Biotech Studies 29(1), 29-37
http://doi.org/10.38042/biost.2020.29.01.04

BIOTECH STUDIES

RESEARCH PAPER

Short-term inhibitory effects of TiO_2 NPs on Anammox process

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Article History
Received 16 May 2020
Accepted 12 June 2020
First Online 15 June 2020

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Keywords
Anammox bacteria
Nanoparticles
Acute inhibition
Nanoparticle toxicity

Abstract

Anammox process has brought about cost-effective, eco-friendly, and innovative technologies to wastewater treatment by reducing the operational cost of treatment plants and decreasing greenhouse gas emissions. Titanium dioxide (TiO_2), as one of the most prevalent nanoparticles (NPs) in the world, is being used in various consumer products and applications. In recent years, studies have focused on potential toxicological impacts of NPs on biological processes due to their endless production and consumption. In this context, the first time in the literature, 24 h acute TiO_2 NPs exposure on Anammox process was investigated. Deterioration on anammox activity gradually increased with increasing applied TiO_2 NPs concentration. At 300 mg/L exposure dose, nitrogen removal rate dramatically decreased to 37.09 ± 0.24 mgN/gVSS.d and a severe inhibition (80.57% ± 1.17%) was observed. Among the several curve fit models, non-linear second order polynomial (quadratic) model was the best fit one with IC_{50} of 154 mg/L. Scanning electron microscope (SEM) images demonstrated the tendency of TiO_2 NPs to aggregate and attach to the surface of the bacteria. Extracellular polymeric substance (EPS) response of anammox bacteria was also investigated and it was found that, the total EPS content gradually decreased by increasing TiO_2 NPs concentration.

Introduction

Nitrogen participates in the structure of many important biomolecules such as ATP, chlorophyll, nucleic acids and vitamins as the most abundant element in the universe. However, nitrogen in dinitrogen gas (N_2) form is inaccessible to most of the living organisms, especially primary producers. Therefore, the nitrogen cycle can be thought as one of the most essential biological processes that ensure the continuity of life (Stein & Klotz, 2016). Nitrification and denitrification are two essential processes involved in the nitrogen cycle. Inspired by the uniqueness of the nature, they have been immensely applied to wastewater treatment systems in order to remove nitrogen and prevent eutrophication. In these processes, ammonia (NH_3) is firstly transformed to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-) by nitrifiers. Thereafter, NO_3^- is reduced into various nitrogen forms including NO_2^-, nitric oxide (NO), nitrous oxide (N_2O) and N_2 by denitrifiers (Heil et al., 2016; Robertson & Groffman, 2015).

Towards the end of the 20th century, global nitrogen cycle has been updated along with the discovery of anaerobic ammonium oxidation (anammox) bacteria by Mulder et al. (1995). Anammox reaction refers to the direct conversion of ammonium to N_2 via nitrite, in the absence of oxygen. Furthermore, Anammox process has brought about cost-effective, eco-friendly and innovative technologies to environmental fields e.g. industrial and domestic wastewater treatment systems (Peeters & van Niftrik, 2019). Remarkable reduction of operational cost is the main advantage of this promising technology over conventional nitrification/denitrification. In this process, there is no need for external carbon source, sludge production is 80% less, and aeration cost decrease by 60% (Cao et al., 2017).

Despite all advantages of the Anammox process and its application in over 200 wastewater treatment

facilities worldwide, anammox bacteria have slow growth rate and low cell yield (Zhang et al., 2016) which make the start-up and operation challenging. They are also susceptible to both inhibitory compounds such as antibiotics, heavy metals, nanoparticles (NPs) (Jin et al., 2012), and environmental factors such as pH, temperature and dissolved oxygen. All the aforementioned factors restrict the application and industrialization of anammox-based processes.

The manipulation of physicochemical properties of NPs (1-100 nm) such as size, shape, charge and coating promotes the widespread implementation of them into various industries including medical, food, military and cosmetic sectors (Bhushan, 2017; Kaphle et al., 2018). The global nanomaterials market was valued USD 7.3 billion in 2016 and is expected to the reach USD 16.8 billion by 2022 (Liu et al., 2019). Titanium dioxide (TiO_2) as one of the most prevalent NPs in the world, is being used in many cosmetic products including sunscreens, beauty products, toothpastes. Dietary supplements, candies, sweets, prosthetic implants, food colorants, paints, plastics, pigment production, anti-fogging car mirrors, ink, coating, photocatalysts are other examples of TiO_2 NPs applications (Chen & Chen, 2017; Mohamed, 2018).

NPs may be released into the ecosystems at every stage of their life cycles. Not only the fate of them in the environment, but also their toxicity levels are determined by their physicochemical properties which undergo some changes in different environment matrices (Liu et al., 2019; Senapati & Kumar, 2018). A significant amount of NPs has been detected in wastewaters and solid waste landfill sites (Keller & Lazareva, 2014; Musee et al., 2011). Therefore, their potential environmental toxicity has become a great concern because of the continuous increment in manufacturing and utilization of NPs (Chen & Chen, 2017). Besides, considering their relatively small size, more freely movement and larger surface area, they can be more toxic than larger particles in the bulk (Gupta & Xie, 2018). This is because entrance of them into the cells is much easier and they can cause cell damage (Liu et al., 2011).

Previously, several studies have been published to reveal the potential impacts of NPs on biological wastewater treatment systems including biological nitrogen and phosphorus removal (Wu et al., 2018; Zheng et al., 2011), anaerobic digestion (Lombi et al., 2012; Mu & Chen, 2011; Zhang et al., 2019) and anammox process (Li et al., 2018; Xu et al., 2019; Z. Z. Zhang et al., 2018a). Currently, there are only 3 studies focusing on the potential impacts of TiO_2 NPs on anammox process. However, 2 of them highlight the chronic (long term) effects of TiO_2 NPs (X. J. Zhang et al., 2018b; Z. Z. Zhang et al., 2018b) while the last one implies the short-term effects of TiO_2 NPs on anammox with the acute exposure tests of 8 h (X. J. Zhang et al., 2018a). A recent study stated that, 12 h exposure of inhibitory compounds is not sufficient to observe the defense mechanisms of anammox bacteria due to their slow growth rate (Song et al., 2018). Therefore, although the studies published in the current literature have enhanced our knowledge about the potential impacts of TiO_2 NPs on anammox process, short-term inhibitory effects of TiO_2 NPs on this process has not clearly been demonstrated yet. In the light of them, this study aimed to investigate the acute impacts of TiO_2 NPs on anammox process. In order to achieve this, seven different TiO_2 NPs doses (1, 10, 50, 75, 100, 200, 300 mg/L) applied to laboratory-scale anammox bioreactor for 24 h. IC_{50} value of TiO_2 NPs was studied with several inhibition models. In addition, extracellular polymeric substance (EPS) response of enriched anammox culture and changes in surface morphology of anammox bacteria were revealed by SEM analysis.

