It is worth

noting that the observed ammonia limitation contradicts our previous batch tests. The batch tests demonstrated the same AD sludge can be acidified to pH 2, relying solely on microbial ammonium oxidation, without any external chemical addition (Wang et al., 2021b). This discrepancy is presumably due to the differing solid concentrations between the two studies. The previous batch test employed diluted AD sludge with a solid concentration of around 15 g/L, in contrast to the concentration of over 30 g/L used in this study. A higher solid concentration would likely induce anoxic zones in the reactor, thereby promoting denitrification which generates alkalinity (Metcalf et al., 1991). Therefore, for the ammonium-based bioleaching process, it is critical to maintain adequate mixing/aeration and to avoid high solids concentration.

Another challenge, which was not identified in previous batch study, is the slow growth of *Ca. Nitrosoglobus* at extremely acidic pH. The slow growth of *Ca. Nitrosoglobus* necessitates an extended SRT, leading to increased capital expenditure. Future studies should focus on elevating the capacity of the ammonium-based bioleaching process. This could be achieved, for example, by retaining *Ca. Nitrosoglobus* in biofilm on carriers (Klaus et al., 2016). Alternatively, the rate of *Ca. Nitrosoglobus* doubles with the temperature increasing from the current 22 °C to 30 °C (Wang et al., 2021a). This elevated temperature could be achieved by harnessing the heat released during aerobic digestion, as demonstrate in the autothermal aerobic digestion process (Jain et al., 2010).

The acidic biosolids produced need neutralisation before final disposal to prevent potential adverse effects on soils and plants. Our previous study indicated that approximately 30 kg lime per ton TS is needed to adjust the pH from 2.0 to 7.0, costing only AUD 1.5 per ton TS processed (Wang et al., 2021b). This low alkali consumption is likely due

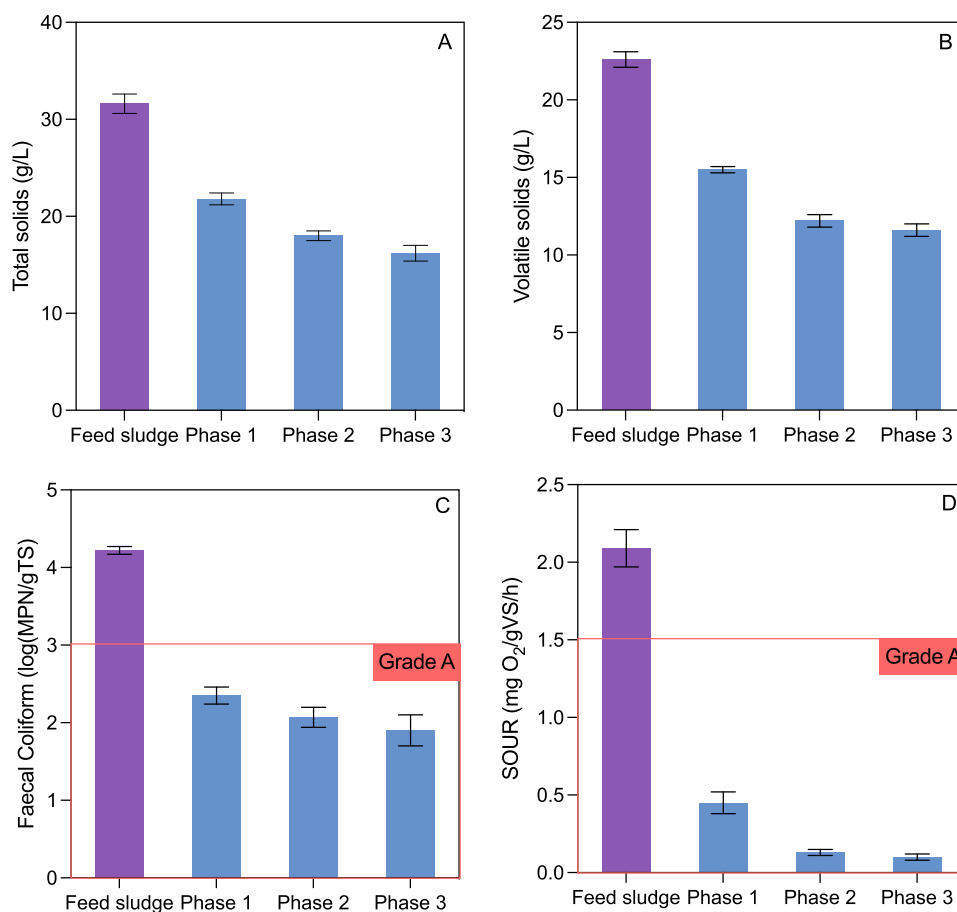


Fig. 4. Total solids (A), volatile solids (B), *Faecal Coliform* abundances (C), and specific oxygen uptake rate (SOUR) (D) in the feed and discharged sludge across three phases. The latter two parameters are compared against the thresholds of Grade A biosolids—the highest quality of biosolids qualified for unrestricted use.

