

Recovery profile of anaerobic ammonium oxidation (anammox) bacteria inhibited by ZnO nanoparticles

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ABSTRACT

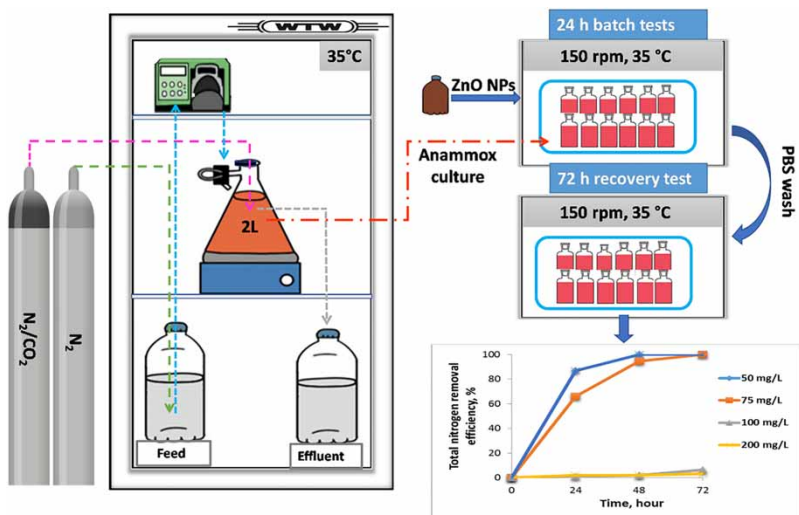
The potential effects of nanoparticles (NPs) on biological treatment processes have become significant due to their increasing industrial applications. The purpose of this research was to investigate the self-recovery ability of anammox bacteria following acute ZnO NPs toxicity. In this context, a 2-liter lab-scale anammox reactor was operated for 550 days to enrich the biomass required to the batch exposure tests. Anammox culture was firstly exposed to four different doses of ZnO NPs (50, 75, 100 and 200 mg/L) for 24 h. Then, the ZnO NPs were removed and self-recovery performance of the anammox bacteria was assessed by evaluating the nitrogen removal capacities for 72 h. Besides the nitrogen removal performance, extracellular polymeric substances (EPS) production was also detected to deeply understand the response of the enriched anammox culture against ZnO NPs exposure. The results revealed that sudden and high load of ZnO NPs (100 and 200 mg/L) resulted in persistent impairment to the nitrogen removal performance of the enriched anammox culture. However, relatively lower doses (50 and 75 mg/L) caused deceleration of the nitrogen removal performance during the recovery period. In addition, EPS content in the reactor decreased along with escalating load of ZnO NPs.

Key words: acute inhibition, anammox bacteria, extracellular polymeric substances, nanoparticle toxicity, self-recovery, zinc oxide nanoparticles

HIGHLIGHTS

- Enriched anammox cultures were exposed to different ZnO NPs doses.
- Total nitrogen consumption per VSS decreased by the escalating burden of ZnO NPs.
- This study takes attention to the self-recovery ability of anammox bacteria against shock ZnO NPs loads.
- Anammox bacteria could not recover their activity following the acute inhibition of high ZnO NPs load.

GRAPHICAL ABSTRACT



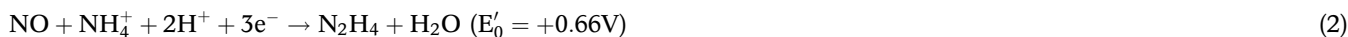
INTRODUCTION

Living things need nitrogen element in order to synthesize many crucial biomolecules such as DNA, chlorophyll, ATP and to maintain their life. Dinitrogen gas (N₂) is transformed into ammonium compound which is the available form of nitrogen for the utilization of (micro) organisms. The fixed ammonia in the soil is then released to the atmosphere by consecutive nitrification and denitrification processes. Basically, ammonia is converted firstly to nitrite and subsequently to nitrate by the nitrification process. In denitrification, nitrate is turned into nitrite and then N₂ gas. Therefore, the global nitrogen cycle represents the natural nitrogen flow in the earth and provides the continuity of life (Stein & Klotz 2016).

Nitrogen-containing compounds such as ammonia and nitrate, are two of the main pollutants in wastewaters. Along with the increase in anthropogenic activities, the removal of these contaminants from wastewaters has become a critical issue. If ammonia is discharged to the ecosystem without treatment, it causes serious environmental problems. The excess amount of ammonia in the water bodies is used by aquatic plants and algae as nutrient and accelerates their growth. The excessive growth of these plants and algae is named eutrophication, which affects the water quality and results in oxygen depletion in water and eventually kills the aquatic organisms (Heisler *et al.* 2008; Luo *et al.* 2015; Wurtsbaugh *et al.* 2019). Therefore, many countries apply stringent nitrogen discharge standards. In European Union, discharge limit of total nitrogen in treated wastewaters is 15 mg/L (10,000–100,000 population equivalent (pe)) and 10 mg/L (>100,000 pe) for areas sensitive to eutrophication (EC 2019). Besides, free nitrous acid can be formed when nitrate accumulates in the environment. Even if ammonia and free nitrous acid exist at low concentrations, they inhibit many microorganisms. Nitrification products may also adversely affect human health (Weralupitiya *et al.* 2021). Drinking water contaminated with nitrate causes blue baby syndrome, which can be fatal in newborn babies (Knobloch *et al.* 2000). Hence, various chemical, physical, and biological treatment processes are applied to remove nitrogen compounds from wastewaters. Inspired by the global nitrogen cycle, conventional nitrification/denitrification processes are mostly applied to treat nitrogen compounds in wastewater (Weralupitiya *et al.* 2021). However, the nitrification process carried out under an oxic environment, leads to high energy requirements due to the aeration cost of the process. Additionally, the need for external organic carbon addition in the denitrification process, excess sludge production and its disposal policy result in high costs (Cao *et al.* 2017; McCarty 2018).

