

Research Article

Mechanistic Insights into Impacts of Microplastics on Anammox

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Abstract

Microplastics (MPs) are pervasive emerging contaminants in wastewater, potentially affecting microbially mediated nitrogen removal process. However, the intricate interactions between MPs and anaerobic ammonium oxidation (Anammox) remain poorly understood. To address the potential threat posed by MPs in municipal wastewater to Anammox process, this study established sequencing batch Anammox systems spiked with colored polyethylene MPs to investigate their impact mechanisms on nitrogen removal efficiency, cellular survival strategies, and microbial community structure dynamics. The results indicated that MPs significantly inhibited nitrogen removal, with $\text{NH}_4^+\text{-N}$ removal efficiency in the aged red MPs group decreased to 71.0%. Toxicological mechanism analysis revealed that aged MPs exacerbated cellular oxidative stress, leading to a malondialdehyde content 7.6 times higher than that of the control. This induced severe membrane damage, reduced energy synthesis, impaired electron transfer, and weakened stress resistance, thereby inhibiting nitrogen removal and Anammox activity. Metagenomic analysis further suggested that aged red MPs reduced the abundance of *Planctomycetota* by 54%, with decreases of 32.3% and 40.3% for *Candidatus Brocadia* and *Candidatus Kuenenia*, respectively, indicating that aged MPs significantly inhibited the proliferation of Anammox microbiome. This study elucidated mechanisms underlying suppression of Anammox by MPs, providing a theoretical basis for MP risk management in wastewater treatment.

Keywords

Anaerobic Ammonium Oxidation, Microplastics, UV Aging, Nitrogen Removal, Microbial Metabolism

1. Introduction

Microplastics (MPs) typically refer to plastic particles, fragments, or fibers with a particle size of less than 5 mm. Plastic products gradually weather and fragment under external forces such as light, high temperature, and mechanical wear to form MPs [1]. Studies have shown that the biological toxicity of MPs is related to their particle size, morphology, surface characteristics, and leaching behavior of chemical additives [2]. MPs typically occur at low abundance levels in the influent of municipal wastewater treatment plants (WWTPs). According to a report

from a secondary treatment plant, MPs in the influent were 126.0 ± 14.0 particles/L, decreasing to 30.6 ± 7.8 particles/L in the effluent, with an overall reduction of approximately 75.7% across the plant. The morphological composition of MPs in wastewater and sludge is dominated by fibers and fragments, typically accounting for 70–90%. Components such as foams, films, and pellets/spheres generally account for a lower proportion, presumably because larger foam/tubular MPs are more easily intercepted by physical units such as screens and grit chambers in the early

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stages, while elongated fibers, due to their physical characteristics of easy entanglement and difficult settling, tend to maintain a higher proportion in the aqueous phase and can still be detected in the effluent [3]. In terms of polymer composition, common types are predominantly polyesters, polyamides, polyethylene terephthalate (PET), and polyethylene (PE), accompanied by minor amounts of polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), and polycarbonate [3].

Anaerobic ammonium oxidation (Anammox) refers to the biological process in which Anammox bacteria (AnAOB) directly convert $\text{NH}_4^+\text{-N}$ to N_2 using $\text{NO}_2^-\text{-N}$ as the electron acceptor under anaerobic conditions [4]. Compared with conventional nitrification-denitrification processes, Anammox can achieve nitrogen removal efficiencies as high as 89%, while reducing aeration energy consumption by more than 60%, saving 100% of organic carbon source, and reducing sludge production by 90% [5]. All currently identified AnAOB belong to the phylum *Planctomycetota*, among which *Candidatus Brocadia* and *Candidatus Kuenenia* are common dominant genera [4], with their combined relative abundance reaching up to 26.9%. In recent years, the potential impact of MPs on the Anammox process has attracted attention. Studies showed that single addition of PVC-MPs had little effect on Anammox, whereas under continuous addition mode, total nitrogen removal dropped to 71.4%, and the abundances of AnAOB and filamentous bacteria decreased by 9.7% and 11.6%, respectively [6]. Studies have shown that the toxicity of MPs is typically significantly enhanced after aging and confirmed that the surface oxygen-containing functional groups of aged NPs increased and could bind with $-\text{OH}$, $-\text{COOH}$, and other groups in sludge, inhibiting microbial metabolic activity and leading to a sharp decrease in extracellular polymeric substance (EPS) content. Therefore, this study conducted a continuous exposure experiment for 30 days by establishing an Anammox granular sludge nitrogen removal system affected by PE-MPs.

