Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jes

JES JOURNAL OF ENVIRONMENTAL SCIENCES

www.jesc.ac.cn

Research Article

The short and long-term effect of polystyrene nanoplastics on nitrifying sludge at high nitrite concentrations

Xiaolong Wang[1], *Jialu Huang*[1], *Zhiqiang Qi*[1], *Ruiqin Kang*[1], *Chao Du*[2], *Da Li*[3,*]

[1] *School of Chemical and Environmental Engineering, Anyang Institute of Technology, Anyang 455000, China*
[2] *Henan Licheng Environmental Technology Co., Ltd., Anyang 455000, China*
[3] *State Key Laboratory of Urban Water Resource and Environment, School of Environment, Harbin Institute of Technology, Harbin 150090, China*

ARTICLE INFO

Article history:
Received 19 November 2022
Revised 12 January 2023
Accepted 14 January 2023
Available online 28 January 2023

Keywords:
Nanoplastics
Nitrifying activity
Extracellular polymeric substance (EPS)
Exposure
Community analysis

ABSTRACT

The effect of nanoplastics (NPs) on nitrite oxidation bacteria (NOB) community in treating high-strength wastewater remains unclear, which seriously affects the stability of nitrogen removal process. In this study, highly active nitrifying sludge was enriched and exposed to 50 nm polystyrene NPs (PS-NPs) for short-term (1, 100, 500, and 1000 mg/L, 1.5 hr) and long-term (1, 10, 100 mg/L, 40 days) at high nitrite concentration. In contrast to previous studies, our results showed that the exposures to PS-NPs had little effect on nitrifying performances. After long-term exposure, the protein/polysaccharide ratios in extracellular polymeric substances (EPS) were positively correlated with PS-NPs concentrations (0.78–0.99). The produced reactive oxygen species (ROS) were gradually removed, and PS-NPs higher than 10 mg/L caused damage to membrane integrity. Long-term exposure for 40 days increased the community diversity and caused significant differences between the control and exposed communities. The control group were dominated by *Nitrobacter* and *Exiguobacterium*, while the exposure group was dominated by *Bacillus, Mycobacterium*, and *Nitrospira*. A noticeable shift in the NOB community from *Nitrobacter* (26.5% to 3.4%) to *Nitrospira* (1.61% to 14.27%) was observed. A KEGG analysis indicated a decrease in cell growth and death, cell motility and energy metabolism. It appeared that NOB could adapt to PS-NPs stress through enhanced secretion and removal of oxidative damage. Overall, this study provided new insights into the response mechanism of NOB to PS-NPs exposure.

Introduction

Global plastic production has increased by 8.4% over the past decade and surpassed 370 million tons in 2019 (Geyer et al., 2017; PlasticsEurope, 2020). A considerable amount of plas-

* Corresponding author.
E-mail: dali@hit.edu.cn (D. Li).

https://doi.org/10.1016/j.jes.2023.01.014
1001-0742/

tic litter breaks down into smaller particles known as microplastics (MPs, 0.1 μm to 5 mm) or nanoplastics (NPs, 100 nm). MPs have been found in almost every environment globally (Joo et al., 2018; Mintenig et al., 2019; Ragusa et al., 2021; Xu et al., 2020; Zhang et al., 2020). Their persistence and widespread occurrence make MPs and NPs (MPs/NPs) emerging environmental pollutants.

The toxicity of NPs has gained increasing attention. Generally, many laboratory-based biological and toxicological studies had suggested that MPs could pose a threat to ecosystems (Du et al., 2020). While there were a variety of concerns regarding NPs. It was assumed that NPs were similar to MPs at first, but NPs had size-dependent properties that distinguish them from MPs, including higher surface area-to-volume ratio, higher adsorption affinity, higher colloidal stability, higher bioavailability, the potential toxicity and leaching additives (Gigault et al., 2021). These properties of NPs can lead to physical injury (e.g., adsorption, blocking, or softening of cell walls), oxidative damage (e.g., oxidation of protein, lipid, or even genome), penetration into biological barriers and lipid membranes, disruption of microbial community structure and gene expression, and inhibition of bacterial physiological activities (Rai et al., 2021; Rossi et al., 2014; Wang et al., 2021; Wei et al., 2020).

Wastewater treatment plants (WWTPs) are the essential collection and distribution center of MPs/NPs from human to natural environments. Studies had found that WWTPs contained high levels of MPs, with concentrations ranging from 0.14×10^4 to 3.14×10^4 items/L (Ali et al., 2021; Mintenig et al., 2017; Wang et al., 2021). Although current treatment technologies were able to remove around 95% of MPs in sewage (Carr et al., 2016; Simon et al., 2018; Talvitie et al., 2017), a significant amount of these MPs were trapped in the excessive sludge. It was reported that the sludge in the thickener, aerobic digester, and dewatering contained 200, 238, and 129 MPs/g dry weight, respectively (Alavian Petroody et al., 2021). As for NPs, there was currently limited information available on their concentrations in wastewater due to limitation in current separation and detection technology. However, it was estimated that up to 11% of MPs may form NPs (Besseling et al., 2018), and the total number of NPs may be up to 10^{14} times higher than that of MPs (Besseling et al., 2018). It was reasonable to speculated that theses high-strength ammonium wastewater contained high concentrations of NPs. The effect of NPs on nitrogen removal must be considered.

Partial nitritation-anaerobic ammonium oxidation (PNA) is effective in removing high levels of ammonium from wastewater (Wang et al., 2022). However, its effectiveness can be seriously affected by the presence of nitrite oxidation bacteria (NOB) (Gao et al., 2018). In order to understand the potential effects of NPs on nitrogen removal in PNA systems, it is important to examine the impact of NPs on NOB consortia. While there is limited information on this topic, and there is no consensus on the effect of NPs on NOB consortia (Yang et al., 2020). It was reported that 300 mg/L of polystyrene (PS) NPs could significantly affect NOB activities but did not impact anaerobic ammonium oxidation bacteria (AOB) activities (Lee et al., 2022). Another study indicated that 10-1000 μg/L of PS-NPs would inhibit both AOB and NOB activities due to the size effect and oxidative damage (Liu et al., 2021). Additionally, it had been reported that 10 mg/L of PS-NPs inhibited AOB and NOB activities in a constructed wetland.

The purpose of this study was to examine the effect of NPs on NOB communities through short-term and long-term exposure. To understand the response of NOB to Ps-NPs exposure, we monitored changes in nitrifying activity, reactive oxygen species (ROS), extracellular polymeric substances (EPS) and microbial community in NOB sludge. The results would be useful in assessing the effect of NOB on PNA systems when exposed to high levels of NPs.

