

Article

Insights into the Differential Impacts of Polystyrene Microplastics and Nanoplastics on Sequencing Batch Reactor Performance: From Physiological Stress to Antibiotic Resistance Gene Dissemination

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Abstract

The widespread occurrence of microplastics (MPs) and nanoplastics (NPs) in wastewater treatment plants (WWTPs) has raised notable ecological concerns. This study investigated the multi-level impacts of polystyrene (PS) MPs and NPs on activated sludge in sequencing batch reactors (SBRs). Although long-term exposure to PS particles (up to 200 mg/L MPs and 50 mg/L NPs) did not compromise the macro-performance of the SBRs (with COD, $\text{NH}_4^+\text{-N}$, and TP removal efficiencies remaining >90%), the physical and biological integrity of the sludge was significantly altered. Sludge volume index (SVI) analysis revealed a decline in settleability, particularly under NP stress, which was corroborated by laser scanning confocal microscopy (LSCM) and transmission electron microscopy (TEM) observations showing the internalization of NPs into microbial cells. Physiologically, PS NPs induced higher levels of reactive oxygen species (ROS) and lactate dehydrogenase (LDH) release, indicating substantial oxidative stress and membrane damage. Furthermore, high-throughput sequencing and qPCR analysis demonstrated that PS particles reshaped the microbial community, enriching the antibiotic-resistant genus *Acinetobacter* and various antibiotic resistance genes (ARGs) such as *sulIII* and *tetG*. Notably, PS NPs exhibited a more pronounced effect on ARG dissemination than MPs, even at lower mass concentrations, by facilitating horizontal gene transfer (HGT) through increased cell membrane permeability. These findings elucidate the distinct toxicological mechanisms of NPs in biological treatment systems and highlight their role as catalysts for the environmental spread of antibiotic resistance.

Keywords: microplastics; nanoplastics; activated sludge; reactive oxygen species; antibiotic resistance genes



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1. Introduction

The widespread application of plastics across industrial, agricultural, and domestic sectors is driven by their chemical stability and convenience [1]. As the demand for plastic

products continues to grow, global plastic production reached 360 million tons in 2018 [2], with projections indicating it could double in the coming decades [3]. However, the majority of plastics cannot be effectively recycled, resulting in significant amounts of plastic waste being released into the environment [4]. Concurrently, larger plastic fragments and fibers undergo biological, mechanical, or chemical degradation, breaking down into smaller particles known as microplastics (MPs; <5 mm) and nanoplastics (NPs; 1–1000 nm) [5]. Due to their durability and resistance to degradation, MPs pose long-term ecological risks, including ingestion by biota, trophic transfer through food webs, and acting as vectors for hydrophobic organic pollutants and pathogenic microorganisms, and have garnered significant attention from researchers worldwide [6,7].

As the primary barrier against environmental pollutant discharge, wastewater treatment plants (WWTPs) act as major sinks for MPs and NPs derived from personal care products, packaging, and other domestic sources [8–10]. While secondary biological treatment processes can achieve removal rates exceeding 90% through adsorption onto activated sludge, this accumulation exerts significant pressure on the microbial ecosystem [11–13]. Previous studies have indicated that MPs can inhibit the abundance of nitrifying and denitrifying bacteria, potentially compromising nitrogen removal [13–15]. However, the comparative toxicological impacts of MPs versus NPs remain poorly understood, particularly in sequencing batch reactors (SBRs). Compared to MPs, the smaller size and larger specific surface area of NPs facilitate enhanced bioavailability and cellular interactions, allowing them to penetrate biological barriers and interact with intracellular components, inducing more pronounced microbial toxicity [16,17].

Antibiotics have been consistently detected in multiple stages of WWTPs [18], and these plants are recognized as a significant source of antibiotic resistance genes (ARGs), which are amplified by the presence of antibiotics [19]. Due to their large surface area, MPs can serve as substrates for microbial colonization, promoting biofilm formation and creating a microhabitat known as the “Plastisphere” [20]. In the plastisphere, horizontal gene transfer (HGT) among microbial communities is enhanced, facilitating the transfer of ARGs from antibiotic-resistant bacteria (ARB) to other bacteria that are not resistant to antibiotics [21]. For instance, Arias-Andres et al. [22] demonstrated that HGT of antibiotic resistance plasmids in the plastisphere occurred at a frequency three orders of magnitude higher than in planktonic communities. Similarly, Wu et al. [23] found that polyvinyl chloride MPs in the plastisphere significantly enriched ARGs and ARB. However, while studies have primarily focused on the transfer and abundance of ARGs within the plastisphere, the effects of MPs versus NPs on ARG distribution in activated sludge, particularly in SBRs, remain underexplored. Furthermore, there is an urgent need to elucidate the distinct mechanisms by which MPs and NPs influence ARG distribution in these systems.