2. Materials and Methods

2.1. Preparation of Virgin and Aged Colored Microplastic Particles

PE-MPs were purchased from Dongguan Huachuang Plastic Co., Ltd., and red, blue, and white powders with a particle size of 200 μm were selected. A 40 W UVB-313 lamp was used for photoaging of the MPs. The virgin PE-MP powders were evenly spread in quartz culture dishes and continuously irradiated under UV light at a wavelength of 313 nm for 20 d to prepare aged PE-MPs [7], with manual mixing every 24 h to ensure uniform irradiation.

2.2. Experimental Setup

This study used seven serum bottle (effective volume 500

mL) experimental systems, including a control group (CK, no MPs), three virgin PE-MP groups of different colors (A1/A2/A3, red/blue/white, 500 mg/L, 200 μm), and three UV-aged PE-MP groups of different colors (A4/A5/A6, red/blue/white, 500 mg/L, 200 μm). Anammox granular sludge with a mixed liquor volatile suspended solids concentration of 8.2 ± 0.3 g/L was used, operated at $35 \pm 0.5^\circ\text{C}$, 120 rpm oscillation, and dark conditions for 30 d, with an initial nitrogen loading rate of 0.15 kg/m³/d. The reactors were operated in sequencing batch mode, with 200 mL of water discharged every 24 h and an equal volume of influent and PE-MPs added: operation in a constant-temperature shaker for 22 h, settling for 0.5 h, and decanting and feeding for 1.5 h.

2.3. Analytical Methods

Influent and effluent samples were collected daily from each treatment group, filtered through 0.45 μm membranes, and the concentrations of $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, and $\text{NO}_3^-\text{-N}$ were measured. The pyridine hemochrome spectrophotometric method was used to determine heme c concentration. The anthrone-sulfuric acid and BCA methods were employed to determine polysaccharide and protein contents in EPS, respectively. Metagenomic sequencing technology was used to analyze the changes in the microbial community structure of Anammox granular sludge under PE-MP stress.

3. Results and Discussion

3.1. Effects of Microplastics on Nitrogen Removal Performance of Anammox Reactors

To systematically evaluate the effects of PE-MPs with different colors and aging stages on Anammox nitrogen removal performance, the effluent concentrations of $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, and $\text{NO}_3^-\text{-N}$ were monitored daily in each treatment group during the 30-d experimental period. The changes in effluent $\text{NH}_4^+\text{-N}$ concentration of each treatment group during the 30-day operation are shown in Figure 1. The effluent $\text{NH}_4^+\text{-N}$ concentration of the CK remained at a relatively low level, indicating that the Anammox system has excellent and stable $\text{NH}_4^+\text{-N}$ removal capacity without MP stress. The effluent $\text{NH}_4^+\text{-N}$ concentrations of all MP treatment groups increased to varying degrees over time, but the variation characteristics differed depending on the color and aging state of MPs. For the virgin groups (A1–A3), at day 30 of operation, the effluent $\text{NH}_4^+\text{-N}$ concentration order was A1 (red) > A2 (blue) > A3 (white), with removal of 71.0%, 80.6%, and 87.0%, respectively, indicating that the color of MPs has a significant effect on $\text{NH}_4^+\text{-N}$ removal, with red MPs showing the strongest inhibition and white the weakest. The effluent $\text{NH}_4^+\text{-N}$ concentrations of the aged groups (A4–A6) were generally higher than those of the corresponding virgin

groups, also showing the trend $A4 > A5 > A6$, with the aged red group (A4) exhibiting the lowest removal. The above results indicated that UV aging significantly enhanced the inhibitory effect of MPs on $\text{NH}_4^+\text{-N}$ removal, and there was a

synergistic enhancement effect between aging and red color. The decrease in $\text{NH}_4^+\text{-N}$ removal can be attributed to impaired AnAOB activity and damaged cell membrane integrity under MP stress [8].