Anaerobic ammonium oxidation (Anammox) enables the direct conversion of ammonium into dinitrogen gas under anoxic conditions. The central metabolism of the anammox reaction has been proposed as three main steps (Equations (1)–(3)) (Strous *et al.* 2006). Therefore, it can be thought of as a shortcut to the classical nitrification/denitrification processes

(Weralupitiya *et al.* 2021).



Anammox bacteria belong to the phylum Planctomycetes, order Brocadiales. Up to now, seven genera of Anammox bacteria have been identified: Kuenenia, Brocadia, Anammoxoglobus, Jettenia, Brasilis, Scalindua, and Anammoximicrobium. Even though they are diversely distributed in the environment such as marine and freshwater ecosystems, the wastewater treatment systems are the main habitat of these bacteria (Pereira *et al.* 2017). Along with the discovery of Anammox at the end of the '90s, this process has been rapidly implemented in different industries. At present, the Anammox process has been applied more than 200 wastewater treatment facilities. The most prominent advantages of this process compared to conventional biological processes are that anammox bacteria use inorganic carbon as a carbon source, reduce the energy requirement by 60%, and produce 80% less waste sludge due to their slow growth rate (Cao *et al.* 2017). Furthermore, it is a more sustainable process, decreasing greenhouse gas emissions by 90% (Manonmani & Joseph 2018). Based on the overall consideration of these factors, the Anammox process has become an alternative treatment technology to conventional nitrification/denitrification processes. Today, the Anammox process has been successfully integrated for ammonia removal from high-strength wastewaters; domestic sewage, sludge liquor, sludge supernatant, sludge digestate, slaughterhouse wastewater, piggery wastewater, synthetic coke-oven wastewater, monosodium glutamate wastewater, semi-conductor factory wastewater, pharmaceutical wastewater, livestock wastewater, tannery wastewater, seafood processing wastewater, digested fish canning effluent, and municipal wastewaters (Adams *et al.* 2020; Aransiola *et al.* 2021). Despite all advantages, certain drawbacks of the process restrict its applications in the industry. Slow-growing anammox bacteria are very vulnerable to not only environmental factors but also several inhibitory compounds including antibiotics, aromatic and organic pollutants, heavy metals, nanoparticles, and so on (Jin *et al.* 2012; Madeira & De Araújo 2021).

Nanotechnology is an emerging technology that encompasses various disciplines including chemistry, physics, material science, engineering, biology, etc. It deals with the matter at a nanoscopic scale (Porter & Youtie 2009). Nanotechnology has gained great attention because the physicochemical properties of nanoparticles (NPs) can easily be altered for a specific purpose (Bhushan 2017). In 2022, the global nanotechnology market value is expected to rise up to \$16.8 billion (Liu *et al.* 2019). Zinc oxide (ZnO) NPs are one of the most commercially used NPs (Liu *et al.* 2019). Paints, ointments, rubber, cement, glass, plastics, adhesives, foods, pigments, fire retardants, ferrites, sealants, batteries, are examples of products containing ZnO NPs (Sabir *et al.* 2014). Because it improves UV protection capability, and acting as anti-acne and aging agent, ZnO NPs have been immensely used in the cosmetic sector (Werkneh *et al.* 2018). ZnO NPs are also gathering unique properties to the semiconductors such as wide band gap, high electron mobility, and good transparency (Sabir *et al.* 2014).

Increasing use of NPs in many consumer products and different industries, and unintentional release of them into the ecosystem at every stage of their life cycles, cause a great concern about their potential toxicological risks on biological systems (Sari *et al.* 2020b). Up to now, the potential toxicity of NPs on biological wastewater treatment processes has been reported by several studies in the current literature (Chen *et al.* 2021). Among the NPs examined by Song *et al.* (2018), ZnO NPs were found to be the most toxic one due to their inhibition mechanisms on bacterial cells (Gutwiński *et al.* 2021). ZnO NPs are among the top 3 most-produced NPs worldwide (Chen & Chen 2017). In the current literature, there are a few studies related to the impacts of ZnO NPs on the Anammox process (Sari *et al.* 2020a). These studies mostly focused on short- and long-term effects of ZnO NPs on the Anammox process, but not on its recovery profile following acute exposure.