1. Materials and methods

1.1. NOB consortia cultivation and synthetic wastewater

The nitrifying sludge was enriched in a lab-scale sequencing batch reactor (SBR) (Appendix A Fig. S1). The SBR had a working volume of 4.5 L, with a diameter of 14 cm and a height of 35 cm. A warm water jacket was used to maintain a constant temperature of 30°C. The dissolved oxygen (DO) was adjusted between 5 and 6 mg/L. The SBR cycle and pH were automatically controlled by a programmable logic controller (PLC). The pH was maintained at 7.5 by adding a solution of 10 mol/L NaOH. An 8-hr SBR cycle involved 12 min of filling, 7.25 hr of aerated reaction, 30 min of settling, and 3 min of the drawing.

The activated sludge used as the inoculum was obtained from the Shouchuang WWTP in Anyang, China, where conventional anaerobic-anoxic-aerobic (AAO) processes were employed. An amount of 860 mg/L of nitrites was fed, and the nitrite removal efficiency was approximately 100%, resulting the nitrite-oxidizing rate was estimated to be 1.26 g/L/day after three weeks. The highly active nitrifying sludge was then used to study the effects of PS-NPs on NOB consortia in both short-term and long-term.

In this study, synthetic wastewater contained (per liter): 0.1 g $CaCl_2$, 0.025 g KH_2PO_4, 0.2 g $MgSO_4$, 0.3 g $NaHCO_3$ and 1 mL trace elements solution. $NaNO_2$ was the only source of nitrite. Synthetic wastewater was initially adjusted to pH 7.5.

1.2. Polystyrene preparation

PS is a kind of common environmental plastic. The PS-NPs chosen for this study have undergone extensive testing and been widely reported in other studies. The monodisperse suspension of 50 nm PS-NPs with a concentration of 25 mg/L was purchased from Mylife Advanced Material Technology in Suzhou, China, and had been dispersed into ultra-pure water to form an emulsion. The PS-NPs stock was sonicated for 1 hr to further improve its dispersion before use.

1.3. Short-term PS-NPs exposure experiment

To investigate the short-term effects of PS-NPs on NOB consortia, four different concentrations of PS-NPs (1, 100, 500, and 1000 mg/L) were added to the batch exposure experiments. These concentrations were chosen because they were high enough to represent a worst-case scenario in theory, as has been done in other studies (Frehland et al., 2020; Song et al.,

2020; Wang et al., 2021). However, serious loss of PS-NPs occurred during aerated reactions due to air flotation effects, the forming bubbles, or adhesion to the beaker walls. As the concentration of added PS-NPs and the aeration duration increased, this issue became more pronounced. Therefore, it was considered appropriate to limit the test concentrations of PS-NPs to 1000 mg/L in short-term tests.

To test the nitrifying activity, an enriched NOB sludge of 200 mL was collected and washed with 10 μmol/L phosphate buffer saline (PBS) at a pH of 7.4. The sludge was then resuspended into 100 mL deionized water in a 500 mL beaker with a 200 mg/L nitrite solution. PS-NPs stock solutions of 0.08, 0.8, 4, and 8 mL were added to the mixture, resulting in final PS-NPs concentrations of 1, 100, 500, and 1000 mg/L, respectively. A control test was also conducted without the addition of PS-NPs.

The bottle containing the mixture was placed in a thermostatic magnetic stirrer and continuous aerated to maintain a DO level of 6 mg/L. A real-time pH feedback control was used to maintain a pH of 7.5 by adding 1 mol/L NaOH. The temperature was maintained at 29°C using a water bath. The mixture was stirred at a rate of approximately 110 r/min to ensure mixing. Mixed liquor samples were collected every 0.5 hr for a total of 1.5 hr to measure the concentrations of NO_2^--N and NO_3^--N. The potential nitrifying activity was calculated as the specific nitrite oxidation rate (SNOR) in unit of mg N/hr/g VSS. A total of three replicates were conducted for each test.

1.4. *Long-term PS-NPs exposure experiment*

Long-term exposure experiments were conducted using two parallel SBRs. Two identical 1 L beakers (with a working volume of 0.5 L) were used to prepare two small SBRs (SBR1 and SBR2). These SBRs were equipped with thermostatic magnetic stirrers, automatic pH controllers, DO meters, and an aeration system. Temperature, pH, DO, and stirring rate was consistent with values obtained in short-term tests. Then 0.5 L of mixed liquor was inoculated into each SBR using the same sampling method described in Section 1.3, with a mixed liquor volatile suspended solids (MLVSS) of 3.7 g VSS/L. The hydraulic retention time (HRT) was 24 hr with a drainage ratio of 50%. Due to the high concentrations of ammonia in targeted sludge digester or dewatering liquor (Ochs et al., 2021), both SBRs were fed with synthetic wastewater containing 980 mg/L of nitrite.

In general, low levels of NPs between 1-1000 μg/L (Liu et al., 2021) and high levels of NPs between 1-100 mg/L (He et al., 2021; Li et al., 2020) were often used to test the long-term toxicology to aquatic organisms. Considering the potential high levels of NPs in WWTPs, the concentrations of 1-100 mg/L of PS-NPs were chosen in the long-term exposure. The two SBRs were run simultaneously for 40 days, divided into four stages. Stage 1 was the baseline stage (day 0–10) where the two SBRs were run without PS-NPs to allow the NOB sludge to acclimate and for convergence to be established. The following three stages were exposure stages, during which SBR2 was exposed to 1 mg/L (day 11-20, stage 2), 10 mg/L (day 21-30, stage 3), and 100 mg/L (day 31-40, stage 4) PS-NPs, respectively. The PS-NPs were added to the influent in each cycle of SBR2 to maintain the exposure concentrations. Meanwhile, SBR1 served as a control system without the addition of PS-NPs. At the end of each stage, sludge samples were collected to test the ROS production and membrane integrity to explore potential toxic mechanisms of PS-NPs. The production of EPS was also extracted and the concentrations of polysaccharides (EPS-S) and proteins (EPS-P) were analyzed. At the end of the long-term PS-NPs exposure experiment, sludge samples were withdrawn, washed with 10 mmol/L PBS for three times, and stored at -24°C for analysis of microbe community structure.

1.5. *Microbial community structure*

The microbial community was analyzed using Illumina Meseq sequencing. For this analysis, following the manufacturer's instructions, 0.2 g (wet weight) biomass was used to extract total genomic DNA (gDNA) by the FastDNA SPIN Kit (MP Biomedicals, CA, USA). The gDNA was separately extracted in triplicate for each biomass sample and then combined into a single mixed gDNA sample for analysis. The quantity and purity of the extracted gDNA were determined using agarose gel electrophoresis and a Nanodrop spectrophotometer (2000, Thermo Scientific, USA).