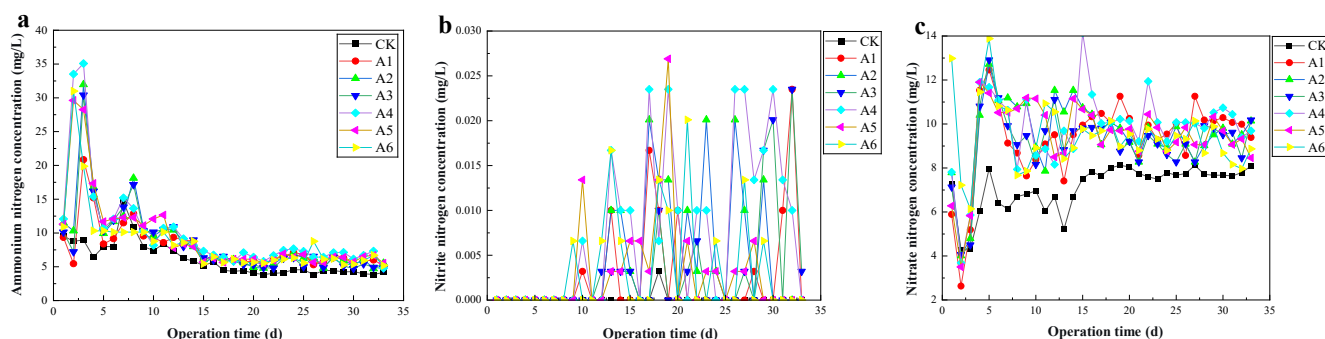


Figure 1. Nitrogen removal performance in Anammox under MP stress.

The effluent $\text{NO}_2^-\text{-N}$ concentration of the CK was always below 1 mg/L, with removal approaching 100%, indicating that the substrate $\text{NO}_2^-\text{-N}$ was almost completely utilized by the Anammox microbial community. In the virgin groups, the effluent $\text{NO}_2^-\text{-N}$ concentration order was $A1 > A2 > A3$, with white MPs (A3) having the least effect on $\text{NO}_2^-\text{-N}$ removal. In the aged groups, A4 showed the most prominent $\text{NO}_2^-\text{-N}$ accumulation. Notably, the increase in $\text{NO}_2^-\text{-N}$ concentration in the aged groups was generally greater than that of $\text{NH}_4^+\text{-N}$. This phenomenon may arise from two reasons: first, after AnAOB activity is inhibited, the decrease in $\text{NO}_2^-\text{-N}$ consumption is greater than that of $\text{NH}_4^+\text{-N}$; second, MP stress may stimulate the activity of some heterotrophic denitrifying bacteria, with additional $\text{NO}_2^-\text{-N}$ consumed through the denitrification pathway [6], but this is insufficient to fully compensate for the loss of the Anammox pathway, resulting in residual $\text{NO}_2^-\text{-N}$ in the effluent [9].

The effluent $\text{NO}_3^-\text{-N}$ concentration of the control group was stable at 8–10 mg/L, basically consistent with the theoretical $\text{NO}_3^-\text{-N}$ production of the Anammox reaction. In contrast, the effluent $\text{NO}_3^-\text{-N}$ concentrations of the MP treatment groups generally showed a decreasing trend, and the decrease amplitude increased with the enhancement of MP toxicity. In the virgin groups, at day 30 of operation, the effluent $\text{NO}_3^-\text{-N}$ concentration order was $A3 < A2 < A1$, with white MPs (A3) having the lowest effluent $\text{NO}_3^-\text{-N}$ concentration, closest to the CK level. In the aged groups, A4 had the highest effluent $\text{NO}_3^-\text{-N}$ concentration. The decrease in $\text{NO}_3^-\text{-N}$ concentration corresponded to the reduction in $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ removal, further confirming that the Anammox reaction was inhibited. Meanwhile, part of the generated $\text{NO}_3^-\text{-N}$ may be additionally consumed through heterotrophic denitrification or dissimilatory nitrate reduction pathways under MP stress [10].