Due to the extremely long doubling time of anammox bacteria (7–20 days) (Kartal *et al.* 2012), challenging start-up period of the process is one of the main obstacles. Therefore, in order to shorten the start-up time of the Anammox process, many researchers have focused on optimizing key parameters such as seeding sludge, operational strategy, biomass immobilization, and so on (Verma *et al.* 2021), and the addition of chemical reagents into the system for stimulating the bacteria (Ganesan & Vadivelu 2019; Miodoński *et al.* 2019). Therefore, in full-scale applications of the Anammox process, in case of a sudden toxicity, recovery would be a better way than restarting it (Ni & Meng 2011). In addition, prior to exploring state-of-art recovery or control strategy in real time, the response of the system itself without any attempt should be well-understood (Arora *et al.* 2021). In this context, this study aimed to figure out the self-recovery profile of the Anammox process following 24 h acute

exposure of ZnO NPs at four different doses in order to assess whether the inhibition of anammox bacteria is persistent or not. In this respect, the current study is the first in the literature to the best of our knowledge. Therefore, this study's primary purpose is investigating acute impacts of the ZnO NPs on the nitrogen treatment capacity of the Anammox process and evaluating the self-recovery ability of the Anammox process immediately after a short-term inhibition period.

MATERIALS AND METHODS

Preparation of synthetic wastewater and ZnO NPs stock solution

Synthetic wastewater was prepared as previously described by Yapsakli *et al.* (2017). Ammonium nitrogen ($\text{NH}_4^+\text{-N}$) and nitrite nitrogen ($\text{NO}_2^-\text{-N}$) was prepared in the forms of $(\text{NH}_4)_2\text{SO}_4$ and NaNO_2 , respectively. The composition of synthetic wastewater is given in Table 1 in detail. The anammox bioreactor was fed daily with synthetic wastewater. Prior to feeding, the synthetic wastewater was purged via dinitrogen gas to remove dissolved oxygen.

Commercially produced ZnO NPs (<100 nm) was prepared as a suspension of 20% ZnO by wt (Sigma Aldrich, USA) by following the procedure described by Mu *et al.* (2012). 0.1 mM sodium dodecylbenzene sulfonate (SDBS) was used to dilute the ZnO suspension in order to procure 1 g/L ZnO stock solution. SDBS, a dispersing agent, assists in the stabilization of NPs in the stock solution (Mu *et al.* 2012; Zhang *et al.* 2017; Song *et al.* 2018). Then, 1 h ultrasonication was employed to the stock solution (25 °C, 40 kHz, 250 W) by Sonopuls Ultrasonic Homogenizer (Bandelin, Germany). Particle size distribution and zeta potential were controlled after 24 h using a Zetasizer (Malvern Panalytical, UK).

Enrichment of anammox bacteria and reactor operation

The experimental setup of the anammox bioreactor was established as previously described by Sari *et al.* (2020a). A lab-scale anammox bioreactor with 2 L working volume was established in order to enrich anammox bacteria for batch tests. The anammox seeding sludge was taken from the up-flow anammox bioreactor that has been operated for more than 10 years in our laboratory. The bioreactor was placed in a TS 606-G/4-i incubator (WTW, Germany) to maintain mesophilic conditions (35 ± 0.5 °C) and operated in sequencing batch reactor (SBR) mode for a 24 h cycle with a 20-min fill time, 22.67-h reaction time, 30-min settling time, and 20-min effluent withdrawal time. Hydraulic retention time (HRT) was set to be 2 days. Therefore, volumetric exchange ratio per cycle was 0.5 during the enrichment period. N_2/CO_2 (90/10%) gas mixture was also introduced to the bioreactor in order to provide anoxic conditions and supply the inorganic carbon requirement. pH of the reactor was kept at 7.5 ± 0.3 . During the enrichment, influent substrate concentrations were kept low at first to help

Table 1 | Composition of synthetic wastewater

Constituent	Quantities in synthetic wastewater
$\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$	1:1.15 ratio
NaHCO_3	1.04 g/L
K_2HPO_4	174.2 mg/L
CaCl_2	73.5 mg/L
MgCl_2	102 mg/L
Trace Element Solution 1	1 ml/L
$\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$: 10 g/L	
FeSO_4 : 5 g/L	
Trace Element Solution 2	1 ml/L
$\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$: 10 g/L	
$\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$: 0.43 g/L	
$\text{CoCl}_2\cdot 6\text{H}_2\text{O}$: 0.24 g/L	
$\text{MnCl}_2\cdot 4\text{H}_2\text{O}$: 0.99 g/L	
$\text{CuSO}_4\cdot 5\text{H}_2\text{O}$: 0.25 g/L	
$\text{NiCl}_2\cdot 6\text{H}_2\text{O}$: 0.19 g/L	
H_3BO_4 : 0.014 g/L	

adaptation of anammox bacteria to their new environment. Thereafter, the substrate concentrations were gradually increased considering the stable nitrogen treatment capacity. Finally, $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations were adjusted to be 100 and 115 mg N/L, respectively, which were the same concentrations used in the short-term exposure tests.