The two mixed gDNA samples for the long-term exposure experiment were sent to MajorBio Company (Shanghai, China) for Illumina MiSeq sequencing following standard protocols, with a primer pair targeting the V4 region of bacterial 16S DNA genes (338F/806R). The raw sequence data of the PS-NPs test and control test had been deposited in the NCBI Sequence Read Archive Database with the accession numbers of SRR19751075 and SRR19751074, respectively. The filtered tags were then clustered into operational taxonomic units (OTUs) at 97% similarity (Uparse v7.0 software). Taxonomic classification was performed using the Ribosomal Database Project (RDP) classifier and the Silva database. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used for annotation to predict the metagenomic functional compositions of bacterial communities (Tax4Fun2 software) based on the 16S information.

1.6. *Chemical and data analytical methods*

The generation of ROS was measured using the 2,7-dichlorofluorescein (DCFH-DA) assay. The integrity of the cell membranes was assessed using the staining method with a red fluorescent probe to label cells with incomplete membranes using the Bacterial Cell Membrane Integrity Detecting Kit (BestBio in Shanghai, China). Generated fluorescein as relative fluorescence unit (RFU) was measured by a 96-well microtiter plate. The detailed procedures attached in Appendix A. The relative fold change of RFU in each stage was normalized by Eq. (1), where the RFU_2 and RFU_1 were the detected RFU value (non-dimensional parameter) in of SBR2 and SBR1, respectively, for the determining ROS or cell membrane integrity.

$$The\ fold\ changes\ of\ RFU\ of\ ROS\ or\ membrane\ damage = RFU_2/RFU_1 \quad (1)$$

EPS was categorized based on the compactness and fastness into Soluble EPS (S-EPS), loosely-bound EPS (LB-EPS), and

tightly-bound EPS (TB-EPS). S-EPS consists of microbial substances that are released into bulk liquid, also called soluble microbial products (SMP). Bound-EPS is located on the cell body surface and is closely integrated with the cell wall. EPS was extracted by the heating method (Appendix A). The protein and polysaccharide contents of EPS tended to vary with the total EPS content, as they made up 70%-80% of the entire EPS content, and P/S ratio as a proxy for the relative hydrophobicity of EPS (Tang et al., 2022). Therefore, the composition of EPS, rather than its absolute content, determined the performance of EPS in sludge. The relative EPS content was first normalized by Eq. (2), where the EPS_2 (mg/L) and EPS_1 (mg/L) were the determined EPS content of SBR2 and SBR1. Then the ratio of protein (EPS-P) to polysaccharide (EPS-S) of EPS (P/S ratio) was calculated to eliminate the variations of EPS contents. The detailed procedures can be found in the supplemental material, including EPS extraction, measurement of ROS and membrane integrity.

$$\text{The relative EPS} = EPS_2/EPS_1 \quad (2)$$

The concentrations of nitrite and nitrate were measured using N-(1-naphthalene)-ethylenediamine photometric method and ultraviolet spectrometry method, respectively. The total suspended solids (TSS) and volatile suspended solids (VSS) were determined using standard methods (Clesceri et al., 1998). The polysaccharide concentration was determined using a colorimetric method with anthrone-sulfuric acid (Roe, 1954). The protein content was determined using a BAC Detection Kit (Solarbio, Beijing, China). DO was measured according to the manufacturer's instructions (Multi 3430, WTW, Germany). The detailed procedures can be found in the supplemental material, including EPS extraction, measurement of ROS and membrane integrity.

1.7. *Calculation and statistical analysis*

A one-way analysis of variance (ANOVA) was performed using SPSS software (v23, IBM) to evaluate the significant differences of the results. The significance level was set at $P<0.05$. Pearson correlation coefficient was calculated using SPSS (v23, IBM, USA). Least significant difference (LSD) was performed for pairwise comparison (SPSS v23, IBM, USA).

2. Results

2.1. *Effects of PS-NPs concentrations on nitrifying activity during short-term exposure*

The effects of different concentrations of PS-NPs on nitrifying activity of NOB consortia during short-term exposure were evaluated in batch tests. Nitrifying activities were measured immediately after mixing with NOB sludge (Fig. 1). With 0, 100, 500, and 1000 mg/L of PS-NPs, the SNOR values were 47.9±4.5, 43.2±8.1, 41.2±5.4, and 48.0±4.3 mg N/hr/g VSS, respectively. Statistical analysis showed that a short-term exposure to PS-NPs at concentrations lower than 1000 mg/L had little effect on nitrifying activity ($P=0.62$) during a 1.5-hr reaction. Despite the high concentration of 1000 mg/L, most of the PS-NPs remained well dispersed in the stirred and aerated culture solutions, resulting in a milk-white appearance of the bulk liquid and NOB sludge. However, part of the PS-NPs was observed to adhere to the inside walls of the beaker and formed bubbles on the liquid surface, leading to a loss of PS-NPs during the short and long-term test.

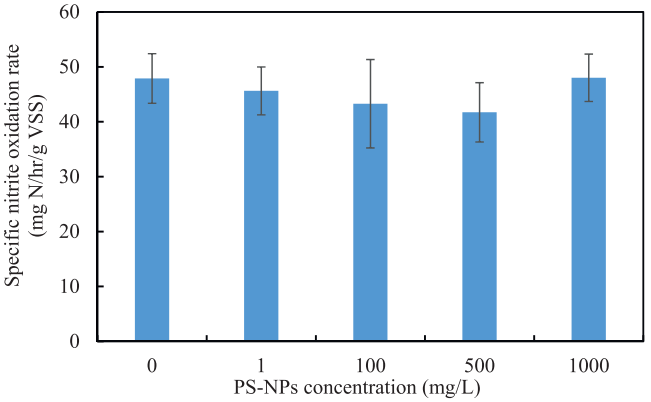

Fig. 1 – Effects of concentrations of PS-NPs on nitrifying activity during short-term exposure. There were no significant differences in the specific nitrite oxidation rate between different PS-NPs concentrations ($P = 0.62$), according to ANOVA test.

2.2. *Long-term effects of PS-NPs on nitrifying performance*

To investigate the long-term effects of PS-NPs on nitrifying performance, two identical SBRs were used in parallel (Fig. 2). During stage 1 (baseline stage, day 0-10) in both SBRs, the nitrite removal efficiency (NRE) maintained 100% and the volumetric nitrite removal rate (VNRR) was 0.49 g N/L/day (5.5 mg N/hr/g VSS), with negligible total nitrogen (TN) removal. Therefore, the convergence in nitrite oxidation performance were established between the two SBRs ($P=0.346$). To match nitrifying activities in both SBRs, the influent nitrite concentration was adjusted to 980 mg/L, as the nitrite loading rate was almost at its maximum capacity in both SBRs. SBR2 was then sequentially exposed to 1, 10, and 100 mg/L of PS-NPs for 10 days while SBR1 served as the control. Interestingly, the addition of PS-NPs for 10 days did have no effect on the nitrite oxidation performance, as there were no significant differences in the effluent concentrations of nitrite and nitrate, as well as NRE, between SBR1 and SBR2 ($P=0.210$) or among different stages ($P=0.146$), with 5.1 and 5.8 mg N/hr/g VSS in SBR1 and SBR2, respectively. The performance remained highly stable throughout the long-term test for both SBRs.