3.2. Toxic Stress Mechanisms of Microplastics on Anammox Microbiome

Heme c is an electron transport protein containing a heme prosthetic group, with the core function of transferring electrons in biological redox chains (Figure 2). Its content can reflect the electron transfer efficiency and metabolic activity of the Anammox microbial community [11]. This study found that PE-MPs with different colors and aging states alter the electron transport chain, thereby exerting toxic effects of varying degrees on AnAOB, ultimately affecting the nitrogen removal performance of the system. Compared with the virgin groups, the heme c concentration of aged red MPs ($0.88 \pm 0.18 \mu\text{mol/g VSS}$) was lower than that of virgin red ($1.00 \pm 0.16 \mu\text{mol/g VSS}$), aged blue MPs ($0.96 \pm 0.19 \mu\text{mol/g VSS}$) was slightly lower than virgin blue ($1.12 \pm 0.11 \mu\text{mol/g VSS}$), and aged white MPs ($0.98 \pm 0.10 \mu\text{mol/g VSS}$) was significantly lower than virgin white ($1.24 \pm 0.15 \mu\text{mol/g VSS}$) and the control. In summary, the color of PE-MPs can affect the electron transport system, and aging further amplifies this toxic effect, thereby significantly reducing the heme c content.

EPS are high-molecular-weight polymer mixtures secreted by microorganisms during growth and metabolism, encapsulating the cell surface, and are a key component of the sludge system [12]. In terms of polysaccharide components, the LB-EPS and TB-EPS polysaccharide concentrations of the CK group were 7.9 and 11.2 mg/g VSS, respectively, at the basal metabolic level. After the introduction of virgin colored PE-MPs (A1–A3), LB-EPS fluctuated slightly between 8.5–9.1 mg/g VSS, but TB-EPS increased significantly from 11.2 in CK to 13.0–18.7 mg/g VSS, with A2 and A3 reaching a peak of 18.7 mg/g VSS, an increase of 66.5% compared with CK. MP stress stimulates polysaccharide synthesis, especially TB-EPS, most evidently in the A3 group [13]. However, the polysaccharide distribution pattern was reversed in the aged MP

groups (A4–A6). The LB-EPS of A4 plummeted to 6.4 mg/g VSS (the lowest point of the entire process), and although it rebounded to 8.2–9.0 mg/g VSS in the A5–A6 groups, TB-

EPS remained consistently lower than that of the A2/A3 groups (maintained at 14.5–17.4 mg/g VSS).

