Evaluation of the inhibitory effects of heavy metals on anammox activity: A batch test study

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HIGHLIGHTS

- Inhibitory effects of Cu(II) and Zn(II) on SAA were evaluated using RSM.
- Pre-exposure to Cu(II) without substrates strongly inhibited anammox activity.
- Pre-exposure to Cu(II) in the presence of NO\textsubscript{2}–N enhanced inhibition.
- The cumulative toxicity of Cu(II) was mitigated by intermittent exposure.

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ABSTRACT

This study evaluated the interactive effect of Cu(II) and Zn(II) on anaerobic ammonium oxidation (anammox) activity using response surface methodology with a central composite design. A regression model equation was developed and validated to predict the normalized anammox activity (NAA) of anammox granules exposed to various heavy metal concentrations. The joint inhibitory effect tended to exacerbate initially and reversed as the concentrations increased and then moderated again. The most severe inhibition, resulting in a NAA of 20.1%, occurred at Cu(II) and Zn(II) concentrations of 16.3 and 20.0 mg L\textsuperscript{-1}, respectively. Notably, the cumulative toxicity was mitigated with the aid of intermittent exposure acclimatization. Additionally, pre-exposure to Cu(II) in the absence of substrates strongly inhibited anammox activity. However, the presence of NO\textsubscript{2}– significantly enhanced Cu(II) inhibition. Therefore, such conditions should be avoided to minimize the disturbance of the anammox process.

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1. Introduction

The discovery of the anaerobic ammonium oxidation (anammox) process revolutionized the removal of nitrogen from ammonium-rich residual streams. In this process, ammonium is directly converted by autotrophic anammox bacteria to dinitrogen gas with nitrite as an electron acceptor under anoxic conditions (Mulder et al., 1995). The anammox-based process has been recognized as an innovative, cost-effective and more sustainable alternative to the conventional nitrification and denitrification processes (Kartal et al., 2010; Lotti et al., 2014; Zhang et al., 2015a,b). To date, over one hundred full-scale anammox plants have been installed for the treatment of municipal and industrial wastewaters, such as anaerobic digestion reject water and waste water produced by the tannery, food-processing, semiconductor, fermentation, yeast, distillery and winery industries (Lackner et al., 2014).

However, because of the slow growth rates, low cell yields and highly variable responses to the external environment, the application and industrialization of anammox-based processes have been limited by the inhibitory substances that are common in nitrogen-rich wastewater (Jin et al., 2012). Certain heavy metals, such as Cu, Zn, Cd, Pb and Ni, are found in high-strength ammonium wastewaters, e.g., municipal landfill leachates, anaerobically digested pig-gery/dairy slurries, effluents from the production of nitrogenous fertilizers, and semiconductor manufacturing wastewater streams (Zhang et al., 2015c). Several publications have focused on the individual effects of heavy metals on the anammox process, e.g., Cu, Zn, Cd, Ag, Pb and Hg (Bi et al., 2014; Lotti et al., 2012; Yang et al., 2013; Zhang et al., 2015d,e). However, the joint effect of heavy metals on the anammox process remains poorly documented (Kimura and Isaka, 2014). At low concentrations, both Cu(II) and Zn(II) are necessary micronutrients and components of many
enzymes and co-enzymes of microorganisms. However, excessive Cu(II) and Zn(II) inhibit or may be toxic to microorganisms in biological wastewater treatment systems (Ochoa-Herrera et al., 2011; Zhang et al., 2015e). Previous studies showed that presence of 1 mg L⁻¹ Cu(II) or Zn(II) improved anammox performance by stimulating anammox activity and that presence of 2 mg L⁻¹ Cu(II) or Zn(II) did not remarkably affect the nitrogen-removal performance of the continuous-flow reactors (Zhang et al., 2015d,e). Unfortunately, the Cu(II) and Zn(II) levels of real industrial wastewaters are expected to be moderate because of the dilution of industrial effluents by wastewaters from residential sources, critical situations (e.g., fluctuations in the flow and composition of wastewaters, anammox bacteria may also experience famine conditions in underloaded bioreactors. The impact of heavy metals on anammox bacteria may vary depending on whether it is being actively metabolized or not. Therefore, two cases should require more attention: the absence of two substrates and the presence of NO₂⁻, which may reduce the tolerance of anammox bacteria to heavy metal inhibition because excessive NO₂⁻ is an inhibitor of anammox bacteria.