2.3. *Variations in EPS during long-term exposure to PS-NPS*

Microbial consortia are enclosed by EPS, which played a crucial role in protecting the cells from harsh environments, facilitating cell-to-cell adhesions and forming sludge structures

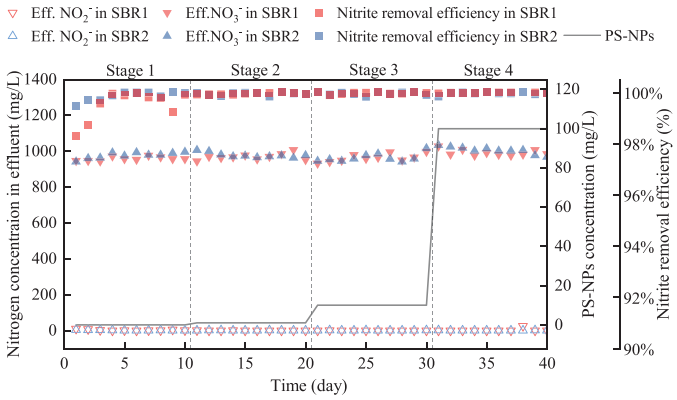

Fig. 2 – Effects of concentrations of PS-NPs on nitrite removal performance during long-term exposure.

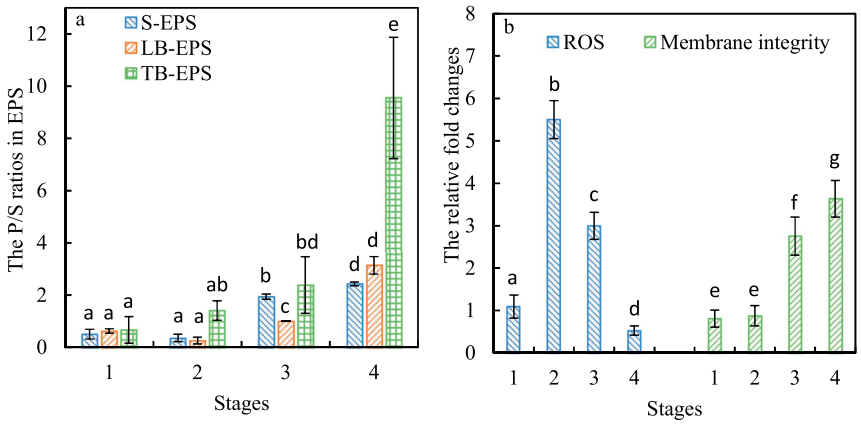

Fig. 3 – The relative P/S ratios changes (a), and the fold changes of ROS or membreane damage (b). Groups with the same letters are not significantly different at $P<0.05$, according to duncan's test.

under survival stress. In this study, the P/S ratios at different stages of long-term exposure were used to assess the effect of PS-NPs concentrations on EPS production (Fig. 3a).

In general, EPS-S content distributed in S-EPS, LB-EPS, and EB-EPS, while EPS-P content mainly distributed in LB-EPS. It was reported that TB-EPS contributed to sludge aggregation, and LB-EPS in the flocculent sludge showed repulsive force, so the NOB sludge in SBR2 had better flocculation capacity than that in SBR1 (Wang et al., 2021). In each stage, the P/S ratio in TB-EPS was higher than that in LB-EPS or S-EPS. At the beginning of stage 1, the average P/S ratio stabilized at 0.60±0.07 for all three types of EPS. However, in stage 2, the P/S ratio increased to 1.41±0.38 in TB-EPS, indicating that EPS-P was more prevalent than EPS-S in TB-EPS. In the following two stages, the P/S ratios continued to increase, with the highest value of 9.54±2.32 in TB-EPS in stage 4. Overall, long-term exposure to PS-NPs resulted in an increase in EPS-P content (from 27.07 to 35.10 mg/L) and a decrease in EPS-S content (from 34.46 to 10.94 mg/L) in SBR2 (Appendix A Fig. S6), resulting in an increase in the P/S ratio. Pearson correlation coefficients between PS-NPs concentrations and the P/S ratios of S-EPS, LB-EPS, and TB-EPS were calculated as 0.78, 0.78, and 0.99, respectively. So it was speculated that nitrifying sludge may adapt PS-NPs stress by increasing the secretion of proteins to EPS and using polysaccharides within EPS. Previous studies had shown that NPs tend to be absorbed with complex EPS before interacting with bacteria cells (Li et al., 2020), which explained the milk-white appearance of the sludge.

2.4. *Variations in ROS and membrane integrity during long-term exposure to PS-NPS*

Exposure to NPs often induces oxidative damage and disrupts cell membranes. Their relative fold changes of RFU in each stage were discussed (Fig. 3b). At the end of stage 1, there were no significant differences between SBR1 and SBR2 in terms of ROS and membrane damage, resulting in their fold change being close to 1. Compared to the control test of SBR1, the fold change of ROS increased considerably in stage 1 (5.49-fold, $P=0.00$), and gradually decreasing to 3.02-fold in stage 3 ($P=0.01$), before returning to a low level of 0.52-fold in stage 4 ($P=0.03$). The bacterial antioxidant systems contributed to the delicate balance between ROS production and elimination, through superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR)), non-enzymatic antioxidant defenses (glutathione, thioredoxin, Vitamin C, Vitamin E), and repair systems (Wang et al., 2017). With the gradually removal

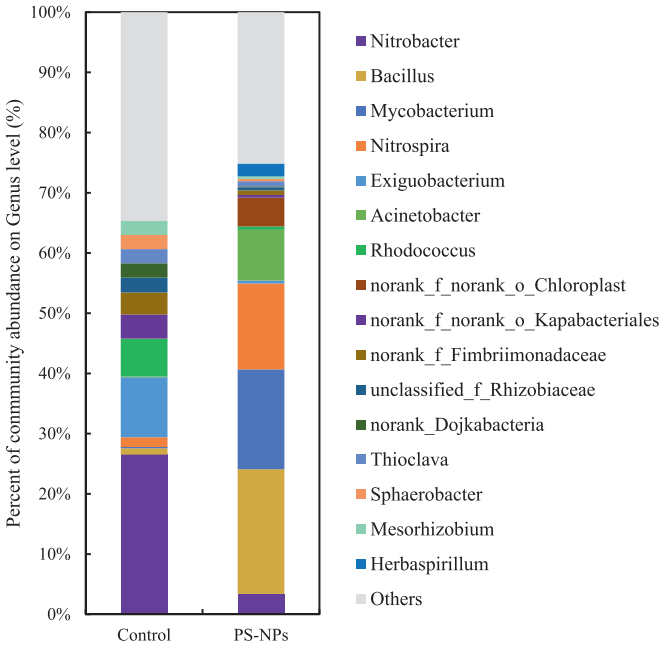

Fig. 4 – The relatvie abundances identified at genus level after long-term exposure experiments. Abundances lower that 0.02 were clusted as Others.

of ROS, it seemed that NOB consortia adapted to the long-term shock of PS-NPs.