Materials and Methods

Anammox seeding sludge and experimental setup

A 2L lab-scale anammox bioreactor was established in order to enrich the anammox bacteria and use for acute exposure tests. Experimental setup is illustrated in Figure 1. Seeding sludge was obtained from an up-flow anammox bioreactor which have been being operated for more than 10 years in our laboratory. The reactor was operated in sequencing batch reactor (SBR)

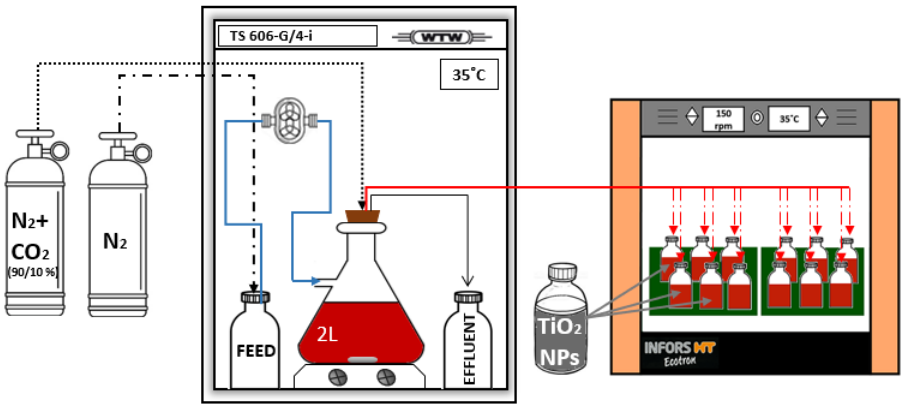

Figure 1. Experimental setup of the study.

mode for 24 h cycle with fill, reaction, settling and effluent withdrawal time of 20 min, 22.67 h, 40 min and 20 min, respectively. Synthetic wastewater to feed the Anammox bioreactor was prepared as previously described by Yapsakli et al. (2017), containing 1:1.15 ratio of NH_4^+-N and NO_2^--N, 0.073 g/L $CaCl_2$, 0.174 g/L K_2HPO_4, 0.102 g/L $MgCl_2$, 1 ml of trace element solution 1 (10 g/L $Na_2EDTA{\cdot}2H_2O$ and 5 g/L $FeSO_4$) and trace element solution 2 (10 g/L $Na_2EDTA{\cdot}2H_2O$, 0.43 g/L $ZnSO_4{\cdot}7H_2O$, 0.24 g/L $CoCl_2{\cdot}6H_2O$, 0.99 g/L $MnCl_2{\cdot}4H_2O$, 0.25 g/L $CuSO_4{\cdot}5H_2O$, 0.19 g/L $NiCl_2{\cdot}6H_2O$, and 0.014 g/L H_3BO_4). Besides, in order to prevent anaerobic environment that may lead to septic conditions, 50 mg/L $NaNO_3$ was introduced to the synthetic feed solution. 1.04 g/L $NaHCO_3$ was also supplied to the synthetic feed to prevent pH changes during the operation. Prior to feeding, in order to get rid of oxygen suppression risk, dissolved oxygen was stripped from the synthetic wastewater solution with N_2 gas.

A TS 606-G/4-i incubator (WTW, Germany) was used to maintain the mesophilic environment (35 ± 0.5 ˚C) to the anammox bioreactor. Hydraulic retention time (HRT) and pH were set to be 2 days and 7.5 ± 0.3, respectively. N_2/CO_2 (90/10%) gas mixture was also supplied to the reactor for inorganic carbon requirement.

Preparation of TiO_2 stock solution

The stock solution containing NPs, was prepared according to Mu et al. (2012). Commercially produced TiO_2 NPs (<25 nm) (Sigma Aldrich, USA) was added to the 0.1 mM sodium dodecylbenzene sulfonate (SDBS) to prepare 1 g/L TiO_2 NPs stock solution in order to provide stability of NPs and prevent agglomeration of them. Thereafter, stock solution was sonicated for 1 h at 25 °C, 40 kHz, 250 W by Sonopuls Ultrasonic Homogenizer (Bandelin, Germany).

Acute exposure tests

In order to conduct batch exposure tests, synthetic wastewater solution, NPs and enriched anammox culture were transferred into amber serum flasks having an effective volume of 50 mL. Synthetic wastewater composition (100 mg/L NH_4^+-N and 115 mg/L NO_2^--N) was the same with that of parent reactor and pH was adjusted to 7.5 ± 0.2. Each serum flask contained 1.5 g/L ± 0.5 g/L volatile suspended solids (VSS). Prior to starting experiment, serum flasks were purged by N_2 gas for 3 min to remove dissolved oxygen. Subsequently, all serum flasks were sealed with rubber stoppers and aluminum crimps. 24 h incubation was performed in Ecotron incubation shaker (INFORS HT, Sweden) at 35 °C and 150 rpm. In order to determine the specific nitrogen removal rates, 0.5 mL well-mixed liquid samples were taken from the flasks every 3 hours. Each exposure experiment was conducted in triplicate. Four different inhibition models were applied by GraphPad Prism (version 7.03) software package to determine the best fit model and estimate the IC_{50} value. Equations of inhibition models are listed below.

Linear regression

$$I\% = m \times NPs + n \quad (1)$$

Modified non-competitive inhibition model

$$I\% = 100 \times \left(1 - \frac{1}{1+\left(\frac{NPs}{a}\right)^b}\right) \quad (2)$$

Non-linear dose-response inhibition models

Inhibitor vs. normalized response

$$A\% = \frac{100}{1+\frac{NPs}{a}} \quad (3)$$

Inhibitor vs. normalized response -- Variable slope

$$A\% = \frac{100}{1+\left(\frac{a}{NPs}\right)^{Hill\ slope}} \quad (4)$$

Non-linear second order polynomial (quadratic) model

$$I\% = B0 + B1 \times NPs + B2 \times NPs^2 \quad (5)$$

Where A% and I% represent the activity and inhibition response of anammox process, respectively, NPs is the applied nanoparticle concentration, a is the value causing 50% inhibition on nitrogen removal rate, b is a fitting parameter and m, B0, B1, B2 are coefficients.