Batch exposure tests

Acute exposure tests were conducted using amber serum flasks having an effective volume of 50 mL. Synthetic wastewater containing 100 mg N/L $\text{NH}_4^+\text{-N}$ and 115 mg N/L $\text{NO}_2^-\text{-N}$, ZnO NPs and biomass were transposed to serum flasks in this order. The volatile suspended solids (VSS) in each serum flask was 1.5 ± 0.5 g/L. The pH was set to 7.5 ± 0.2 . In the batch exposure tests, anammox bacteria were subjected to four different ZnO NPs dosages of 50, 75, 100 and 200 mg/L. N_2 gas was used to purge the serum flasks for 3 min to prevent oxygen suppression before starting the experiment. Afterwards, each flask was sealed with a rubber stopper and aluminum crimp. All flasks were kept in an Ecotron incubation shaker (INFORS HT, Sweden) at 35 °C and 150 rpm for 24 h incubation period. Each condition was conducted in triplicate. A 0.5 mL sample was collected from each serum flask at 3 h intervals in order to analyze total nitrogen concentrations and determine total nitrogen removal efficiencies.

Recovery tests

The recovery ability of the anammox bacteria exposed to the ZnO NPs was observed during recovery tests. ZnO NPs attached to the bacteria were removed by applying the washing procedure described by Zhang *et al.* (2017) with some modifications. Biomass in serum flasks was transferred into 50 mL centrifuge tubes and centrifuged at 35 °C and 3,000 g for 10 min using a Universal 320R centrifuge (Hettich, Germany). After removing the supernatant, 30 mL of 10 mM phosphate buffer solution (PBS) was added onto the pellets, and tubes were shaken at 35 °C and 150 rpm for 30 minutes using an Ecotron incubation shaker (INFORS HT, Sweden). The centrifugation step was repeated twice more. Finally, the washed anammox sludge was transferred into new serum flasks containing fresh synthetic wastewater. For the preparation of serum flasks in the recovery tests, the same procedure was followed with that of exposure tests, except with ZnO NPs addition. Besides, the operation time in the recovery test was 72 h, and the liquid samples were collected from the flasks at 24 h intervals.

Analytical methods

pH was daily tested by HQ40D digital portable multimeter kit (HACH, USA). VSS (SM 2540E), ammonia nitrogen (SM 4500- $\text{NH}_3\text{-A}$), and nitrite nitrogen (SM 4500- $\text{NO}_2\text{-B}$) were determined according to Standard Methods (APHA/AWWA/WEF 2005). The Anthrone method and modified Lowry method was applied to detect polysaccharide (PS) and protein (PN) concentrations in EPS content, respectively. During the EPS analysis, bovine serum albumin and glucose were used as standard solutions for the PN and PS analysis, respectively. A modified heat extraction method was performed as previously described by Morgan *et al.* (1990) for EPS extraction. Subsequently, supernatant parts were filtered through 0.45 μm membrane filter and stored at -20 °C. A UV-2450 Spectrophotometer (Shimadzu, Japan) was used for all EPS quantification experiments. Each measurement was performed with two independent samples and each sample was tested in duplicate.

Statistical analysis

The results of the experiments were demonstrated as the mean \pm standard error using Microsoft Excel.

RESULTS AND DISCUSSION

Enrichment of anammox bacteria

In the current study, the established anammox bioreactor was operated for 550 days, and start-up period of the bioreactor was divided into three phases. Figure 1 represents the nitrogen components and the treatment capacity of the bioreactor during the start-up and enrichment period. Following the inoculation (Phase I: Day 1–53), mild conditions were provided by adding relatively lower substrate concentrations in order to adapt anammox bacteria to their new environment. Numerically, $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations within the bioreactor were kept at 39.32 ± 12.36 mg N/L and 41.92 ± 12.54 mg N/L, respectively, during the start-up period. In the initial days of Phase I, the anammox bioreactor showed poor nitrogen treatment capacity with inconsistent low nitrogen removal efficiencies. Along with observing satisfactory nitrogen removal performances (>80%), phase II (Day 54–255) was initiated. In Phase II, initial $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations were increased stepwise to enhance the microbial activity and enrich the anammox culture. On days 166–234, total nitrogen concentration within the bioreactor reached its maximum level (358.51 ± 35.36 mg N/L). Furthermore, the final phase (Phase III)