A central composite design (CCD) followed by response surface methodology (RSM), a statistics-based method, constitutes a powerful tool to determine optimal process parameters. These statistical methods have been widely used for bioprocess optimization to enhance the production of value-added products, although few reports describe the application of RSM to the inhibitory effects of toxicants (Chen et al., 2014; Xing et al., 2014).

Therefore, the purpose of this study is to evaluate (i) the interactive effects of Cu(II) and Zn(II) on anammox activity using RSM with CCD; (ii) the cumulative toxicity of Cu(II) and Zn(II) on anammox granules; and (iii) the pre-exposure effects of Cu(II) on anammox bacteria in the absence of substrates or the presence of NO₂⁻.

2. Methods

2.1. Origin of the biomass

Anammox biogranules were used in all batch experiments. Bacterial sludge was cultivated and maintained in a laboratory-scale (2-L) up-flow anaerobic sludge blanket (UASB) reactor fed with a synthetic medium at a loading rate of 10 kgN L⁻¹ d⁻¹. The sludge was dominated by anammox bacteria of the genus Candidatus Kuenenia stuttgartiensis (Zhang et al., 2015a). These mature anammox granules possessed excellent settleability (settling velocity over 70 m h⁻¹) and superior granule diameter (5.1 ± 1.6 mm on average). The specific anammox activity (SAA) of the granules was 444.4 ± 26.1 mgTN g VSS⁻¹ d⁻¹.

2.2. Acute exposure bioassays and anammox activity determination

Batch exposure assays were performed in serum flasks with a total volume of 160 mL and a liquid-phase volume of 120 mL. A total of 100 mL of basal mineral medium and 1.25 mL of trace element solutions I and II per liter of sterilized water were introduced, resulting in a composition similar to that of the synthetic wastewater. The mineral medium composition was 10 mg L⁻¹ KH₂PO₄, 5.6 mg L⁻¹ CaCl₂·2H₂O, 300 mg L⁻¹ MgSO₄·7H₂O, and 1250 mg L⁻¹ KHC₂O₃. The composition of trace element solution I was 5 g L⁻¹ EDTA and 9.14 g L⁻¹ FeSO₄·7H₂O, and trace element solution II was composed of 15 g L⁻¹ EDTA, 0.014 g L⁻¹ H₂BO₃, 0.99 g L⁻¹ MnCl₂·4H₂O, 0.25 g L⁻¹ CuSO₄·5H₂O, 0.43 g L⁻¹ ZnSO₄·7H₂O, 0.21 g L⁻¹ NiCl₂·6H₂O, 0.22 g L⁻¹ NaMoO₄·2H₂O and 0.24 g L⁻¹ CoCl₂·6H₂O. Fresh biomass was withdrawn from the nursing reactor, washed and re-suspended in mineral medium. Then, the flask were subsequently inoculated with approximately 10 mL of the anammox biomass (2.0 gVSS L⁻¹). (NH₄)₂SO₄, NaNO₂, CuCl₂·2H₂O and ZnCl₂·2H₂O were added to the mineral medium as needed (Fig. 1, Protocols 1 and 2). Initially, the pH was fixed at approximately 7.5 by adding 0.1-M hydrochloric acid, and then, the headspace and liquid phase were gasified with argon to remove oxygen. The serum flasks were sealed with rubber stoppers and aluminum crimp seals and were incubated on an orbital shaker (180 rpm) in the dark at 35 ± 1 °C. Three-milliliter test samples were periodically collected using a syringe with a needle and were stored at 4 °C for the determination of NH₄⁺–N and NO₂⁻–N. The SAA was calculated from the maximum slope of the time course of the NH₄⁺–N and NO₂⁻–N concentrations in the bulk and was expressed as mgTN g VSS⁻¹ d⁻¹ (Yang and Jin, 2013). The activity of each experiment was normalized with respect to the activity of a control that was not subjected to inhibitory conditions: normalized anammox activity (NAA, %) = SAA inhibited/SAA control × 100.