Analytical procedures

All analytical procedures including VSS, total suspended solids (TSS), NH_4^+-N and NO_2^--N were determined according to Standard Methods (APHA, 2005). pH was measured by HQ40D digital portable multimeter (HACH, USA).

EPS analysis

Protein (PN) and polysaccharides (PS) contents account for 75-89% of total EPS content (Tsuneda et al., 2003). Therefore, PN and PS concentrations in the samples were determined by modified Lowry method and Anthrone method, respectively. Prior to EPS quantification, modified heat extraction method, which was previously described by Morgan et al. (1990), was performed to extract EPS from mixed-liquor sludge samples. Subsequently, supernatant of the samples was filtered through 0.45 µM and stored at -20 °C. Each measurement was performed with two independent samples and each sample was tested in duplicate. A UV-2450 Spectrophotometer (Shimadzu, Japan) was used to conduct EPS measurements.

SEM analysis

Mixed-liquor sludge samples were firstly centrifuged at 3000 rpm for 10 min. Following the removal of supernatant, the pellet parts were washed three times for 3 min by 0.1 M phosphate buffer to get

rid of the unbounded materials. Thereafter, the pellets were fixed by fixation solution containing 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer solution (PBS) at 4˚C for 4 h. In order to remove fixation solution, washing procedure was applied again. Finally, the pellets were dehydrated in 50%, 70%, 80%, 90% and 100% ethanol solutions, respectively for 15 min and transferred on grids. Following air drying, the samples were coated with platinum and the images were obtained by environmental scanning electron microscope (Philips XL30 ESEM-FEG /EDAX).

Statistical analysis

The results of the experiments were represented as the mean ± standard deviation using Microsoft Excel. Paired t-test was also performed considering $P < 0.05$ as statistically significant.

Results and Discussion

Acute exposure of TiO_2 NPs on nitrogen removal performance of anammox process

SDBS, which is known as an anionic surfactant, was used as a dispersing agent in order to enhance the NPs stability and prevent their aggregation in the stock NPs solution. Previously published studies revealed no negative impacts of SDBS at low concentrations on biological processes including anaerobic treatment and anammox systems (Mu & Chen, 2011; Mu et al., 2012; Qiao et al., 2016; Zhang et al., 2017). In the current study, it was observed that, 0.1 mM SDBS has no statistically significant inhibitory impact on nitrogen removal efficiency of Anammox process (α=0.05, P = 0.25 > 0.05 for μ1=μ2) (Figure 2).

In the scope of exposure tests, anammox bacteria were exposed to seven different TiO_2 NPs dosages from 1 mg/L to 300 mg/L for 24 h incubation in order to observe the acute effects of TiO_2 NPs on nitrogen removal performance. Nitrogen concentrations in amber serum flasks were analyzed at every 3 h.

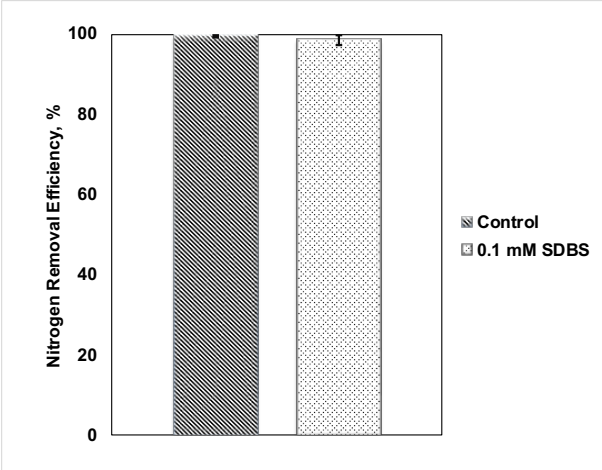

Figure 2. The effect of 0.1 mM SDBS on nitrogen removal efficiency. Data indicate average, and error bars represent standard deviations of the results from three independent sampling.

Thereafter, nitrogen removal rates (NRR) of the enriched anammox culture were calculated for each dose using the maximum slope of nitrogen removal vs time curves. In the absence of TiO_2 NPs (0 mg/L), NRR of anammox system was detected to be 190.94 ± 12.93 mgN/ gVSS.d. During the acute exposures of relatively small TiO_2 NPs dosages (1 mg/L and 10 mg/L), NRR was not significantly affected (α=0.05, P > 0.05 for μ1=μ2) (Figure 3a). As the applied NPs dosage increased, the inhibition percentage also increased (Figure 3b). Along with the increase of NPs dose to 50 mg/L, percentage inhibition reached to 20.62% ± 1.70% and a statistically significant decrease was observed in NRR (α=0.05, P = 0.02 < 0.05 for μ1=μ2). When the applied dose was risen to 100 mg/L and/or higher loads, extremely significant deteriorations were observed in NRR (α=0.05, P < 0.01 for μ1=μ2). In the presence of 300 mg/L TiO_2 NPs dosage, NRR was dramatically decreased to 37.09 ± 0.24 mgN/ gVSS.d and a severe inhibition (80.57 ± 1.17%) was observed (Figure 3a & 3b).

In order to examine the response of anammox bacteria to NPs stress, several inhibition models namely,

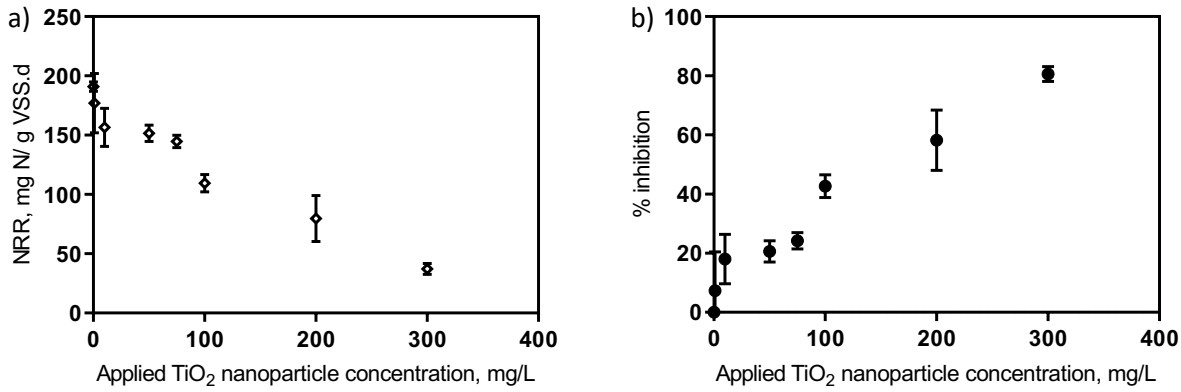

Figure 3. Acute effects of TiO_2 NPs on anammox activity. a) nitrogen removal rate in the presence of TiO_2 NPs b) percent inhibition (%) response of the applied TiO_2 NPs dosage. Data indicate average, and error bars represent standard deviations of samples measured in triplicate.