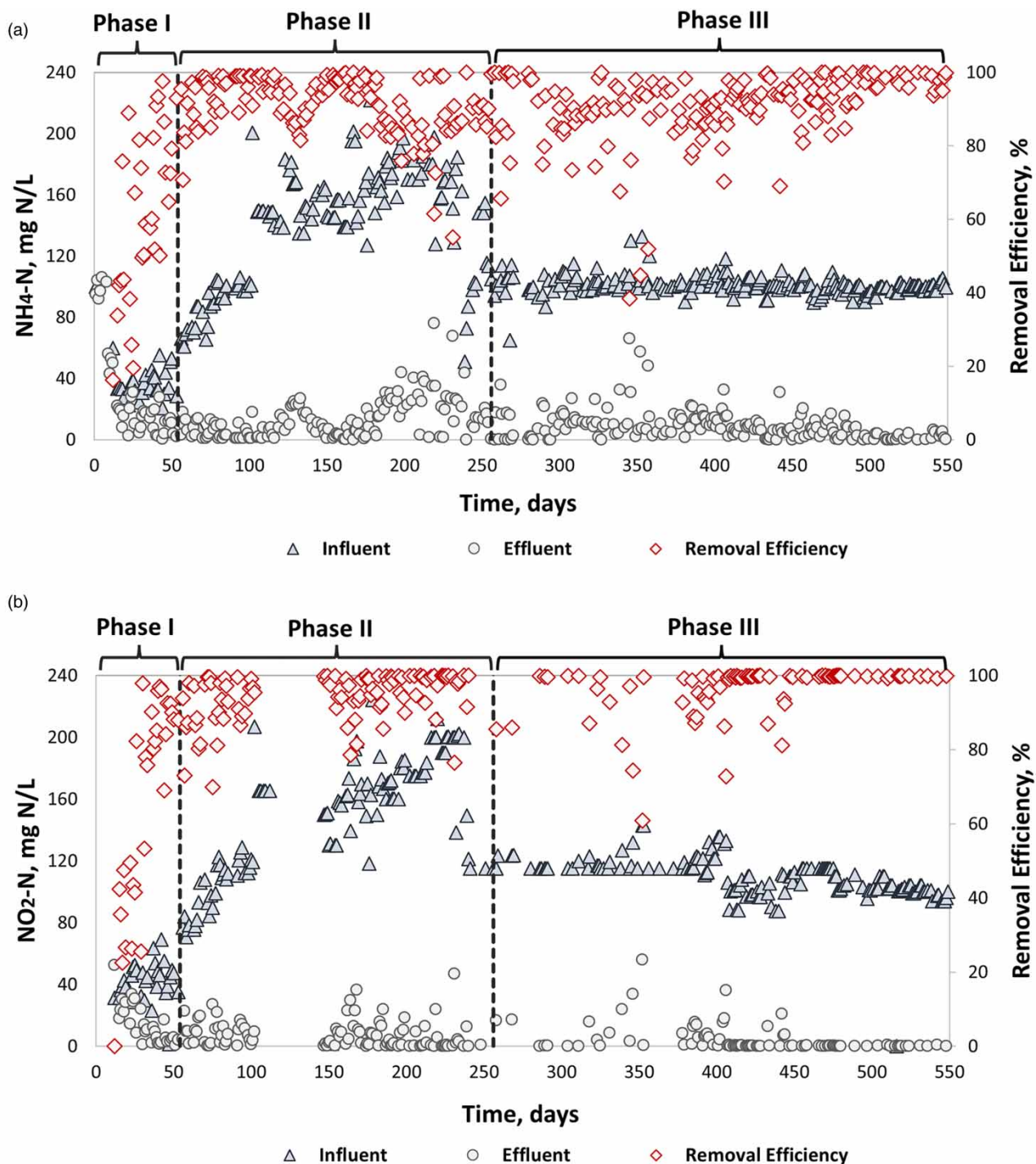


Figure 1 | Nitrogen treatment capacity of the anammox bioreactor. (a) ammonium; (b) nitrite removal during the start-up period. Phase I: Days 1–53; Phase II: Days 54–255; Phase III: Days 256–550.

comprises the days from 256 to 550. In this phase, the main purpose was to create the conditions to be applied in the short-term exposure tests and acclimatize the enriched anammox culture to the conditions. Therefore, influent $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations were adjusted to be 100 and 115 mg N/L, respectively, in the final phase. From day 458, satisfying nitrogen treatment performances were observed. During this period, the removal efficiencies of $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ was obtained as $95.87 \pm 4.21\%$ and $99.79 \pm 0.22\%$, respectively.

Recovery profile of anammox bacteria following the acute exposure of ZnO NPs

The ZnO NPs toxicity on anammox activity in a short-term manner has already been reported by a few studies in the current literature. However, the observed inhibition effects of ZnO NPs show differences in these studies due to the different experimental setups, operational strategies, microbial dynamics, and the sludge type (Sari *et al.* 2020a). Besides, to the best of our knowledge, no study has assessed whether the acute toxicity of ZnO NPs on anammox bacteria is persistent or not. Therefore, this study has focused on enlightening the recovery profile of the enriched anammox culture following the acute ZnO NPs toxicity. In order to achieve this, anammox bacteria were exposed to 50, 75, 100, and 200 mg/L ZnO NPs for 24 h. During the 24 h incubation period, the changes in substrate concentrations were observed, and the total nitrogen (the sum of ammonium and nitrite) removal efficiencies were calculated.

During the incubation period, total nitrogen concentration diminished over time in all experiment groups (Figure 2(a)). In control groups, which were not exposed to any NPs dosage, 90.91 ± 7.44% of TN had been removed from the system within