2.3. Experimental design and data analysis

A CCD was used to study the interactive effects of two heavy metals, i.e., Cu (X₁) and Zn (X₂), on the responses of anammox activity. Both variables were assessed at five different coded levels, represented as −2.12, 0.±1, and ±2. A total of 13 experiments, including five replications at the center points, were conducted as per the design. The full experimental design and the coded and natural (uncoded) values of all variables are shown in Table 1. The experimental levels were based on the preliminary experiments, in which the 50% inhibition concentrations (IC₅₀) for Cu and Zn were 30 and 25 mg L⁻¹, respectively. Design-Expert software version 8.0 (STAT-EASE Inc., Minneapolis, USA) was used for the design of the CCD experiment, regression analysis of the experimental data and plotting of the response surface and contour plots. The independent variables Cu (X₁) and Zn (X₂) and their mathematical relationships with the response Y (NAA) can be approximated by the quadratic polynomial equation, as shown in Eq. (1) (Daverey et al., 2015)

\[ Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_i X_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j \]  

(1)

where Y is the predicted response, k is the number of factor variables, X is the coded levels of the independent variables, \( \beta_0 \) is the offset term, \( \beta_i \) is the ith linear coefficient, \( \beta_{ij} \) is the ith quadratic coefficient, and \( \beta_{ij} \) is the ith interaction coefficient. These coefficient parameters were estimated through a multiple linear regression analysis performed using the Design-Expert 8.0 program. Analysis of variance (ANOVA) was used to evaluate the interaction between the process variables and the response, and p < 0.05 was considered statistically significant. The quality of the fit of the quadratic model was expressed via the coefficient of determination, \( R^2 \), and the statistical significance was determined by an F-test in the same program. The significance of the regression coefficient was tested via a t-test (Xing et al., 2014).

2.4. Assessment of joint toxicity

The joint toxicities consist of four main types: independent effect (INE), additive effect (ADE), synergistic effect (SYE) and antagonistic effect (ANE) (Ding et al., 2015). Isobole plot (IP) is a...
2.5. Cumulative toxicity bioassays

The cumulative toxicity bioassays were conducted in serum flasks with a total volume of 650 mL and a liquid-phase volume of 500 mL. The basal mineral medium and trace element solutions were the same as in the acute exposure bioassays. During every 24-h cycle, NH$_4^+$–N and NO$_2^-$–N were supplemented every 12 h at a final concentration of 100 mg L$^{-1}$. Anammox granules were exposed to heavy metals (equimolar Cu(II) and Zn(II)) for 12 h, followed by decanting the bulk. The granules were then used for the 12-h determination of SAA. This intermittent exposure test was repeated four times. Samples were periodically collected using a syringe with a needle and were stored at 4 °C for the determination of NH$_4^+$–N and NO$_2^-$–N. Three dosages of heavy metals were selected as low (1.25 mg HM g$^{-1}$ VSS), moderate (6.25 mg HM g$^{-1}$ VSS) and high level (12.5 mg HM g$^{-1}$ VSS). The cumulative impact was analyzed by the cumulative coefficient ($K_n$), as shown in Eq. (2)

$$K_n = \frac{ED_{50(n)}}{ED_{50(1)}}$$

where $ED_{50(n)}$ is the cumulative dosage causing half inhibition under multiple exposures to heavy metals and $ED_{50(1)}$ is the dosage causing half inhibition under single exposure to heavy metals.
cumulative inhibition was classified as severe \((0 < K < 1)\), obvious \((1 < K < 3)\), medium \((3 < K < 5)\) and slight \((K > 5)\) \citep{Zhang2015}

The values of \(ED_{50(\%)}\) were calculated using an extended non-competitive inhibition model (Eq. (3)), which was fitted to the experimental data by means of the minimum squared errors method

\[
IR = 100 \times \frac{1}{1 + \left(\frac{[HM]}{K_i}\right)^b}
\]

where \(IR (\%)\) is the inhibition response, \([HM]\) is the dosage of heavy metals (mgHM g\(^{-1}\) VSS), \(K_i\) is the inhibition constant (mgHM g\(^{-1}\) VSS) and \(b\) is the inhibition order (dimensionless). Parameter \(K_i\) is the model estimation of the \(ED_{50}\) (dosage causing 50\% inhibition).