Compared to the control test of SBR1, the fold of membrane damage stabilized at 0.88±0.07 in stage 2 (P=0.02), and continued to increase with the increasing concentrations of PS-NPs, reaching a final value of 3.64-fold in stage 4. Pearson correlation coefficient between PS-NPs concentrations and the folds of membrane damage was calculated as 1.00, indicating a strong correlation. At concentrations higher than 10 mg/L, PS-NPs were found to damage the integrity of bacterial cell membranes, leading to the decrease of MLVSS from 3.7 to 3.5 g/L in SBR2, compared to the increase of MLVSS from 3.7 to 4.0 g/L in SBR1.

2.5. *Long-term effects of PS-NPs on the community structure*

After conducting long-term exposure experiments, the community structures (Fig. 4) and KEGG pathways (Fig. 5) of the two SBRs were analyzed. Sequencing produced 35,468 and 38,100 effective sequences for SBR1 and SBR2, respectively, which were then clustered into 305 and 427 OTUs with an average length of 417 bp. Of these, 305 OTUs were shared between the two SBRs (Appendix A Fig. S3). Their coverages for SBR1 and SBR2 were 99.8% and 99.9, respectively (Appendix A Fig. S2). The exposure of PS-NPs resulted in an increase in alpha diversity indexes, including observed species, ACE, Chao1, and Shannon in SBR2, due to the higher numbers of OTU and higher richness. In contrast, the Simpson index decreased in SBR1 because it was more sensitive to the evenness in community (Appendix A Table S1). Other research had also indicated that the exposure to PS-NPs could increase the alpha diversity of functional genes in freshwater biofilms (Miao et al., 2022).

After the long-term exposure, significant differences were noticed between the two communities. The most commonly identified bacterial phyla were *Proteobacteria, Firmicutes, Nitrispirota*, and *Actinobacteriaota*. The control microbial community was dominated by *Nitrobacter* (26.5%) and *Exiguobacterium* (10.0%). In contrast, the microbial community in SBR2 was dominated by *Bacillus* (20.7%), *Mycobacterium* (16.6%), and *Nitrospira* (14.3%), which made up 51.6% of the community overall. The exposure to PS-NPs resulted in an increase in *Bacillus, Mycobacterium, Nitrospira, Acinetobacter, Chloroplast*, and *Herbaspirillum*.

Nitrobacter and *Nitrospira* are two different types of nitrifiers that are commonly found in nitrogen removal systems. In this study, the *Nitrobacter* community decreased from 26.5% to 3.4% while *Nitrospira* community increased from 1.61% to 14.27. This caused the dominant NOB community to shift from *Nitrobacter* to *Nitrospira*. The ecology of these two nitrite oxidizers and their response to disturbances had significant differences. *Nitrobacter* is typically classified as an r-strategist, meaning it can quickly reproduce, while *Nitrospira* is considered a K-strategist, so *Nitrobacter* was often found in high nitrite environments (Blackburne et al., 2007). This was the reason why *Nitrobacter* would likely be more prevalent in SBR1 with a high influent nitrite concentration of 980 mg/L. The shift between *Nitrospira* and *Nitrobacter* were reported due to different responses to factors such as sulfide, NO, Biochar and other inhibitors (Delgado Vela et al., 2018; Wang et al., 2022; Zhao et al., 2021). So we speculated that the PS-NPs may have enhanced the increase of *Nitrospira*. It was reported that the relative abundance of *Nitrospira* declined at 1 mg/L PS-NPs (Yang et al., 2020; Zhou et al., 2022), which was opposite to this study. However, others indicated that 100 mg/L polyamide MPs can increase the abundance of *Nitrospira* in aerobic granular sludge (Wang et al., 2021).

In SBR1, *Exiguobacterium* and *Rhodococcus* were frequently found alongside nitrogen removal bacteria and linked to organic degradation and heterotrophic nitrification. *Rhodococcus* was also noted for its high tolerance to high nitrite levels (Blasco et al., 2001). In SBR2, *Bacillus, Mycobacterium,* and *Acinetobacter* were present in high abundance. These taxa were linked to potentially pathogenic species and were related to organic degradation and heterotrophic nitrification, which tend to be accumulated by MPs (Maity and Pramanick, 2020).

2.6. *Long-term effects of PS-NPs on the KEGG pathways*

The KEGG analysis conducted by Tax4Fun2 found that both SBRs had similar relative abundances of functional genes (Fig. 5). These functional genes were clustered into four groups: Metabolism (62.8% and 63.2%), Environmental information processing (18.1% and 17.5%), Genetic information process (11.5% to 12.0%), Cellular processes (5.1% and 3.8%), and Human diseases (1.8% and 2.3%). The Metabolism pathway dominated in both SBRs. It was also estimated that the metabolic abundance would make up about 80% of the common activated sludge (Tang et al., 2022). As shown in Fig. 5, functional genes with high abundances in the KEGG pathway level 2 in both SBRs were amino acid metabolism, carbohy-

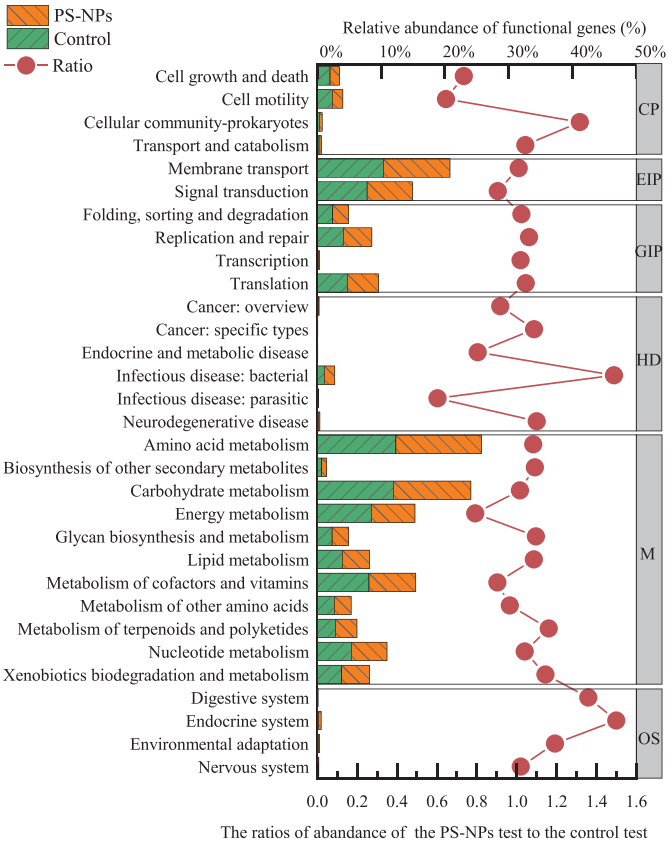

Fig. 5 – The KEGG analysis of predictive abundances of metagenomic functional genes (>0.02%) on pathway level 1 and level 2 after long-term exposure experiment in SBR1 and SBR2. The pathway level 1 included Cellular Processes (CP), Environmental Information Processing (EIP), Genetic Information Processing (GIP), Human Diseases (HD), Metabolism (M) and Organismal Systems (OS).