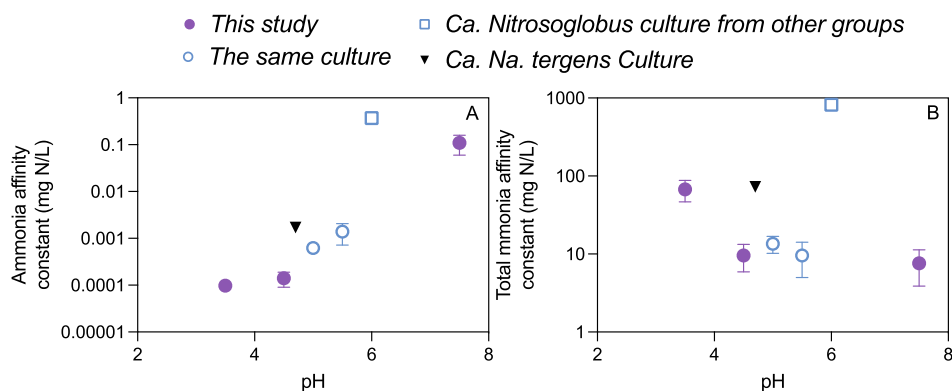


Fig. 5. Comparison of the affinities for ammonia (A) and total ammonia (B) across a range of pH among known acid tolerant ammonia-oxidizing bacteria (AOB).

to the limited buffering substances remaining post bioleaching. The resulting supernatant contains soluble metals requiring removal/recovery. While alkalization via alkali addition or denitrification would induce effective metals precipitation, these approaches lack selectivity. Alternatively, electrodialysis (Zeng et al., 2022) or membrane separation processes (Yesil et al., 2021a; Yesil and Tugtas, 2019) can be employed for metal removal/recovery.

Recently, fermentation-based anaerobic bioleaching has been proposed as an attractive method for heavy metal removal (Meulepas et al., 2015; Molaey et al., 2021b; Yesil et al., 2021a). This process leverages the dissolved organic matters and VFAs, which are produced during sludge hydrolysis and fermentation, as soluble organic ligands to mobilize heavy metals from solid to liquid (Molaey et al., 2021a). Compared to aerobic processes, anaerobic bioleaching requires neither aeration nor additional chemicals, representing a cost-effective option. Nevertheless, its overall metal solubilization efficiencies are lower. For instance, it was reported that alkaline fermentation of sewage sludge led to only 8–12 % total metals removal (Yesil et al., 2021b), compared to over 60 % achieved in iron-, sulfur-, and ammonium-based aerobic bioleaching processes (Babel and del Mundo Dacera, 2006; Pathak et al., 2009; Wang et al., 2021b).

In previous studies, a sulfur-based bioleaching process was proposed, leveraging the indigenous SOB in sludge (Chan et al., 2003; Jain and Tyagi, 1992; Mehrotra et al., 2016). This sulfur-based approach demonstrated comparable performance in terms of solids reduction, sludge stabilization, and toxic metal removal, akin to the results of this study. However, the sulfur-based processes entail the addition of sulfur powders (usually 30–50 kg S per ton dry sludge) (Sreerishnan et al., 1993), leading to additional operational costs. Furthermore, up to half of the added sulfur remains in sludge, potentially causing soil acidification if applied to lands (Blais et al., 1992). These limitations of the sulfur-based process can potentially be mitigated through the integration with ammonium-based bioleaching. Concurrently, the inclusion of sulfur-based processes could address the slow growth of acid-tolerant AOB. It would be intriguing to investigate the potential of combining sulfur- and ammonium-based bioleaching processes, and the synergy between SOB and AOB. In sum, this study provides an alternative or complementary option to the existing sulfur-based technology for simultaneous sludge digestion and metal leaching.

## 5. Conclusion

- An ammonia-based bioleaching process processing AD sludge was stably operated in a continuous reactor at a pH of  $2.5 \pm 0.3$  without chemicals addition.
- The acid tolerant AOB *Ca. Nitrosoglobus* has an abnormally high total ammonia affinity constant at pH 3.5 ( $67.3 \pm 20.7$  mg N/L) compared to pH 4.5–7.5 (7.6 – 9.6 mg N/L), suggesting that

inadequate ammonium is a key challenge limiting bioleaching rates in continuous processes.

- The ammonia-based bioleaching process successfully upgraded AD sludge, an important by-product of STPs, from Grade C to Grade A biosolids. These upgraded biosolids are suitable for unrestricted reuse, promoting the principles of a circular economy.

## CRedit authorship contribution statement

**Zhiyao Wang:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Xi Lu:** Data curation. **Xueqin Zhang:** Writing – review & editing. **Zhiguo Yuan:** Writing – review & editing. **Min Zheng:** Writing – review & editing, Conceptualization. **Shihu Hu:** Writing – review & editing, Project administration, Funding acquisition.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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