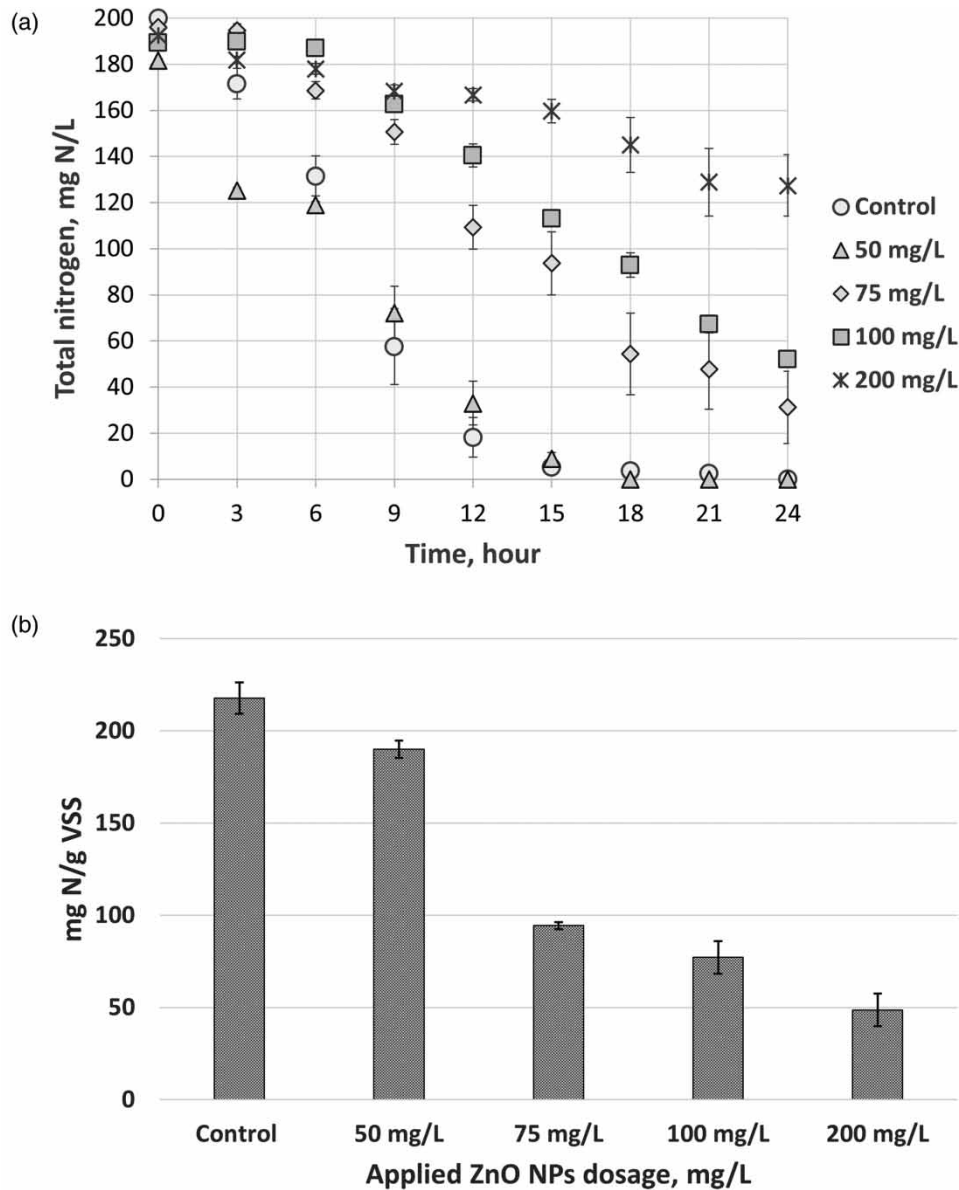


Figure 2 | Impacts of 50, 75, 100, 200 mg/L ZnO NPs on anammox bacteria during 24 h incubation period. (a) total nitrogen concentrations; (b) the consumed total nitrogen per VSS (mg N/g VSS) after 24 h. Data indicate average, and error bars represent standard error of the results from three independent samplings.

12 h. The experimental groups exposed to 50 mg/L ZnO NPs dose showed similar nitrogen removal trends with the control groups. Numerically, the TNRE was detected to be $81.83 \pm 9.10\%$ in 12 h, and all nitrogen was treated within 18 h. As the applied ZnO NPs dosages increased, the nitrogen treatment capacity of the enriched anammox culture considerably decreased. During 75 mg/L ZnO NPs exposure, $44.04 \pm 9.50\%$ of TN was removed from the system in 12 h, and the TNRE was found to be $83.70 \pm 14.65\%$ at the end of the experiment. Moreover, the TNRE was detected to be $72.53 \pm 1.86\%$ as a result of the load of 100 mg/L ZnO NPs in 24 h. As for 200 mg/L ZnO NPs exposure, only $33.96 \pm 10.49\%$ of the total nitrogen was removed by the enriched anammox culture within 24 h. In order to be able to comment more accurately, the consumed amount of TN per VSS was calculated for each group (Figure 2(b)). Even though similar nitrogen removal performances were observed in both control and the 50 mg/L ZnO experiment groups, the TN consumed per VSS was lower in the 50 mg/L exposure group (190.00 ± 8.26 mg N/g VSS) than that of the control group (217.79 ± 12.03 mg N/g VSS). Additionally, a sharp decrease in TN consumption per VSS was observed when the exposure dose of ZnO NPs increased. In the acute exposures of 75, 100, and 200 mg/L ZnO NPs doses, the consumed TN per VSS was found to be 94.37 ± 3.31 , 77.19 ± 15.34 , 48.70 ± 15.24 mg N/g VSS, respectively.