drate metabolism, energy metabolism, metabolism of cofactors and vitamins metabolism, membrane transport, and signal transduction. This suggesting that the consortia in both SBRs were actively engaged in total carbon and nitrogen cycling (Tang et al., 2022). After the long-term exposure to PS-NPs, the relative abundances of functional genes at pathway level 2 related to cell growth and death, cell motility and energy metabolism decreased (ratios < 0.8), while the bacterial infectious disease and cellular community-related genes increased (ratios >1.2). The decrease of cell growth and death was corresponded to the decrease of MLVSS from 3.7 to 3.5 g/L. In pathway level 3, ko00910 pathway linked to nitrogen metabolism was identified as needing further discussion. Based on the predicted enzyme abundance, module of denitrification and assimilatory nitrate reduction dominated ko00910 in SBR1 and SBR2, respectively, and the nitrification module increased from 0.006% to 0.013% after long-term exposure to PS-NPs, which guaranteed good nitrifying performance under stress from PS-NPs. The reduced relative abundance of ko02030 (Bacterial chemotaxis) and ko02040 (Flagellar assembly), as well as the increased ko05111 (Biofilm formation - Vibrio cholerae), suggested that PS-NPs may facilitate bacterial adhesion. The relative abundance of genes related to bacterial infectious diseases also increased in proportion to the pathogenic species.

3. Discussion

3.1. *High PS-NPs level had no inhibition effects on nitrifying performance*

The effect of NPs on nitrification in wastewater treatment are not yet well understood due to the lack of information on their presence, concentration, toxicity and behavior in the environment. Currently, most studies on this topic had primarily focused on the effect of MPs/NPs on the nitrification process (AOB and NOB consortia) in activated sludge. These studies had reported that MPs/NPs would inhibit AOB and NOB activity at different concentrations of NPs (0.01, 1, 10 mg/L NPs) (Tang et al., 2022; Zhou et al., 2022).

In contrast, other studies claimed that nitrifying activity would not be significantly affected by low levels of NPs in short-term. For example, polyvinyl chloride (PVC)-MPs (0-10000 particles/L) inhibited the efficiency of the partial nitritation process by 0.49 times compared to the control, while did not affect the nitrate accumulation (Song et al., 2020). Similarly, PS-NPs (1-1000 μg/L) did not significantly affected the ammonia oxidation or nitrate generation rates as reported (Liu et al., 2021). However, another study found that 300 mg/L of PS-NPs had no significant impact on the AOB activity, but

did negatively affect NOB activity (Lee et al., 2022). These findings indicated that NPs may affect nitrification by causing oxidative damage, membrane damage, changes in membrane potential, alterations in nitrifying enzymatic activity and bacterial community (Lee et al., 2022; Tang et al., 2022; Wei et al., 2020).

This study specifically focused on the effects of PS-NPs on NOB consortia. To enhance sensitivity and ensure robust nitrifying activity, active NOB consortia were cultured with high SNOR of 47.9±4.5 mg N/hr/g VSS and fed directly with 980 mg/L nitrite. This SNOR was higher than the reported range of approximate 3-30 mg N/hr/g VSS in activated sludge (Bae et al., 2001; Li et al., 2018). According to our findings, high concentrations of PS-NPs did not inhibit nitrifying performance during short-term or long-term exposure, which had not been previously reported.

Despite this, the long-term exposure to PS-NPs had a significant impact on various aspects of the NOB consortia. Several possible reasons may contribute to adaptation of NOB consortia to PS-NPs stress in this study. Firstly, the P/S ratio of TB-EPS was 14 times higher in stage 4 compared to stage 1. The protein in EPS was primarily composed of hydrophobic substances, while the polysaccharides were mostly hydrophilic. Increasing the protein content would help to limit the diffusion of NPs and prevent NPs from entering cells (Feng et al., 2018; Lee et al., 2022). The apparent increase in the P/S ratio assisted in the adaptation of the NOB consortia to PS-NPs stress. Additionally, because S-EPS and LB-EPS were derived from TB-EPS, and the LB-EPS attached to the cell membrane surface was relatively stable, which may have contributed to the resistance to PS-NPs.

Additionally, non-modified PS-NPs had a negative charge, making them relatively loosely attracted to bacterial cells compared to other positive charges (Sun et al., 2018). The uneliminated ROS would accumulate over time and may persist for a considerable period of time. ROS accumulation often caused oxidative DNA damage and lipid oxidation, and apoptosis. In this study, the ROS content did not accumulate and had recovered to the initial level at stage 4, indicating that the ROS were gradually removed to maintain an intracellular reduction state. Additionally, EPS had been reported to have antioxidant scavenging properties (Grassi et al., 2020). Finally, *Nitrospira* replaced *Nitrobacter* in SBR2 to compensate for nitrite oxidation performance to achieve a stable SNOR. However, doses of PS-NPs higher than 10 mg/L caused damage to the cell membrane in NOB consortia, leading to cell apoptosis and contributing to organic matter and carbon metabolism. This could be a potential threat to long-term stability.

3.2. *Possible influences of NPs to nitrogen removal treatment*

In WWTPs, a large amount of MPs/NPs are often retained as waste sludge and usually disposed of via anaerobic digestion. The PNA process is known for its ability to treat high-ammonia digestion. However, the success of the PNA process relies on the out-selection of NOB, as they can compete with oxygen and nitrite with anammox bacteria, leading to the conversion of more ammonia into nitrate rather than nitrogen gas. Therefore, it is essential to consider the effects of NPs contamination on treating high-ammonia wastewater using the PNA process.

Several reports indicated that a concentration of 100 mg/L of PVC-MPs reduced the relative abundance of Ca. *Brocadia* after 40 days of operation (Tang et al., 2021). In addition, long-term exposure to 1 mg/L of PS-NPs was found to significantly inhibit anammox activity, resulting in a 57.6% decrease (Xu et al., 2022). Most studies had found that NPs would inhibit nitrification by affecting AOB activities. Therefore, NPs may reduce the activity of partial nitritation and anammox (Song et al., 2020). According to our results, if NOB were not affected by NPs, the overgrowth of NOB would deteriorate the PNA process and result in a low nitrogen removal rate. In addition to MPs/NPs stress, instability should also be considered as a factor that may negatively impact PNA operations.