**2.6. Pre-exposure bioassays**

The procedures of the pre-exposure bioassays were based on those used for the acute exposure bioassays. However, the addition of NO\(_2\)-N, NH\(_4\)-N and Cu(II) (30 mg L\(^{-1}\)) to the bioassays was performed following the protocols described below and is depicted in Fig. 1.

Protocol 1 was the standard SAA determination using the fresh anammox granules in the absence of inhibitors.

Protocol 2 was the acute exposure test, in which NH\(_4\)-N, NO\(_2\)-N and Cu(II) were added simultaneously, followed by SAA determination.

Protocol 3: The biomass was pre-exposed to Cu(II) for different durations up to 48 h. After the pre-exposure period, bioassays were spiked with NH\(_4\)-N and NO\(_2\)-N, followed by SAA determination.

Protocol 4: Bioassays were supplemented with Cu(II) and NO\(_2\)-N (100 mg L\(^{-1}\)) and pre-incubated for different time periods up to 48 h. Then, the bioassays were spiked with NH\(_4\)-N, followed by SAA determination.

Protocol 5: The biomass was pre-exposed to Cu(II) for 24 or 48 h. Then, the biomass was allowed to settle, and the liquid was decanted (for Protocol 6) and replaced with 100 mL of mineral medium containing no N-compounds. This process was repeated twice to ensure that no Cu(II) remained on the surface of the granules (“washed granules”). Subsequently, the washed granules were supplemented with mineral medium, NH\(_4\)-N and NO\(_2\)-N, followed by SAA determination.

Protocol 6: Fresh anammox biomass was incubated with the liquid medium decanted from the pre-exposure assay. Subsequently, NH\(_4\)-N and NO\(_2\)-N were supplemented for SAA determination.

Protocol 7: The biomass was pre-exposed to Cu(II) and NO\(_2\)-N (100 mg L\(^{-1}\)) for 24 or 48 h. Then, the biomass was allowed to settle, and the liquid was decanted (for Protocol 8) and replaced with 100 mL of mineral medium containing no N-compounds. This process was repeated twice to ensure that no Cu(II) or NO\(_2\)-N remained on the surface of the granules (“washed granules”). Subsequently, the washed granules were supplemented with mineral medium and NH\(_4\)-N for SAA determination.

Protocol 8: Fresh anammox biomass was incubated with the liquid medium decanted from the pre-exposure assay. Subsequently, NH\(_4\)-N and NO\(_2\)-N were supplemented for SAA determination.

During the SAA determination, the initial NH\(_4\)-N and NO\(_2\)-N concentrations were set at 100 mg L\(^{-1}\).

**2.7. Analytical methods**

The NH\(_4\)-N, NO\(_2\)-N, pH and volatile suspended solids (VSS) levels were determined using standard methods \citep{APHA2005}. The Cu concentration was determined after acid digestion (HNO\(_3\)) via atomic absorption spectrometry using an AA6300C spectrometer (SHIMADZU, Japan) with an air/acetylene flame \citep{Zhang2015}.

**3. Results and discussion**

**3.1. Model fitting and statistical analysis**

Thirteen experiments were performed in a random manner to minimize the interference of uncontrolled variables on the obtained responses. A quadratic polynomial equation, shown in Eq. (4), obtained by applying multiple regression analysis on the experimental data was found to explain the interactive effects of Cu(II) and Zn(II) on anammox activity

\[
NAA = 149 - 6.59 \times [Cu] - 6.63 \times [Zn] + 0.0412 \times [Cu] \times [Zn] + 0.177 \times [Cu]^2 + 0.129 \times [Zn]^2
\]

Statistical tests involving the quadratic model for NAA were performed with the F-test for ANOVA, and the results are shown in Table 2. The linear and square terms of the regression model were significant, whereas the interaction term was insignificant. The model F-value of 49.23 indicated that the model was highly significant \((p < 0.0001)\), with probability values of less than 0.05 for a 95\% confidence interval. The “Lack of fit F-value” of 1.04 indicated that the lack of fit was not significant relative to the pure error. The “Pred R-Squared” of 0.8894 was in reasonable agreement with the “Adj-R-Squared” of 0.9723.
a random plot of residuals indicated homogeneous error variances across the observed values. As shown in Fig. 2b, a random distribution was observed for the residual plots for the models and the NAA dataset.