linear regression, modified non-competitive inhibition model, non-linear dose-response inhibition models, and non-linear second order polynomial (quadratic) model were tested (Figure 4). In all models, the IC_{50} of TiO_2 NPs on anammox activity was calculated. Compliance of the experimental data and determining the reliable curve fitting were evaluated by the R^2 value. The best fit curve was obtained with the non-linear second order polynomial (quadratic) model. In this model, the IC_{50} was determined to be 154 mg/L (R^2= 0.964).

In the literature, there is only one study that highlights the short-term effects of TiO_2 NPs on Anammox process (X. J. Zhang et al., 2018a) with an exposure time of 8 h. In that study, it is reported that, 1 mg/L NPs concentration enhanced the anammox activity while 5-20 mg/L dosages inhibited the anammox activity. It was also emphasized that 50 mg/L TiO_2 NPs dose showed lower suppression. On the contrary, this study demonstrated that acute exposure of TiO_2 NPs caused inhibition on Anammox process. The inhibition severity also rose as the applied dose was increased. The difference in the incubation period of acute exposure tests may be the most probable reason for different outcomes. In a recently published study, it was stated that, less than 12 h exposure period is not adequate to examine the anammox response against the inhibitory factors (Song et al., 2018). Besides these, not only the experimental setups but also the use of anammox granules/flocs and/or dominant anammox species in the enriched anammox culture may lead to the variations in the findings.

Attitudes of NPs in Anammox reactor

At the end of each acute exposure test, surface morphology of anammox bacteria was observed by SEM images. TiO_2 NPs mainly exist in solid phases because of their highly insoluble characteristic (Zheng et al., 2011). They tend to adsorb on biological surfaces or form aggregates with each other rather than the ion form (Cervantes-Aviles et al., 2017; Kokalj et al., 2018). As shown in Figure 5, TiO_2 NPs made clusters and attached to the surface of the bacteria. Moreover, when the applied dosage increased, amount of attached NPs also increased. Therefore, the substrate transfer rate between the environment and bacteria was possibly eliminated and NRR of Anammox process decreased. On the other hand, it has already been known that the larger the particle size is, the lower the toxicity. Hence,

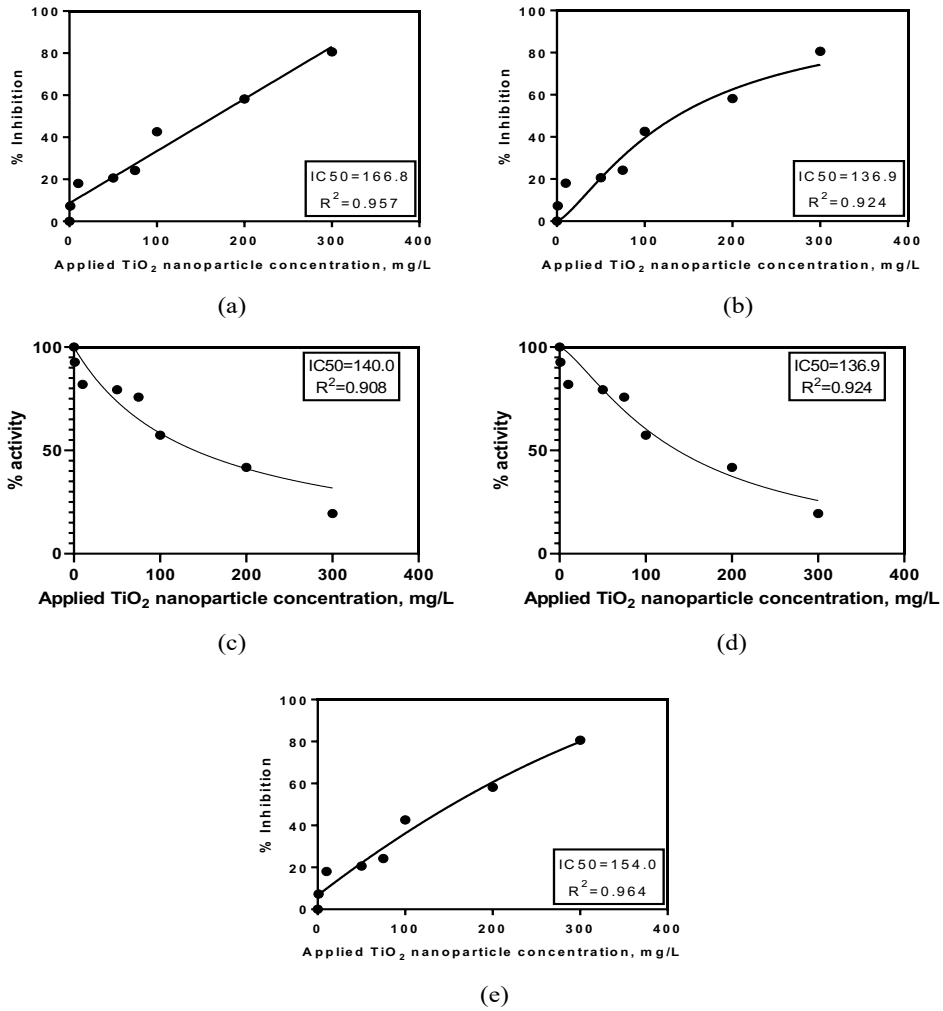

Figure 4. Inhibition response of the applied TiO_2 NPs dosage with different inhibition models. a) linear regression; b) modified non-competitive model; c) Inhibitor vs. normalized response; d) Inhibitor vs. normalized response - Variable slope; e) non-linear second order polynomial (quadratic) model.

due to the clustering of TiO_2 NPs, particle size may increase, which may lead to lower toxicity. As a different perspective, the EPS produced by the bacteria may trap the NPs and protect the bacteria assisting the aggregation of NPs. Even though, it was formerly speculated that TiO_2 NPs cannot be accumulated in high quantities on not only bacterial cell wall but also EPS due to electrostatic repulsions (Huangfu et al., 2019), SEM analysis exhibited the aggregation of TiO_2 NPs on anammox bacteria. A previous study also demonstrated the deposition of significant amount of metal oxide NPs including TiO_2 on the outer layer of anammox granules (Z. Z. Zhang et al., 2018b).