Prior to the self-recovery test, the anammox bacteria were firstly washed to get rid of ZnO NPs from the serum flasks as described in the materials and method part. Following the washing procedure, all serum flasks were fed with 100 mg N/L $\text{NH}_4^+\text{-N}$ and 115 mg N/L $\text{NO}_2^-\text{-N}$ concentrations. Thereafter, the nitrogen treatment capacities of the anammox systems were examined. During the 72 h recovery period, TN concentrations in the serum flasks were measured at 24 h time intervals and total nitrogen removal efficiencies were determined (Figure 3). The inhibition tests demonstrated that the application of 50 mg/L ZnO NPs did not have any impairment on the nitrogen removal performance of the anammox bacteria. Even all ammonium and nitrite nitrogen had been removed within 18 h. However, after removing ZnO NPs from the anammox system, the treatment of total nitrogen took more than 24 h during the recovery period. In addition, it has already been demonstrated that, 24 h exposure time of 75 mg/L ZnO NPs caused deterioration of nitrogen removal performance of anammox bacteria by the loss of $16.30 \pm 14.65\%$ nitrogen removal capacity. As for the recovery period following the 75 mg/L ZnO NPs application, it is much longer than that of the 50 mg/L ZnO NPs application. During the recovery experiment, 65.93% of TN has been treated in the first 24 h, and the TN removal efficiency reached 94.51% in 48 h. This indicates that increasing dose of NPs worsens the nitrogen removal performance. As the NPs dose rose to 100 mg/L, 27.47 \pm 1.86% of TN was not removed from the system in the exposure period. However, it was observed that only 6.32% of TN was removed by enriched anammox culture during the recovery period. When experiment groups of 75 and 100 mg/L are compared, although there was a slightly similar nitrogen removal performance between them during the exposure period, anammox bacteria couldn't recover their activities even after the 72 hour recovery period following the 100 mg/L ZnO NPs exposure. The 200 mg/L ZnO NPs load to the anammox system substantially impacted the nitrogen removal performance,

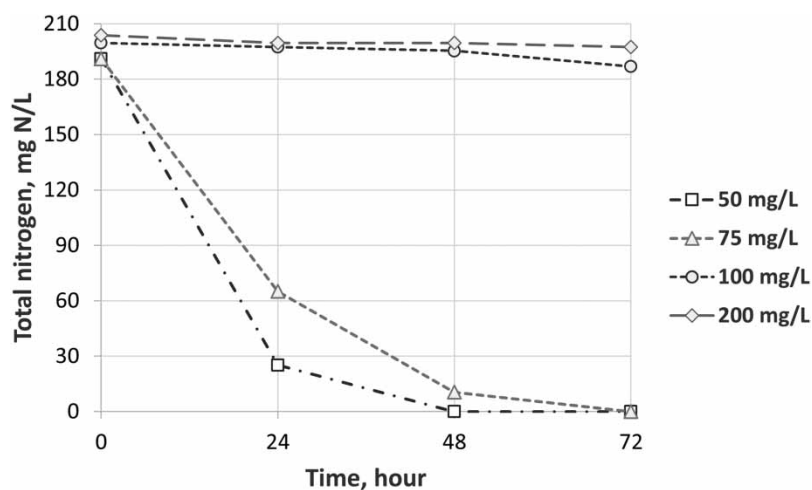


Figure 3 | Recovery profile of anammox bacteria following the acute exposure tests.

and the TNRE of the anammox bacteria was detected to be $33.96 \pm 10.49\%$. After 200 mg/L NPs exposure, anammox bacteria showed almost no nitrogen removal performance (3.09%) during its recovery period.

The recovery profiles following the exposure tests strongly exhibited that sudden and high load of ZnO NPs (100 and 200 mg/L) resulted in persistent impairment on the nitrogen removal performance of the enriched anammox culture. However, relatively lower doses (50 and 75 mg/L) caused deceleration of nitrogen removal performance during the recovery period. The most likely reason for this may be the amount of Zn^{2+} released from the ZnO NPs (Liu *et al.* 2011; Mu & Chen 2011; Zheng *et al.* 2011). It has already been reported that free metal ions dissolved from the surface of NPs are one of the major inhibition mechanisms (Ee *et al.* 2018; Song *et al.* 2018). In Zn-overloaded environments, the binding capacity of EPS, which is a defense mechanism of microorganisms (Tang *et al.* 2018), might be exceeded and Zn ions might penetrate into the cells (Zhang *et al.* 2018a). In such case, the zinc ions may chelate to sulfhydryl groups and disrupt heme c and protein bonds. Eventually, free Zn ions may lead to disorder in the cell metabolism by disrupting the function of intracellular proteins (Zhang *et al.* 2019). Therefore, an increment in the released Zn cations corresponding to the applied ZnO NPs doses may gradually increase inhibitive effects on anammox systems. Additionally, not only dissolved ion toxicity, but also the undissolved NPs might be the reason for the loss of nitrogen removal capacity, because undissolved NPs may lead to pits forming on the cell membrane. Smaller NPs may also penetrate to the cells and increase the permeability of the cell membrane. Additionally, reactive oxygen species (ROS) may be produced at the ZnO NPs surface owing to the electrostatic properties. ROS generation causes oxidative stress and the viability of anammox bacteria might have decreased (Zhao *et al.* 2019). It has also been reported that, when the ZnO NPs dosage increases, more NPs accumulate on the biomass (Sari *et al.* 2020a).