To verify the validity of the regression model, five additional confirmation experiments were conducted, as described in Table 1. The experimental values of NAA were close to the model predictive values \( R^2 = 0.9509 \), which supports the validity of the model under the investigated conditions. Therefore, the model is suitable for the design space.

### 3.2. Interactive effects of Cu and Zn on anammox activity

Fig. 3 shows the three-dimensional response surface and two-dimensional contour plots for the output values of NAA. The contour plot was elliptical, indicating a significant interaction between the two heavy metals. The inhibitory effect of Cu(II) and Zn(II) on anammox activity tended to exacerbate initially and reversed as the concentrations increased and then moderated again. The surface confined in the smallest curve of the contour plot can be used to identify the minimum NAA. When the Cu(II) and Zn(II) concentrations were 16.3 and 20.0 mg L\(^{-1}\), respectively, a valley value was observed to induce the most severe inhibition, with a NAA of 20.1%.

Since the joint toxicity of multicomponent mixtures is usually different from the toxicities of the individual chemicals (Ding et al., 2015), IP was used to determine the joint toxicities of Cu(II) and Zn(II) mixtures (shown in Fig. 4). As the concentrations increased, the joint toxic effect of the Cu(II) and Zn(II) mixtures shifted from synergistic to additive, then to independent, and finally to antagonistic.

Generally, metal uptake, including extracellular sorption, transmembrane transport, and intracellular accumulation, occurs in many microorganisms and depends on the metal concentrations and biomass viability (Hu et al., 2003). Previous studies revealed that most heavy metals (>80%) accumulated in anammox granules via adsorption because extracellular polymeric substances (EPS) and anammox bacterial cell surfaces contain a variety of binding sites, including amino, carboxylic, hydroxyl, and phosphate functional groups. The Cu migrated from soluble EPS to bound EPS over time and then accumulated on the cell surfaces, entered the cells or converted to inert cores during long-term exposure (Zhang et al., 2015c). Due to the multi-layer structure of the anammox granules including two distinct regions, the portion of Cu arrived on the cell surfaces was much lower than the total Cu in the bulk liquid during several hours of short-term exposure. In

Therefore, the anammox bacteria in the granules was not inactivated after several hours of exposure.

During the acute exposure batch tests in the preliminary experiments, the inhibitory effects of Cu(II) and Zn(II) on anammox activity were simulated using the modified non-competitive inhibition model, i.e., Eq. (5) and Eq. (6), where the individual IC\(_{50}\) values for Cu(II) and Zn(II) were calculated to be 30 and 25 mg L\(^{-1}\), respectively. The NAA decreased as the heavy metal concentrations increased. This dose-dependent inhibitory effect was confirmed in the ranges of 5–45 mgCu L\(^{-1}\) and 5–40 mgZn L\(^{-1}\) (Zhang et al., 2015d,e)

\[
\begin{align*}
\text{NAA} &= 100 \times \left[ 1 + \left(\frac{\text{Cu}}{30}\right)^{1.8} \right]^{-1} \\
\text{NAA} &= 100 \times \left[ 1 + \left(\frac{\text{Zn}}{25}\right)^{1.2} \right]^{-1}
\end{align*}
\]

Since the joint toxicity of multicomponent mixtures is usually different from the toxicities of the individual chemicals (Ding et al., 2015), IP was used to determine the joint toxicities of Cu(II) and Zn(II) mixtures (shown in Fig. 4). As the concentrations increased, the joint toxic effect of the Cu(II) and Zn(II) mixtures shifted from synergistic to additive, then to independent, and finally to antagonistic.

- **Table 2** ANOVA for the applied response surface models.

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<th>F value</th>
<th>p-Value</th>
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<tr>
<td>C.V.%</td>
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<td>R(^2) pred</td>
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the case of low concentrations of heavy metals, specific metal transport can be induced via various transporter families, such as those driven by ATP, because heavy metals are indispensable micronutrients and components of many enzymes and coenzymes (Hu et al., 2003). Metals also play a significant role in stimulating the metabolism of microorganisms (Zhang et al., 2015b). However, Zn is also known as a potent inhibitor of the electron transport chain for *Nitrosococcus mobilis* (Zhang et al., 2015e). Because heme c connects proteins via two sulfhydryl groups, copper ions, once inside the anammox bacteria cell, are thought to chelate sulfhydryl groups, causing metabolic disorders in metabolic pathways that depend on intracellular proteins and enzymes (Zhang et al., 2015c). Meanwhile, Cu-induced cytoplasmic membrane rupture may accelerate the accumulation of other heavy metals, e.g., Zn(II) in this study. Therefore, their joint toxicity might be synergistic.