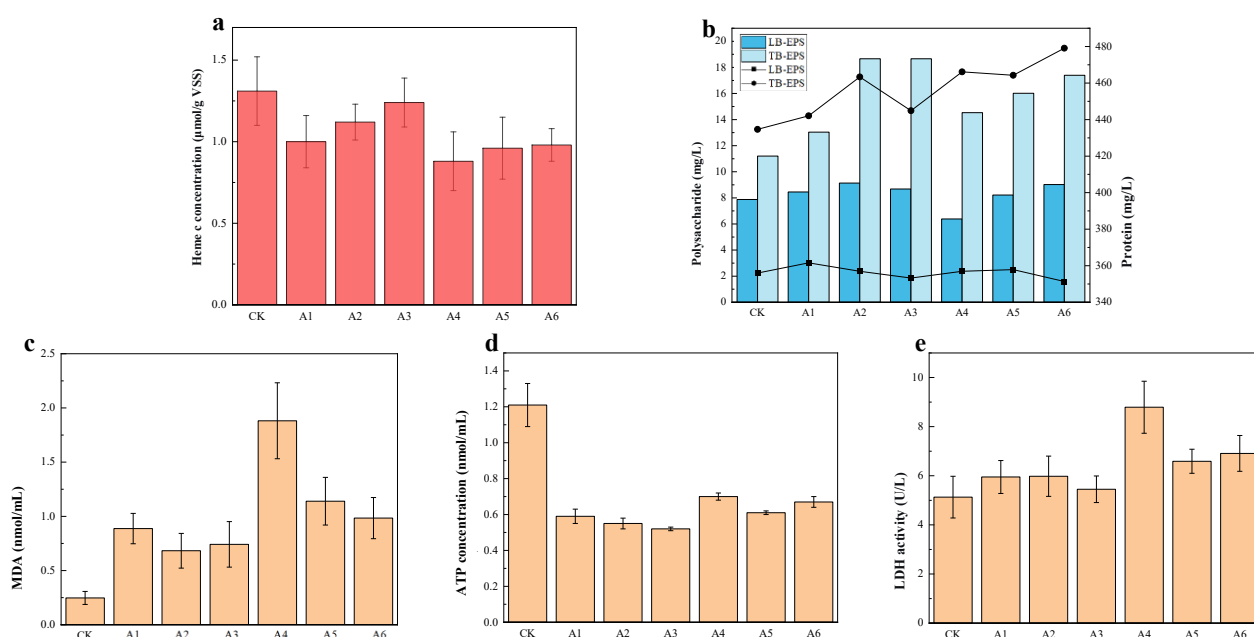


Figure 2. Changes of physiological indicators in Anammox under MP stress.

Malondialdehyde (MDA) is the end product of lipid peroxidation triggered by reactive oxygen species (ROS) attacking polyunsaturated fatty acids in cell membrane phospholipids. The results showed that the MDA concentration of the CK was 0.247 ± 0.06 nmol/mL, at a low level, indicating mild lipid peroxidation of the Anammox microbial cell membrane without MP stress. The MDA concentrations of all MP treatment groups were significantly higher than that of the CK group ($p < 0.05$), with the virgin red group (A1) at 0.887 ± 0.14 nmol/mL, virgin blue group (A2) at 0.683 ± 0.16 nmol/mL, and virgin white group (A3) at 0.742 ± 0.21 nmol/mL; the aged red group (A4) at 1.882 ± 0.35 nmol/mL, aged blue group (A5) at 1.140 ± 0.22 nmol/mL, and aged white group (A6) at 0.984 ± 0.19 nmol/mL. The MDA concentrations of the treatment groups in descending order were: A4 > A5 > A6 > A1 > A3 > A2 > CK. In summary, the increase in MDA content is direct evidence of oxidative damage caused by MPs to Anammox bacterial community [14]. This damage to cell membrane integrity may impair the proton motive force and the function of membrane-bound proteins, disrupt intracellular homeostasis, and ultimately lead to decreased cell viability and even cell death.

Adenosine triphosphate (ATP) is the core carrier of energy metabolism in living cells, and its content directly reflects the level of metabolically active biomass in the microbial community. The ATP concentration of the control group sludge was 1.21 ± 0.12 nmol/mL, within the typical active range of a stable Anammox system, indicating vigorous energy metabolism of the microbial community without MP pressure. The

ATP concentrations of all MP treatment groups were significantly lower than that of the CK group ($p < 0.05$), showing a pattern highly consistent with nitrogen removal performance and cell damage indicators: aged groups < virgin groups, red groups < blue groups < white groups. Specifically, in the virgin groups, the ATP concentrations of the red group (A1), blue group (A2), and white group (A3) were 0.59 ± 0.04 , 0.55 ± 0.03 , and 0.52 ± 0.01 nmol/mL, respectively, decreasing by 51.2%, 54.5%, and 57.0% compared with CK; in the aged groups, the ATP concentrations of red (A4), blue (A5), and white (A6) further decreased to 0.70 ± 0.02 , 0.61 ± 0.01 , and 0.67 ± 0.03 nmol/mL, decreasing by 42.1%, 49.6%, and 44.6% compared with CK, respectively. Among them, the virgin white group (A3) had the most severe ATP depletion, at only 43% of CK, indicating that the energy synthesis system of Anammox bacteria in this group was most severely damaged. Virgin white MPs, due to their strongest physical roughness and chemical activity, play the most critical role in this cascade effect. The significant decrease in ATP concentration directly confirms the inhibitory effect of colored and aged MPs on the metabolic activity of Anammox bacteria, with virgin white MPs showing the most significant inhibition, providing an energy-level mechanistic explanation for the decline in nitrogen removal efficiency.