EPS response of Anammox bacteria

EPS, an organic matrix containing proteins, carbohydrates, DNA and lipids (Tang et al., 2018), is secreted by various microorganisms in anammox granules. Not only it protects microorganisms from adverse conditions as a first barrier against to environmental stress such as heavy metals and NPs, but also it plays an important role in accelerating the granulation process, improving stability of the matrix structure and granules (Yang et al., 2013; Zhang et al., 2015, 2017). Therefore, it is necessary to track EPS released by enriched anammox culture after acute exposure tests in order to understand the role of EPS in the resistance of anammox system against TiO_2 NPs.

Type of biological systems and the properties of NPs specify the potential effects of metal NPs (Xu et al., 2019). Thus, there have been various outcomes about the EPS responses of microorganisms. Decrement in PN content of anaerobic granular sludge against elevating ZnO NP concentration from 10 to 200 mg/g TSS was reported by Mu et al. (2012) while some studies were stated increment in EPS secretion in the existence of metal NPs including CuO, CeO_2 (Hou et al., 2015; Ma et al., 2013; You et al., 2017). Furthermore, some studies revealed that, NPs (ZnO, NiO) at lower concentrations firstly could rise up the EPS amount while higher loads of the same NPs decrease the EPS amount (He et al., 2017; Xu et al., 2019).

In the current study, the total EPS content gradually decreased with escalating burden of TiO_2 NPs

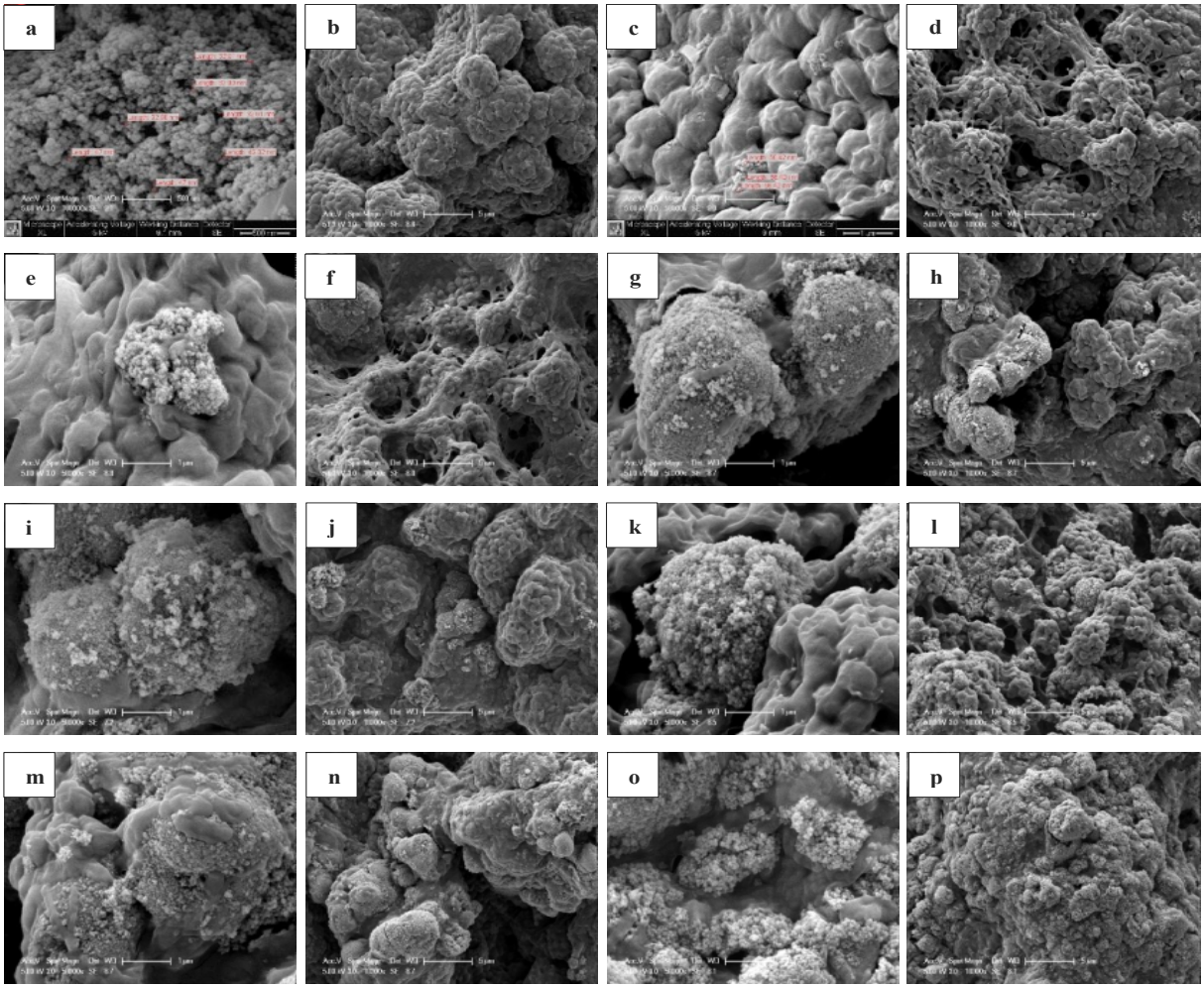

Figure 5. SEM images of anammox sludges after acute exposure tests a) TiO_2 NPs stock solution; b) anammox sludge; c) and d) 1 mg/L TiO_2 NPs exposure; e) and f) 10 mg/L TiO_2 NPs exposure; g) and h) 50 mg/L TiO_2 NPs exposure; i) and j) 75 mg/L TiO_2 NPs exposure; k) and l) 100 mg/L TiO_2 NPs exposure; m) and n) 200 mg/L TiO_2 NPs exposure; o) and p) 300 mg/L TiO_2 NPs exposure.

dosage (Figure 6). Numerically, 49.73% and 92.19% decrements in total EPS content was determined after the acute exposures of 50 mg/L and 300 mg/L TiO_2 NPs dosages, respectively. During all the exposure experiments, PN production was greater than PS production. Therefore, it could be interpreted that, sensitivity of PN to TiO_2 NPs was more than that of PS. Similar findings also revealed by other studies focused on other NPs (Xu et al., 2019; Xu et al., 2018).