Cu(II) can adsorb on both low-energy and high-energy adsorption sites in soils (Zhang et al., 2015b). As its concentration increases, Zn(II) may compete for the adsorption sites of the EPS and anammox bacterial cell surfaces. When one heavy metal can decrease the absorption and accumulation of other heavy metals, the joint toxicity can be antagonistic (Ding et al., 2015). Moreover, inhibition is not always a function of the total analytical metal concentration but is strongly correlated with the free cation concentration (Hu et al., 2002). Because the pH and synthetic wastewater composition play important roles in metal precipitation due to their influence on the saturation indices (SIs) of different minerals, a Visual MINTEQ (VMINTEQ.v3.1) model was used to estimate the possible minerals precipitated in the exposure bioassays. The calculation results showed that the SIs of the minerals and the species of precipitates increased as the heavy metal concentration increased (Supplementary materials).

### 3.3. Cumulative toxicity of heavy metals on anammox granules

When some heavy metals are present in excess, they can accumulate through rapid and relatively nonspecific metal transport systems, which are constitutively expressed. Therefore, the cumulative inhibition or toxicity of heavy metals is a concern. Fresh anammox granules withdrawn from the reactor were intermittently exposed to various dosages of heavy metals 4 times within 4 days; the cumulative total nitrogen removal (CTNR) capacities are depicted in Fig. 5a. After the first exposure, the CTNRs of the anammox granules exposed to 1.25, 6.25 and 12.5 mg HM g⁻¹ VSS were 0.565, 0.265 and 0.153 g TN g⁻¹ VSS, respectively. Therefore, the inhibition response was dose dependent, as shown in Fig. 5b. The corresponding EC_{50(1)} of 3.26 ± 0.03 mg HM g⁻¹ VSS was
monitored using an extended non-competitive inhibition model ($R^2 = 0.9999$). The inhibition responses after the second exposure were 68.8%, 34.2% and 20.7%, respectively, and the corresponding $EC_{50(2)}$ was estimated to be 5.96 ± 0.10 mgHM g$^-1$ VSS ($R^2 = 0.9998$). $K_{(2)}$ was 1.83, indicating a cumulative toxicity of heavy metals on the anammox granules. After the third exposure, the $EC_{50(3)}$ was 13.1 ± 1.42 mgHM g$^-1$ VSS ($R^2 = 0.9880$), and the corresponding $K_{(3)}$ was 4.02, suggesting a moderate cumulative toxicity. Similarly, the $EC_{50(4)}$ and $K_{(4)}$ were 14.9 ± 0.65 mgHM g$^-1$ VSS ($R^2 = 0.9982$) and 4.57, respectively, indicating moderate cumulative toxicity. Therefore, the cumulative toxicity was mitigated as the exposure times increased. Two potential underlying reasons should be considered: (i) Anammox bacteria gain self-adaptation through acclimatization, e.g., secreting more EPS for self-protection. This behavior can be considered a survival strategy of anammox cells to reduce the uptake of heavy metals, i.e., the so-called adaptive stress response mechanism. (ii) Anammox cells implement a self-healing strategy by actively pumping excessive heavy metals out of the sensitive regions of the cells, i.e., active efflux mechanisms.

3.4. Pre-exposure to heavy metals strongly inhibits anammox activity

The impact of heavy metals on anammox bacteria may vary depending on whether it is being actively metabolized. Thus, the absence of substrates may affect the tolerance of anammox bacteria to heavy metal inhibition. In experiments where NH$_4^+$ and NO$_2^-$ were fed simultaneously from the beginning, the NAA was 47.1% in the presence of 30 mgCu L$^{-1}$. However, in experiments where the biomass was first pre-exposed to Cu(II) for 12 h prior to NH$_4^+$ and NO$_2^-$ addition, 18.4% activity remained after the subsequent addition of substrates. Further experiments were designed to determine how rapidly the Cu(II) pre-exposure inflicts full impact. As shown in Fig. 6a, this effect was more pronounced.
when the pre-exposure time was extended, and the residual activity of anammox granules pre-exposed to Cu(II) for 48 h was as low as 15.8%.