Lactate dehydrogenase (LDH) is a key cytoplasmic enzyme in the microbial glycolysis pathway, catalyzing the conversion between pyruvate and lactate and coupling NAD^+/NADH electron transfer. Among them, the aged red group (A4) had the highest LDH activity, increasing by 71.3% compared with

CK, and completely corresponding to the MDA peak (1.882 nmol/mL), indicating that the colorant system of red MPs (possibly containing transition metal components) catalyzed more severe lipid peroxidation during aging, leading to membrane structure disintegration. LDH leakage has a direct causal relationship with the decline in cellular physiological functions. On the one hand, LDH efflux indicates the loss of cytoplasm and intracellular enzyme reserves, directly affecting glycolysis efficiency, which is related to the significant decrease in ATP content (A4 group decreased by 42.1% compared with CK), confirming that membrane damage leads to the collapse of the intracellular metabolic network and hinders energy synthesis. On the other hand, the increase in LDH occurs simultaneously with the decrease in cytochrome c, indicating that membrane damage not only causes leakage of cytoplasmic enzymes but also disrupts the membrane-bound environment of the electron transport chain, leading to impaired denitrification function. The significant increase in supernatant LDH activity directly confirms the membrane integrity disruption effect of colored and aged PE-MPs on Anammox granular sludge. Aged red MPs, due to their dual toxicity of physical scraping and chemical oxidation, are the strongest factor inducing membrane damage in this study, providing a mechanism explanation at the cell membrane level for the decline in denitrification performance.

3.3. Effects of Aged Colored Microplastics on Microbial Community

According to the metagenomic sequencing results, aged colored PE-MPs significantly interfered with the microbial community structure and functional metabolism of Anammox granular sludge, and this interference exhibited obvious color dependence and aging synergistic effects [15]. *Chloroflexota* (average relative abundance 13.12%) and *Planctomycetota* (average relative abundance 9.26%) were the dominant phyla (Figure 3), but they exhibited differentiated response patterns in different treatment groups. Among them, *Planctomycetota*, which contains AnAOB, was most sensitive to MP stress, with its abundance decreasing from 9.8% in the control group (CK) to 7.2% in the virgin red group (A1), and further sharply decreasing to 4.5% in the aged red group (A4). This declining trend completely coincided with the decay pattern of $\text{NH}_4^+\text{-N}$ removal (71.0%), directly confirming that the loss of Anammox functional bacteria is the fundamental reason for the decline in nitrogen removal efficiency. In contrast, *Chloroflexota*, as a facultative anaerobic fermentative bacterium, showed small abundance fluctuations under stress (13.1% (CK) to 12.3% (A4)), but its metabolic function may have changed.