In a recent study, Z. Z. Zhang et al. (2018b) investigated the long-term effects of TiO_2 NPs on Anammox process at three different NPs dosages (1 mg/L, 50 mg/L, 150 mg/L) for a month each and reported the enhancement of EPS production during the exposure period and highlighted the EPS secretion as adaptation strategy. In this study, however, the anammox biomass was exposed to shock loads of NPs within a short time. Hence, anammox bacteria may not be able to adapt to the changing environment and could not produce enough EPS to protect the cellular structure against the inhibitory compound. This phenomenon may also be explained by the longer lag phase of EPS secretion in anammox population compared to the heterotrophs because of their relatively slow growth rates (Song et al., 2018). Besides, the differences in exposure periods of the same NPs may change the EPS responses. Zhao et al. (2019) pointed out 24 h short-term exposure of ZnO NPs (5, 50, 150 mg/L) caused gradually decrement in EPS release by anammox granules. However, Sari et al. (2020) emphasized that, long-term exposure of ZnO NPs increased the EPS secretion in anammox reactor up to 40 mg/L NPs dosage. Consequently, in the current study, the EPS production gradually decreased inversely proportional to the increase in inhibition percentage.

Conclusion

Acute response of TiO_2 NPs on Anammox process at seven different dosages (1, 10, 50, 75, 100, 200, 300 mg/L) was examined for 24 h incubation period. When the concentration was risen to 50 mg/L, a significant deterioration on NRR was obtained. Inhibition percentage on anammox activity was gradually increased by escalating the burden of applied TiO_2 NPs dosage. At 300 mg/L exposure dose, NRR was dramatically decreased from 190.94 ± 12.93 to 37.09 ± 0.24 mgN/ gVSS.d and a severe inhibition (80.57% ± 1.17%) was observed. Several inhibition models were tested in order to estimate the IC_{50} value. Non-linear second order polynomial (quadratic) model was the best fit model with $R^2 = 0.964$. The IC_{50} value was determined to be 154 mg/L with this model. At the end of the acute exposure tests, surface morphology of anammox bacteria was also observed by SEM images. The results revealed that, TiO_2 NPs tended to form cluster and attached to the surface of the bacteria. Moreover, as the applied NPs dosage increased, amount of attached NPs also increased. Finally, EPS secretion by enriched anammox culture was tracked in order to analyze the response of the anammox system. The total EPS content gradually decreased by increasing TiO_2 NPs dosage which is inversely proportional to the increase in inhibition percentage of anammox activity.

Acknowledgements

This study was funded by the Scientific and Technological Research Council of Turkey (TUBITAK-Project No: 117Y214) and Marmara University Scientific Research Committee (BAPKO- Project No: FEN-C-YLP-

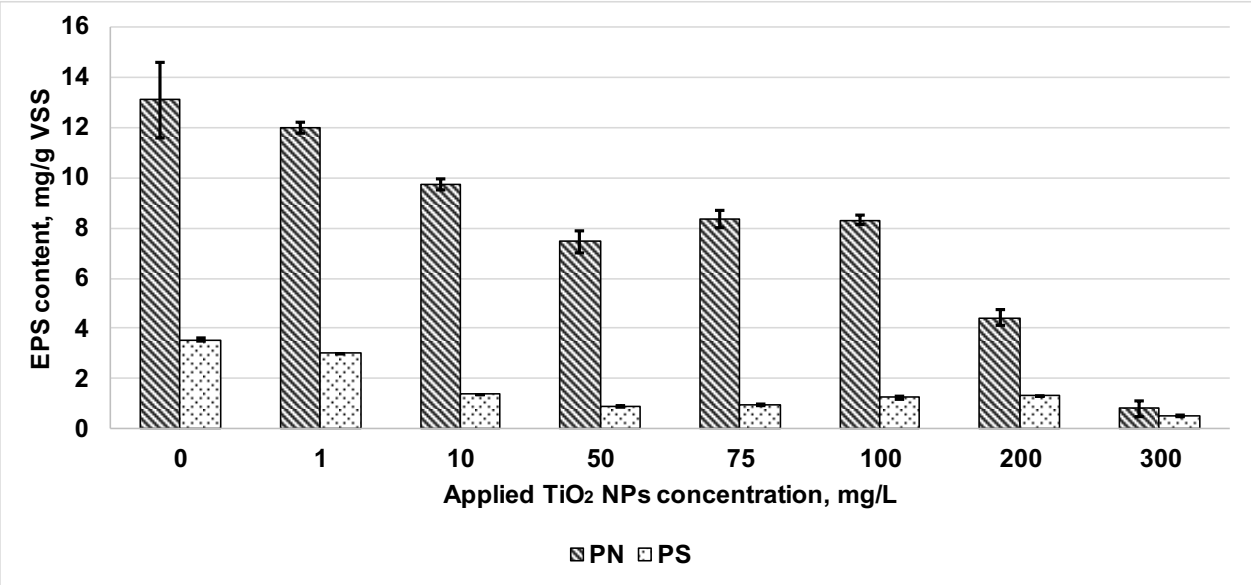

Figure 6. EPS secretion by enriched anammox culture exposed to TiO_2 NPs. Data indicate average, and error bars represent standard deviation of the results from two independent sampling, each tested in duplicate.

090217-0054). The authors express their gratitude to Prof. Bulent Mertoglu for sharing his laboratory infrastructure and Prof. Kozet Yapsakli for her helps in model studies.

Credit

Portions of this paper were presented at the ICETI Conference held in Sarajevo Bosnia and Herzegovina, March 22-26, 2017.

References

Bhushan, B. (2017). Introduction to Nanotechnology In B. Bhushan (Ed.), Springer Handbook of Nanotechnology (4 ed., pp. 1-18). Berlin, Germany: Springer.

Cao, Y. S., van Loosdrecht, M. C. M., & Daigger, G. T. (2017). Mainstream partial nitritation-anammox in municipal wastewater treatment: status, bottlenecks, and further studies. *Applied Microbiology and Biotechnology*, 101(4), 1365-1383.

Cervantes-Aviles, P., Pinas, N. C., Ida, J., & Cuevas-Rodriguez, G. (2017). Influence of wastewater type on the impact generated by TiO_2 nanoparticles on the oxygen uptake rate in activated sludge process. *Journal of Environmental Management*, 190, 35-44.