4. Conclusions

This study investigated the effect of PS-NPs on the enriched NOB consortia at high nitrite level. In contrast to previously studies, results indicated that nitrifying activity was not affected by short-term or long-term exposure to high levels of PS-NPs. During the long-term exposure, the P/S ratios of EPS increased while the ROS accumulation was removed. However, PS-NPs at concentrations above 10 mg/L did damage the cell membranes. The community of NOB shifted from *Nitrobacter* to *Nitrospira* during long-term exposure, with *Nitrobacter* and *Exiguobacterium* dominating the SBR1 while *Bacillus, Mycobacterium* and *Nitrospira* dominating SBR2. KEGG analysis revealed a decrease in cell growth and death, cell motility, and energy metabolism after exposure to PS-NPs. It was suggested that PS-NPs stress would cause unstable performance when treating high-strength wastewater.

Declaration of Competing Interest

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

Acknowledgments

This work was supported by the Science and Technology Project of Henan Province (No. 212102310510), the Doctoral Scientific Research Foundation of Anyang Institute of Technology (No. BSJ2019026) and the Anyang Municipal Major Special Project (No. 201928).

Appendix A Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2023.01.014.

REFERENCES

Petroody, Alavian, S, S., Hashemi, S.H., van Gestel, C.A.M., 2021. Transport and accumulation of microplastics through wastewater treatment sludge processes. Chemosphere 278, 130471.

Ali, I., Ding, T., Peng, C., Naz, I., Sun, H., Li, J., et al., 2021. Micro- and nanoplastics in wastewater treatment plants: occurrence, removal, fate, impacts and remediation technologies - a critical review. Chem. Eng. J. 423, 130205.

Bae, W., Baek, S., Chung, J., Lee, Y., 2001. Optimal operational factors for nitrite accumulation in batch reactors. Biodegradation 12 (5), 359–366.

Besseling, E., Redondo-Hasselerharm, P., Foekema, E.M., Koelmans, A.A., 2018. Quantifying ecological risks of aquatic Micro- and Nanoplastic. Crit. Rev. Env. Sci. Technol. 49 (1), 32–80.

Blackburne, R., Vadivelu, V.M., Yuan, Z., Keller, J., 2007. Kinetic characterisation of an enriched nitrospira culture with comparison to nitrobacter. Water Res 41 (14), 3033–3042.

Blasco, R., Martínez-Luque, M., Madrid, M.P., Castillo, F., Moreno-Vivián, C., 2001. Rhodococcus Sp. Rb1 grows in the presence of high nitrate and nitrite concentrations and assimilates nitrate in moderately saline environments. Arch. Microbiol. 175 (6), 435–440.

Carr, S.A., Liu, J., Tesoro, A.G., 2016. Transport and fate of microplastic particles in wastewater treatment plants. Water Res 91, 174–182.

Clesceri, L.S., Greenberg, A.E., Eaton, A.D., 1998. Standard methods for the examination of water and wastewater. 20th Ed, 20st. American Public Health Association, American Water Works Association. Water Environment Federation, Washington, DC.

Delgado Vela, J., Dick, G.J., Love, N.G., 2018. Sulfide inhibition of nitrite oxidation in activated sludge depends on microbial community composition. Water Res 138, 241–249.

Du, J., Xu, S., Zhou, Q., Li, H., Fu, L., Tang, J., et al., 2020. A review of microplastics in the aquatic environmental: distribution, transport, ecotoxicology, and toxicological mechanisms. Environ. Sci. Pollut. Res. 27 (11), 11494–11505.

Feng, L.J., Wang, J.J., Liu, S.C., Sun, X.D., Yuan, X.Z., Wang, S.G., 2018. Role of extracellular polymeric substances in the acute inhibition of activated sludge by polystyrene nanoparticles. Environ. Pollut. 238, 859–865.

Frehland, S., Kaegi, R., Hufenus, R., Mitrano, D.M., 2020. Long-term assessment of nanoplastic particle and microplastic fiber flux through a pilot wastewater treatment plant using metal-doped plastics. Water Res 182, 115860.

Gao, D., Wang, X., Liang, H., Wei, Q., Dou, Y., Li, L., 2018. Anaerobic Ammonia oxidizing bacteria: ecological distribution, metabolism, and microbial interactions. Front. Env. Sci. Eng. 12 (3), 10.

Geyer, R., Jambeck, J.R., Law, K.L., 2017. Production, use, and fate of all plastics ever made. Sci. Adv. 3 (7), e1700782.

Gigault, J., El Hadri, H., Nguyen, B., Grassl, B., Rowenczyk, L., Tufenkji, N., et al., 2021. Nanoplastics are neither Microplastics nor engineered nanoparticles. Nat. Nanotechnol. 16 (5), 501–507.

Grassi, G., Gabellieri, E., Cioni, P., Paccagnini, E., Faleri, C., Lupetti, P., et al., 2020. Interplay between Extracellular Polymeric Substances (Eps) from a marine diatom and model nanoplastic through eco-corona formation. Sci. Total Environ. 725, 138457.

He, Z.W., Yang, W.J., Ren, Y.X., Jin, H.Y., Tang, C.C., Liu, W.Z., et al., 2021. Occurrence, effect, and fate of residual microplastics in anaerobic digestion of waste activated sludge: a state-of-the-Art review. Bioresour. Technol. 331, 125035.

Joo, P.D.C., Pao, A., Santos, P.S.M., Duarte, A.C., Rocha-Santos, T., 2018. Microplastics in soils: assessment, analytics and risks. Environ. Chem. 16, 18–30.

Lee, J., Jeong, S., Long, C., Chandran, K., 2022. Size dependent impacts of a model microplastic on nitrification induced by interaction with nitrifying bacteria. J. Hazard. Mater. 424 (Pt B), 127363.

Li, H., Xu, S., Wang, S., Yang, J., Yan, P., Chen, Y., et al., 2020. New insight into the effect of short-term exposure to polystyrene nanoparticles on activated sludge performance. J. Water Process Eng. 38, 101559.

Li, L., Liu, D., Li, Z., Song, K., He, Y., 2020. Evaluation of microplastic polyvinylchloride and antibiotics tetracycline Co-effect on the partial nitrification process. Mar. Pollut. Bull. 160, 111671.

Li, S., Fei, X., Chi, Y., Jiao, X., Wang, L., 2018. Integrated temperature and do effect on the lab scale A2o process: performance, kinetics and microbial community. Int. Biodeterior. Biodegrad. 133, 170–179.

Liu, Q., Li, L., Zhao, X., Song, K., 2021. An evaluation of the effects of nanoplastics on the removal of activated-sludge nutrients and production of short chain fatty acid. Process Saf. Environ. Prot. 148, 1070–1076.

Maity, S., Pramanick, K., 2020. Perspectives and challenges of micro/nanoplastics-induced toxicity with special reference to phytotoxicity. Global Change Biol 26 (6), 3241–3250.