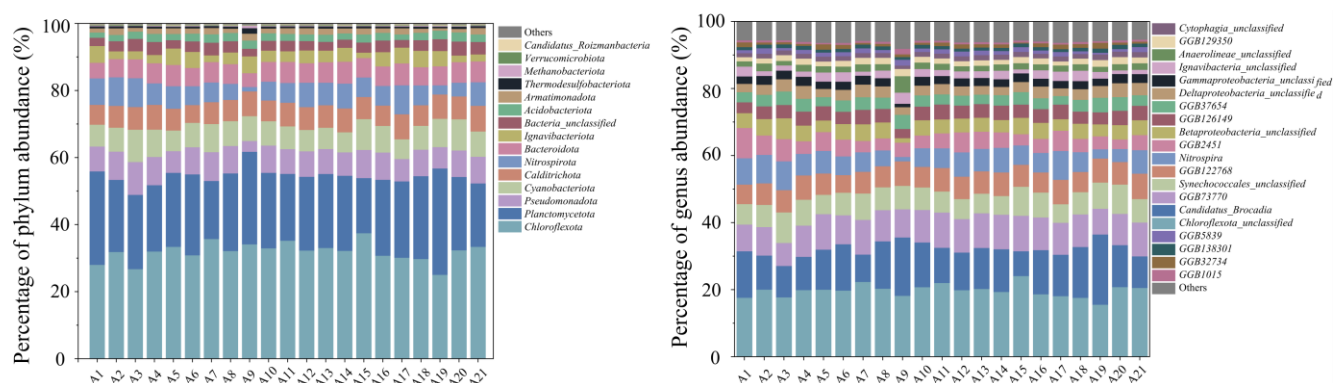


Figure 3. Microbial community changes in Anammox under MP stress.

At the finer genus level, previously dominant key functional genera (such as *GGB1015*, *GGB32734*, and *GGB138301*, all with abundances exceeding 15%) were inhibited under the stress of aged red MPs, with their abundances typically dropping below 8%; in contrast, tolerant genera with strong environmental adaptability, such as *Simplicispira*, showed a significant growth trend. *Simplicispira* may resist the physical wear of MPs by secreting viscous substances, while the enrichment of *Methanoxthrix* indicates that the carbon flow in the system may shift from denitrification to methanogenesis, and this functional microbial replacement effect further reduces the denitrification efficiency of the system. At the Anammox functional genus level, *Candidatus Brocadia*

was the stably dominant AnAOB genus, with a relative abundance of $6.8 \pm 0.7\%$ in the CK group, but significantly decreased to $3.9 \pm 0.4\%$ in the aged red PE-MP group (A4) (a decrease of 42.6% compared with CK), completely consistent with the trend of $\text{NH}_4^+\text{-N}$ removal decreasing from 85.2% to 71.0%. In addition, aged red MPs caused a 40.3% decrease in the abundance of *Candidatus Jettenia*. In summary, aged colored PE-MPs weakened the stability of the Anammox system by reducing the abundance of Anammox functional bacteria, among which aged red MPs, due to the dual attack of physical and chemical toxicity, constitute a high-risk environmental factor in the operation of this process [16].

4. Conclusions

This study systematically revealed the toxic effects and mechanisms of PE-MPs on the nitrogen removal performance, cellular physiology, and microbial community of the Anammox system through a 30-d experiment. MPs significantly inhibited the nitrogen removal performance of the Anammox system. Aged MPs damaged the Anammox microbial community through physical, chemical, and biological pathways. Metagenomic analysis revealed the high sensitivity of Anammox functional bacteria to MP stress, manifested as a sharp decrease in the abundance of *Candidatus Brocadia*.

Abbreviations

Anammox	Anaerobic Ammonium Oxidation
AnAOB	Anaerobic Ammonium-oxidizing Bacteria
MP(s)	Microplastic(s)
PE-MPs	Polyethylene Microplastics
PVC-MPs	Polyvinyl Chloride Microplastics
NPs	Nanoplastics
WWTPs	Wastewater Treatment Plants
PET	Polyethylene Terephthalate
PE	Polyethylene
PP	Polypropylene
PS	Polystyrene
PVC	Polyvinyl Chloride
UV	Ultraviolet
UVB	Ultraviolet B
EPS	Extracellular Polymeric Substances
LB-EPS	Loosely Bound Extracellular Polymeric Substances
TB-EPS	Tightly Bound Extracellular Polymeric Substances
BCA	Bicinchoninic Acid Assay
VSS	Volatile Suspended Solids
CK	Control Group
MDA	Malondialdehyde
ROS	Reactive Oxygen Species
ATP	Adenosine Triphosphate
LDH	Lactate Dehydrogenase
NAD ⁺	Oxidized Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinucleotide
NH ₄ ⁺ -N	Ammonium Nitrogen
NO ₂ ⁻ -N	Nitrite Nitrogen
NO ₃ ⁻ -N	Nitrate Nitrogen
N ₂	Nitrogen Gas

Author Contributions

Linlin Zhang: Conceptualization, Data curation, Visualization, Writing – original draft

Yongxin Zhao: Formal Analysis, Writing – review & editing

Conflicts of Interest

The authors declare no conflicts of interest.

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