Since NO$_2^-$ is a substrate and inhibitor of anammox bacteria, the presence of NO$_2^-$ may also affect the tolerance of anammox bacteria to heavy metal inhibition. The residual activity of anammox granules pre-exposed to Cu(II) and NO$_2^-$ for 12 h was 87% lower than that of granules pre-exposed to Cu(II). Moreover, this effect was more pronounced when the pre-exposure time was extended, and the residual activity of anammox granules pre-exposed to Cu(II) for 48 h was as low as 11.8%, 74% lower than granules that were pre-exposed to Cu(II). Although a NO$_2^-$ concentration of 100 mgN L$^{-1}$ has been reported to be a safe limit for anammox bioreactor operation (Jin et al., 2012), here, the inhibitory effect of Cu(II) was significantly enhanced in the presence of NO$_2^-$ ($p < 0.01$).

Fig. 6a shows a steep activity-deterioration process in the first 12 h of pre-exposure, followed by gradual decline over the following 36 h. This two-step activity-deterioration process may be attributed to the multi-layer structure of the anammox granules including two distinct regions. The outer region, which is glued by the readily-extractable EPS, is a dispersible part in which the particles are entangled by weak interactions, that is, ion bridging by EPS through multivalent ions and van der Waals interactions. However, the inner region, which is glued by the relatively compact part of the EPS, is stable, and the particles are entangled by strong interactions, such as polymer entanglement (Zhang et al., 2015a). Therefore, the anammox biomass in the granules cannot be inactivated by several days of short-term exposure, and further increasing the inhibition will require more time.

3.5. Role of the liquid medium pre-incubated with heavy metals

The strong inhibition observed following the exposure of anammox bacteria to Cu(II) pre-exposure could result from the formation of toxic byproducts during pre-incubation. To test this hypothesis, fresh anammox bacteria were exposed to decanted culture media obtained from bioassays pre-exposed to Cu(II) (Protocol 5). Similarly, the medium of anammox bacteria pre-exposed to Cu(II) was decanted (Protocol 6), the biomass was washed and the liquid was replaced with fresh medium to determine whether the washing reversed the toxicity or whether the anammox cells were damaged by the Cu pre-exposure.

Fig. 6b compares the NAA of biomass pre-exposed to Cu(II), biomass washed after pre-exposure to Cu(II), and fresh biomass exposed to the medium decanted from pre-exposure. Washing the biomass after 24 h of pre-exposure increased the NAA from 16.1% to 88.0%, resulting in a significant relief in the inhibition; thus, most of the anammox cells stopped metabolism instead of being inactivated after the 12 h of pre-exposure. However, the detoxification effect of washing disappeared when the pre-exposure was extended to 48 h, indicating that most of the anammox cells were damaged by Cu(II) pre-exposure for 48 h. In the absence of external sustenance, starved anammox bacteria primarily undergo endogenous processes, i.e., maintenance processes linked to the utilization of intracellular polymers (glycogen) and EPS, which maintain cellular integrity and activity. Meanwhile, no energy released from the anammox reaction was used to resist the Cu(II) invasion by secreting more EPS as a self-protection behavior or to actively pump toxic Cu(II) out of the sensitive regions of the cells as a self-healing behavior. Therefore, pre-exposure to heavy metals strongly inhibits anammox activity in the absence of two substrates, and this inhibitory effect was more pronounced when the pre-exposure time was extended.

As shown in Fig. 6c, washing the biomass after the pre-exposure to Cu(II) and NO$_2^-$ for 24 h moderately ameliorated the inhibition because the NAA increased from 13.7% to 23.5% ($p > 0.05$). The joint toxicity of Cu(II) and NO$_2^-$ was only partially reversible by washing, confirming that a large portion of the toxicity resulted from lasting cellular damage. Indeed, the NAA was as low as 11.2% and could not recover any more by washing after pre-exposure for 48 h, indicating that most anammox cells became inactivated rather than stopping their metabolism during further exposure.