Chen, R., & Chen, C. (2017). Environment, Health and Safety Issues in Nanotechnology. In B. Bhushan (Ed.), Springer Handbook of Nanotechnology (pp. 1559-1586). Berlin, Germany: Springer.

Gupta, R., & Xie, H. (2018). Nanoparticles in Daily Life: Applications, Toxicity and Regulations. *Journal of Environmental Pathology Toxicology and Oncology*, 37(3), 209-230.

He, Q. L., Gao, S. X., Zhang, S. L., Zhang, W., & Wang, H. Y. (2017). Chronic responses of aerobic granules to zinc oxide nanoparticles in a sequencing batch reactor performing simultaneous nitrification, denitrification and phosphorus removal. *Bioresource Technology*, 238, 95-101.

Heil, J., Vereecken, H., & Bruggemann, N. (2016). A review of chemical reactions of nitrification intermediates and their role in nitrogen cycling and nitrogen trace gas formation in soil. *European Journal of Soil Science*, 67(1), 23-39.

Hou, J., Miao, L. Z., Wang, C., Wang, P. F., Ao, Y. H., & Lv, B. W. (2015). Effect of CuO nanoparticles on the production and composition of extracellular polymeric substances and physicochemical stability of activated sludge flocs. *Bioresource Technology*, 176, 65-70.

Huangfu, X. L., Xu, Y. H., Liu, C. H., He, Q., Ma, J., Ma, C. X., & Huang, R. X. (2019). A review on the interactions between engineered nanoparticles with extracellular and intracellular polymeric substances from wastewater treatment aggregates. *Chemosphere*, 219, 766-783.

Jin, R. C., Yang, G. F., Yu, J. J., & Zheng, P. (2012). The inhibition of the Anammox process: A review. *Chemical Engineering Journal*, 197, 67-79.

Kaphle, A., Nagaraju, N., & Daima, H. K. (2018). Contemporary Developments in Nanobiotechnology: Applications, Toxicity, Sustainability, and Future Perspective. In A. Dhawan, S. Singh, A. Kumar, & R. Shanker (Eds.), Nanobiotechnology: Human Health and the Environment (pp. 1-34). Boca Raton, Florida: CRC Press.

Keller, A. A., & Lazareva, A. (2014). Predicted Releases of Engineered Nanomaterials: From Global to Regional to Local. *Environmental Science & Technology Letters*, 1(1), 65-70.

Kokalj, A. J., Drobne, D., & Novak, S. (2018). Nanotoxicology: Toxicity Evaluation, Risk Assessment and Management. Boca Raton, Florida: CRC Press.

Li, H., Chi, Z. F., & Yan, B. X. (2018). Insight into the impact of Fe3O4 nanoparticles on anammox process of subsurface-flow constructed wetlands under long-term exposure. *Environmental Science and Pollution Research*, 25(29), 29584-29592.

Liu, G. Q., Wang, D. M., Wang, J. M., & Mendoza, C. (2011). Effect of ZnO particles on activated sludge: Role of particle dissolution. *Science of the Total Environment*, 409(14), 2852-2857.

Liu, W. Z., Weng, C. Z., Zheng, J. Y., Peng, X. Q., Zhang, J., & Lin, Z. (2019). Emerging investigator series: treatment and recycling of heavy metals from nanosludge. *Environmental Science-Nano*, 6(6), 1657-1673.

Lombi, E., Donner, E., Tavakkoli, E., Turney, T. W., Naidu, R., Miller, B. W., & Scheckel, K. G. (2012). Fate of Zinc Oxide Nanoparticles during Anaerobic Digestion of Wastewater and Post-Treatment Processing of Sewage Sludge. *Environmental Science & Technology*, 46(16), 9089-9096.

Ma, J. Y., Quan, X. C., Si, X. R., & Wu, Y. C. (2013). Responses of anaerobic granule and flocculent sludge to ceria nanoparticles and toxic mechanisms. *Bioresource Technology*, 149, 346-352.

Mohamed, H. R. H. (2018). Genotoxicity and Carcinogenicity of Daily Used Nanoparticles: In Vivo Studies. In V. Kumar, N. Dasgupta, & S. Ranjan (Eds.), Nanotoxicology: Toxicity Evaluation, Risk Assessment and Management (pp. 193-218). Boca Raton, Florida: CRC Press.

Morgan, J. W., Forster, C. F., & Evison, L. (1990). A Comparative-Study of the Nature of Biopolymers Extracted from Anaerobic and Activated Sludges. *Water Research*, 24(6), 743-750.

Mu, H., & Chen, Y. G. (2011). Long-term effect of ZnO nanoparticles on waste activated sludge anaerobic digestion. *Water Research*, 45(17), 5612-5620.

Mu, H., Zheng, X., Chen, Y. G., Chen, H., & Liu, K. (2012). Response of Anaerobic Granular Sludge to a Shock Load of Zinc Oxide Nanoparticles during Biological Wastewater Treatment. *Environmental Science & Technology*, 46(11), 5997-6003.

Mulder, A., Vandegraaf, A. A., Robertson, L. A., & Kuenen, J. G. (1995). Anaerobic Ammonium Oxidation Discovered in a Denitrifying Fluidized-Bed Reactor. *Fems Microbiology Ecology*, 16(3), 177-183.

Musee, N., Thwala, M., & Nota, N. (2011). The antibacterial effects of engineered nanomaterials: implications for wastewater treatment plants. *Journal of Environmental Monitoring*, 13(5), 1164-1183.

Peeters, S. H., & van Niftrik, L. (2019). Trending topics and open questions in anaerobic ammonium oxidation. *Current Opinion in Chemical Biology*, 49, 45-52.

Qiao, S., Zheng, N., Tian, T., Yu, C., & Zhou, J. T. (2016). Effects of short-term exposure to linear anionic surfactants (SDBS, SLS and SDS) on anammox biomass activity. *Rsc Advances*, 6(58), 53004-53011.

Robertson, G. P., & Groffman, P. M. (2015). Nitrogen Transformations. In E. A. Paul (Ed.), Soil microbiology,

ecology and biochemistry (4 ed., pp. 421-446). Burlington, Massachusetts, USA: Academic Press.

Sari, T., Can, S., & Akgul, D. (2020). Assessment of Anammox process against acute and long-term exposure of ZnO nanoparticles. *Science of the Total Environment*, 727.