In the current literature, there is only a study investigating the reversibility of ZnO NPs inhibition on the Anammox process following long-term NPs addition (524 days) (Sari *et al.* 2020a). Contrary to the findings in the current study, the previously published study reported that anammox bacteria recovered themselves in 120 days. The most possible reason for this difference is the slow metabolism of the anammox bacteria. In this study, anammox bacteria were not allowed to adapt ZnO NPs and the bacteria were suddenly exposed to a huge amount of ZnO NPs dosage. Afterward, the recovery period only lasted for 72 h. Hence, anammox bacteria might not be able to secrete EPS to protect themselves and could not protect and/or recover their cell integrity under these conditions (Song *et al.* 2018). Therefore, it is noteworthy to mention that the Anammox process without any additional recovery strategy is vulnerable in the face of a sudden and massive amount of ZnO NPs loads to the wastewater treatment facilities. Therefore, the discharge limits of wastewaters containing ZnO NPs should be carefully determined considering the worldwide ZnO NPs utilization.

EPS response of the enriched anammox culture after 24 h acute exposure of ZnO NPs

EPS, an organic matrix, is an important defense mechanism of microorganisms against many inhibitors including nanoparticles (Tang *et al.* 2018). Plenty of studies have reported an increment in the EPS production when environmental stress increases (He *et al.* 2017; You *et al.* 2017). Therefore, the EPS production may be an adaptation strategy developed by the microbial community (Zhang *et al.* 2018c). On the other hand, some studies have revealed opposite results (Mu *et al.* 2012; Zhao *et al.* 2019; Sari *et al.* 2020b). Therefore, there are many different outcomes related to EPS secretion in the current literature. In this study, the EPS content was examined to understand the response of the enriched anammox culture exposed to several ZnO NPs dosages.

The EPS composition varies depending on the microbial dynamics. Besides, proteins (PN) and polysaccharides (PS) have mainly been found in EPS content (Tsuneda *et al.* 2003). It has been pointed out that in complex microbial communities, such as enriched anammox culture, PN secretion is considerably higher than the PS secretion (Tang *et al.* 2018). In the current study, far more PN content was produced than PS content. Moreover, both PN and PS production decreased as the applied NPs dosage increased (Figure 4). This phenomenon may have two possible reasons. Firstly, anammox bacteria are slow-growing bacteria, and they may need a longer lag phase for the EPS secretion to protect themselves (Song *et al.* 2018). However, in this study, the bacteria had been shocked with a high load of ZnO NPs in only 24 h incubation period. Therefore, they may not have been able to produce enough EPS to adapt to changing environmental stress. Secondly, the migration and absorption of Zn^{2+} ions released from ZnO NPs might play a role in the potential inhibition of ZnO NPs on the anammox process (Zhao *et al.* 2019). In this case, cells may utilize their energy to provide cell integrity rather than EPS secretion (Zhang *et al.* 2018b). Thereby, it might lead to a decrement in EPS secretion.

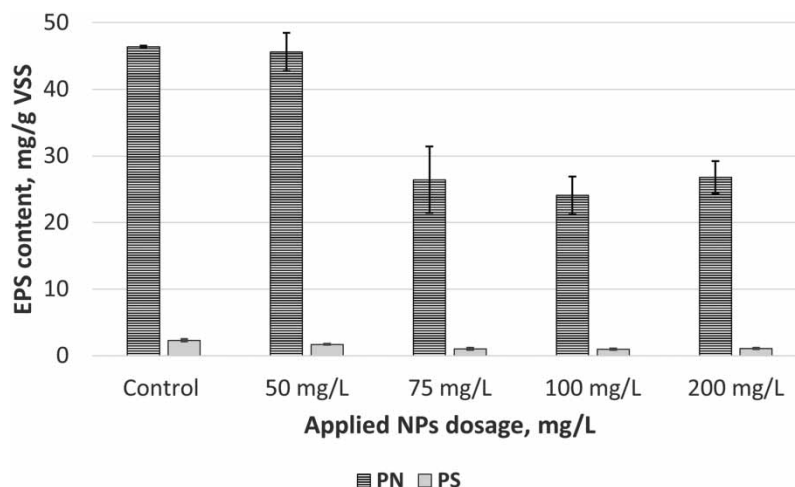


Figure 4 | EPS response of the enriched anammox culture after 24 h exposure of ZnO NPs. Data indicate average, and error bars represent standard error of the results from two independent samplings, each tested in duplicate.

CONCLUSION

This study was carried out to exhibit recovery performance of the anammox system in case of shock ZnO NPs loads to the wastewater treatment facilities. Acute exposure tests revealed that TNRE of the anammox bacteria and the consumed TN per VSS decreased as the applied dosage of ZnO NPs increased. The anammox bacteria could not recover their nitrogen removal performance even in a 72 h recovery period in consequence of the acute exposure of 100 and 200 mg/L ZnO NPs. The most likely reason for this may be the amount of Zn released from the ZnO NPs. In other words, as the dose of ZnO NPs increases, the dissolved toxic Zn ion concentration increases, which may lead to more deteriorative effects on the nitrogen removal capacity. Furthermore, the adsorption of undissolved NPs and even their penetration to the cells might be another reason. Besides, escalating the burden of applied ZnO NPs dosage resulted in a decline in the EPS content that is proportional to the deterioration of nitrogen removal performance of the anammox bacteria.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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