Miao, L., Guo, S., Wu, J., Adyel, T.M., Liu, Z., Liu, S., et al., 2022. Polystyrene nanoplastics change the functional traits of biofilm communities in freshwater environment revealed by Geochip 5.0. J. Hazard. Mater. 423 (Pt B), 127117.

Mintenig, S.M., Int-Veen, I., Loder, M.G.J., Primpke, S., Gerdts, G., 2017. Identification of microplastic in effluents of waste water treatment plants using focal plane array-based micro-Fourier-transform infrared imaging. Water Res 108, 365–372.

Mintenig, S.M., M.G.J, L., Primpke, S., Gerdts, G., 2019. Low numbers of microplastics detected in drinking water from ground water sources. Sci. Total Environ. 648, 631–635.

Ochs, P., Martin, B.D., Germain, E., Stephenson, T., van Loosdrecht, M., Soares, A., 2021. Ammonia removal from thermal hydrolysis dewatering liquors Via three different Deammonification technologies. Sci. Total Environ. 755, 142684.

PlasticsEurope, 2020. Plastics-the Facts 2020: an analysis of European plastics production, demand and waste data. PlasticEurope 4321, 64.

Ragusa, A., Svelato, A., Santacroce, C., Catalano, P., Notarstefano, V., Carnevali, O., et al., 2021. Plasticenta: First Evidence of Microplastics in Human Placenta. Environ. Int. 146, 106274.

Rai, P.K., Lee, J., Brown, R.J.C., Kim, K.-H., 2021. Micro- and Nano-Plastic pollution: behavior, microbial ecology, and remediation technologies. J. Cleaner Prod. 291, 125240.

Roe, J.H., 1954. The Determination of dextran in blood and urine with anthrone reagent. J. Biol. Chem. 208, 889–896.

Rossi, G., Barnoud, J., Monticelli, L., 2014. Polystyrene nanoparticles perturb lipid membranes. J. Phys. Chem. Lett. 5 (1), 241–246.

Simon, M., van Alst, N., Vollertsen, J., 2018. Quantification of microplastic mass and removal rates at wastewater treatment plants applying focal plane array (Fpa)-based Fourier transform infrared (Ft-Ir) imaging. Water Res 142, 1–9.

Song, K., Li, Z., Liu, D., Li, L., 2020. Analysis of the partial nitrification process affected by polyvinylchloride microplastics in treating high-ammonia anaerobic digestates. ACS Omega 5 (37), 23836–23842.

Sun, X., Chen, B., Li, Q., Liu, N., Xia, B., Zhu, L., et al., 2018. Toxicities of polystyrene nano- and microplastics toward

marine bacterium halomonas alkaliphila. Sci. Total Environ. 642, 1378–1385.
Talvitie, J., Mikola, A., Koistinen, A., Setälä, O., 2017. Solutions to microplastic pollution – removal of microplastics from wastewater effluent with advanced wastewater treatment technologies. Water Res 123, 401–407.
Tang, L., Su, C., Chen, Y., Xian, Y., Hui, X., Ye, Z., et al., 2021. Influence of biodegradable polybutylene succinate and non-biodegradable polyvinyl chloride Microplastics on Anammox sludge: performance evaluation, suppression effect and metagenomic analysis. J. Hazard. Mater. 401, 123337.
Tang, S., Qian, J., Wang, P., Lu, B., He, Y., Yi, Z., et al., 2022. Exposure to nanoplastic induces cell damage and nitrogen inhibition of activated sludge: evidence from bacterial individuals and groups. Environ. Pollut. 306, 119471.
Wang, L., Wu, W.M., Bolan, N.S., Tsang, D.C.W., Li, Y., Qin, M., et al., 2021. Environmental fate, toxicity and risk management strategies of nanoplastics in the environment: current status and future perspectives. J. Hazard. Mater. 401, 123415.
Wang, X.-F., Li, J., Li, G., Zhang, G.-L., Wang, Z.-W., Zhi, Y.-C., et al., 2022. Biochar application affects nitrobacter rather than nitrospira in plastic greenhouse vegetable soil. Appl. Soil Ecol. 175, 104449.
Wang, Y., Wang, J., Liu, Z., Huang, X., Fang, F., Guo, J., et al., 2021. Effect of Eps and its forms of aerobic granular sludge on sludge aggregation performance during granulation process based on Xdlvo theory. Sci. Total Environ. 795, 148682.
Wang, Y., Wu, Y., Wang, Y., Xu, H., Mei, X., Yu, D., et al., 2017. Antioxidant properties of probiotic bacteria. Nutrients 9 (5), 521.
Wang, Z., Gao, J., Dai, H., Zhao, Y., Li, D., Duan, W., et al., 2021. Microplastics affect the ammonia oxidation performance of aerobic granular sludge and enrich the intracellular and extracellular antibiotic resistance genes. J. Hazard. Mater. 409, 124981.
Wang, Z., Zheng, M., Duan, H., Yuan, Z., Hu, S., 2022. A 20-year journey of partial nitritation and Anammox (Pn/a): from sidestream toward mainstream. Environ. Sci. Technol. 56 (12), 7522–7753.
Wei, W., Hao, Q., Chen, Z., Bao, T., Ni, B.J., 2020. Polystyrene Nanoplastics reshape the anaerobic granular sludge for recovering methane from wastewater. Water Res 182, 116041.
Xu, S., Li, H., Wang, G., Liu, Z., Long, M., Guo, J., et al., 2022. Effects of long-term exposure to low-concentration Ps-Nps on Anammox granular sludge: resistance and inhibition depend on Ps-Np accumulation. J. Cleaner Prod. 365, 132902.
Xu, S., Ma, J., Ji, R., Pan, K., Miao, A.J., 2020. Microplastics in aquatic environments: occurrence, accumulation, and biological effects. Sci. Total Environ. 703, 134699.
Yang, X., He, Q., Guo, F., Sun, X., Zhang, J., Chen, M., et al., 2020. Nanoplastics Disturb nitrogen removal in constructed wetlands: responses of microbes and Macrophytes. Environ. Sci. Technol. 54 (21), 14007–14016.
Zhang, Y., Kang, S., Allen, S., Allen, D., Gao, T., Sillanpää, M., 2020. Atmospheric Microplastics: a review on current status and perspectives. Earth Sci. Rev. 203.
Zhao, J., Zhao, J., Xie, S., Lei, S., 2021. The role of hydroxylamine in promoting conversion from complete nitrification to partial nitrification: no toxicity inhibition and its characteristics. Bioresour. Technol. 319, 124230.
Zhou, C.S., Wu, J.W., Ma, W.L., Liu, B.F., Xing, D.F., Yang, S.S., et al., 2022. Responses of nitrogen removal under microplastics versus nanoplastics Stress in SBR: toxicity, microbial community and functional genes. J. Hazard. Mater. 432, 128715.