Senapati, V. A., & Kumar, V. (2018). Nanoparticles and the Aquatic Environment: Application, Impact and Fate. In A. Dhawan, S. Singh, S. Kumar, & R. Shanker (Eds.), Nanobiotechnology: Human Health and the Environment (pp. 299-323). Boca Raton, Florida: CRC Press.

Song, Y. X., Chai, L. Y., Tang, C. J., Xiao, R. Y., Li, B. R., Wu, D., & Min, X. B. (2018). Influence of ZnO nanoparticles on anammox granules: The inhibition kinetics and mechanism analysis by batch assays. *Biochemical Engineering Journal*, 133, 122-129.

Stein, L. Y., & Klotz, M. G. (2016). The nitrogen cycle. *Current Biology*, 26(3), R94-R98.

Tang, J., Wu, Y. H., Esquivel-Elizondo, S., Sorensen, S. J., & Rittmann, B. E. (2018). How Microbial Aggregates Protect against Nanoparticle Toxicity. *Trends in Biotechnology*, 36(11), 1171-1182.

Tsuneda, S., Aikawa, H., Hayashi, H., Yuasa, A., & Hirata, A. (2003). Extracellular polymeric substances responsible for bacterial adhesion onto solid surface. *Fems Microbiology Letters*, 223(2), 287-292.

Wu, Q., Huang, K. L., Sun, H. H., Ren, H. Q., Zhang, X. X., & Ye, L. (2018). Comparison of the impacts of zinc ions and zinc nanoparticles on nitrifying microbial community. *Journal of Hazardous Materials*, 343, 166-175.

Xu, J. J., Cheng, Y. F., Xu, L. Z. J., Liu, Y. Y., Zhu, B. Q., Fan, N. S., Huang, B. C., & Jin, R. C. (2019). The revolution of performance, sludge characteristics and microbial community of anammox biogranules under long-term NiO NPs exposure. *Science of the Total Environment*, 649, 440-447.

Xu, J. J., Zhu, X. L., Zhang, Q. Q., Cheng, Y. F., Xu, L. Z. J., Zhu, Y. H., Ji, Z. Q., & Jin, R. C. (2018). Roles of MnO_2 on performance, sludge characteristics and microbial community in anammox system. *Science of the Total Environment*, 633, 848-856.

Yang, G. F., Ni, W. M., Wu, K., Wang, H., Yang, B. E., Jia, X. Y., & Jin, R. C. (2013). The effect of Cu(II) stress on the activity, performance and recovery on the Anaerobic Ammonium-Oxidizing (Anammox) process. *Chemical Engineering Journal*, 226, 39-45.

Yapsakli, K., Aktan, C. K., & Mertoglu, B. (2017). Anammox-zeolite system acting as buffer to achieve stable effluent nitrogen values. *Biodegradation*, 28(1), 69-79.

You, G. X., Wang, P. F., Hou, J., Wang, C., Xu, Y., Miao, L. Z., Lv, B., Yang, Y., Liu, Z., & Zhang, F. (2017). Insights into the short-term effects of CeO_2 nanoparticles on sludge dewatering and related mechanism. *Water Research*, 118, 93-103.

Zhang, L. L., Zhang, Z. X., He, X., Zheng, L., Cheng, S. K., & Li, Z. F. (2019). Diminished inhibitory impact of ZnO nanoparticles on anaerobic fermentation by the presence of TiO_2 nanoparticles: Phenomenon and mechanism. *Science of the Total Environment*, 647, 313-322.

Zhang, X. J., Zhou, Y., Ma, Y. P., Zhang, H., Li, Y. Z., Yang, J. C., Xie, X. Y., & Pang, Q. (2018a). Short-Term Effects of CuO, ZnO, and TiO_2 Nanoparticles on Anammox. *Environmental Engineering Science*, 35(12), 1294-1301.

Zhang, X. J., Zhou, Y., Xu, T. F., Zheng, K. W., Zhang, R. R., Peng, Z. X., & Zhang, H. Z. (2018b). Toxic effects of CuO, ZnO and TiO_2 nanoparticles in environmental concentration on the nitrogen removal, microbial activity and community of Anammox process. *Chemical Engineering Journal*, 332, 42-48.

Zhang, Z. Z., Deng, R., Cheng, Y. F., Zhou, Y. H., Buayi, X., Zhang, X., Wang, H. Z., & Jin, R. C. (2015). Behavior and fate of copper ions in an anammox granular sludge reactor and strategies for remediation. *Journal of Hazardous Materials*, 300, 838-846.

Zhang, Z. Z., Zhang, Q. Q., Xu, J. J., Deng, R., Ji, Z. Q., Wu, Y. H., & Jin, R. C. (2016). Evaluation of the inhibitory effects of heavy metals on anammox activity: A batch test study. *Bioresource Technology*, 200, 208-216.

Zhang, Z. Z., Xu, J. J., Shi, Z. J., Cheng, Y. F., Ji, Z. Q., Deng, R., & Jin, R. C. (2017). Short-term impacts of Cu, CuO, ZnO and Ag nanoparticles (NPs) on anammox sludge: CuNPs make a difference. *Bioresource Technology*, 235, 281-291.

Zhang, Z. Z., Cheng, Y. F., Bai, Y. H., Xu, L. Z. J., Xu, J. J., Shi, Z. J., Zhang, Q. Q., & Jin, R. C. (2018a). Enhanced effects of maghemite nanoparticles on the flocculent sludge wasted from a high-rate anammox reactor: Performance, microbial community and sludge characteristics. *Bioresource Technology*, 250, 265-272.

Zhang, Z. Z., Cheng, Y. F., Xu, L. Z. J., Bai, Y. H., Xu, J. J., Shi, Z. J., Shen, Y. Y., & Jin, R. C. (2018b). Evaluating the effects of metal oxide nanoparticles (TiO_2, Al_2O_3, SiO_2 and CeO_2) on anammox process: Performance, microflora and sludge properties. *Bioresource Technology*, 266, 11-18.

Zhao, J., Zhang, B. W., & Zuo, J. E. (2019). Response of anammox granules to ZnO nanoparticles at ambient temperature. *Environmental Technology & Innovation*, 13, 146-152.

Zheng, X., Chen, Y. G., & Wu, R. (2011). Long-Term Effects of Titanium Dioxide Nanoparticles on Nitrogen and Phosphorus Removal from Wastewater and Bacterial Community Shift in Activated Sludge. E*nvironmental Science & Technology*, 45(17), 7284-7290.