The use of a medium pre-incubated with Cu(II) for 48 h stimulated the activity of fresh biomass by 20%. Previous study showed that 1 mg L$^{-1}$ Cu(II) improved anammox performance by stimulating the apparent metabolic activity. Therefore, the stimulating effect observed here may be partly attributed to the remaining Cu(II) in the medium (1.8 mg L$^{-1}$). The use of a medium pre-incubated with Cu(II) for 24 h stimulated the activity of fresh biomass by 57% ($p < 0.05$), and 3.9 mg L$^{-1}$ Cu(II) remained, which cannot be explained by the results of the acute exposure batch tests. The mechanism remains unclear and requires further investigation.

However, the use of liquid medium recovered from the pre-incubation with Cu(II) and NO$_2^-$ for 24 h and 48 h inhibited the activity of fresh biomass by 23.5% and 10.6%, respectively. These results did not agree with the Cu(II) level of the medium. Some toxic by-products may have been formed during the pre-incubation period. NO and intermediates produced by NO$_2^-$ reduction can potentially generate other toxic products, such as nitrogen dioxide or peroxynitrite anion, which are highly reactive with biomolecules, including DNA, lipids, and proteins (Carvajal-Arroyo et al., 2014). These by-products showed considerable toxicity to anammox granules, similar to the direct pre-exposure to Cu(II) and NO$_2^-$, indicating that the inhibition likely occurred via the inactivation of the biomass by Cu(II) and the formation of soluble toxic intermediates resulting from nitrite stress.

3.6. Implications of this work

The copper levels of real industrial wastewaters are considerably high. Although the copper levels in municipal wastewaters are expected to be lower due to the dilution of industrial effluents by wastewater from residential sources, critical situations (e.g., fluctuations in the influent) are inevitable in field engineering and can cause heavy metal overload shock. The joint toxicities of heavy metals are complex and variable, however, anammox cells in the granules were not inactivated by several hours of exposure in the presence of substrates because of the multi-layer structure of anammox granules. Once the overload shock of heavy metal occurs, an in-situ remediation strategy should be implemented to minimize the duration of the disturbance. Washing as quickly as possible using the buffer medium was confirmed to be an effective strategy in this study. Depending on the metal loading and contamination age, accelerating the external diffusion of Cu, such as by ethylenediamine tetraacetic acid (EDTA) washing and ultrasound-enhanced EDTA washing, could be an alternative strategy (Zhang et al., 2015c). Furthermore, the cumulative toxicity of Cu(II) was mitigated by acclimatization via intermittent exposure. This regulation strategy may represent an important and useful method for wastewater treatment plants. Additionally, the anammox process is always applied in combination with partial nitrification, in which approximately half of the NH$_4^+$ is oxidized to NO$_2^-$. This anammox-based process can be achieved using one-stage (CANON, OLAND, DEMON) or two-stage configurations (SHARON–ANAMMOX). Pre-exposure to Cu(II) in the absence of substrates strongly inhibited anammox activity. Events that cause underloading or starvation of the one-stage reactor require special attention and include the following: seasonal wastewater flows, oversized bioreactors, and mechanical failures. On the other hand, the presence of NO$_2^-$ significantly enhanced Cu(II) inhibition.
plete oxidation of NH$_4^+$ to NO$_2^-$ during the nitritation step of the two-stage process could lead to failure of the anammox process. Certainly, the failure of NH$_4^+$ delivery pump could also result in severe inhibition if NH$_4^+$ and NO$_2^-$ are pumped into an anammox reactor from two different sources.

4. Conclusions

The joint inhibitory effect of Cu(II) and Zn(II) tended to exacerbate initially and reversed as the concentrations increased and then moderated again. The most severe inhibition, resulting in a NAA of 20.1%, occurred at Cu(II) and Zn(II) concentrations of 16.3 and 20.0 mg L$^{-1}$, respectively. Notably, the cumulative toxicity was mitigated by intermittent exposure acclimatization. Additionally, pre-exposure to Cu(II) in the absence of substrates strongly inhibited anammox activity, and the presence of NO$_2^-$ significantly enhanced copper inhibition. Therefore, such conditions should be avoided to minimize the disturbance of the anammox process.

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Appendix A. Supplementary data

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References