In this study, we aim to investigate the impacts of micron- and nano-sized polystyrene (PS) particles on activated sludge in SBRs. PS MPs and NPs were selected due to their widespread use in plastic products and frequent presence in WWTPs [24]. We systematically evaluated the operational stability of SBRs, sludge settleability, and microbial physiological responses (ROS, ATP, and LDH). Crucially, laser scanning confocal microscopy (LSCM) and transmission electron microscopy (TEM) were employed to visualize the subcellular distribution and internalization of PS NPs. Furthermore, the correlation between community succession, membrane damage, and the abundance of ARGs and mobile genetic elements (MGEs) was elucidated. This study aims to provide a mechanistic understanding of how nanoscale plastics escalate biosafety risks in biological wastewater treatment systems by bridging the gap between physical internalization and genetic dissemination.

2. Materials and Methods

2.1. Chemicals and Activated Sludge

The sodium acetate (NaAc), ammonia chloride (NH_4Cl), monopotassium phosphate (KH_2PO_4) and anhydrous calcium chloride (CaCl_2) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). The micron-sized PS (100–200 μm) was purchased from Rongfu Technology Co., Ltd (Suzhou, China), according to the common dimensions of MPs detected in the WWTPs [25]. The PS NPs (80–100 nm) with green fluorescence attributes were purchased from Ruige Technology Co., Ltd (Suzhou, China). As shown in Figure 1, the sizes of PS MPs/NPs (PS: 100–200 μm , NPs: 80–100 nm) were verified by a scanning electron microscope (SEM) and TEM. The purity of standards was >98%. The activated sludge fed to the SBRs was gathered from a sludge thickener at a municipal WWTP located in Jiangsu Province, China.

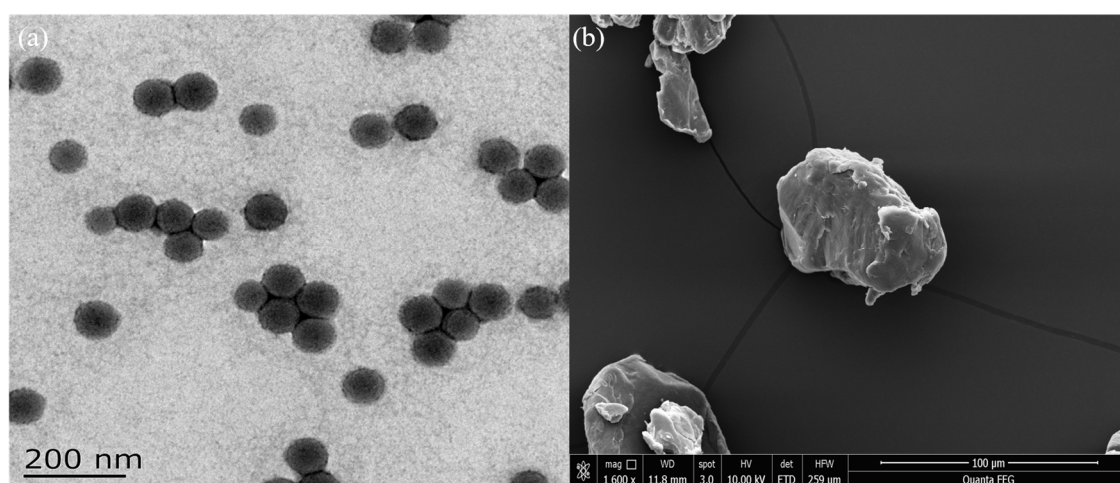


Figure 1. Images of (a) PS-NPs and (b) PS-MPs obtained by the TEM and SEM, respectively.

2.2. Sequencing Batch Reactor (SBR) Long-Term Culture Experiment

To investigate the effects of PS MPs/NPs on activated sludge in SBRs, four parallel reactors were established. The influent in all SBRs simulated urban wastewater, with an experimental duration consisting of 5 days of acclimatization without pollutant addition, followed by 40 days of pollutant exposure. During the exposure phase, the reactors were designated as Control, PS-1, PS-2, and PS-Nano, with 0, 50, and 200 mg/L micro-PS and 50 mg/L PS NPs added, respectively (Table 1). The high concentration of 200 mg/L micro-PS was based on previous studies [14], which indicated that MPs can accumulate in wastewater treatment processes at elevated concentrations. To ensure accurate evaluation, the PS particles were added to the reactors in a single dose. The operation cycle of SBRs was set as 6 h, which consisted of flooding water (10 min), stirring (75 min), aeration (225 min), settling (30 min), drainage (10 min) and disengagement (10 min). The time switches were used to convert different running phases. Moreover, the concentration of dissolved oxygen (DO) was controlled in the range of 0.3–0.5 mg/L and 3–4 mg/L in the stirring and aeration periods, respectively. The SBRs were built as cylinders measuring 15 cm × 30 cm in diameter and height, respectively, with a 3 L effective experiment volume. The operating temperature was maintained at 25 ± 2 °C, and the pH was maintained at 7.0–7.5.

The influent composition for all SBRs was consistent, containing carbon (NaAc), nitrogen (NH_4Cl), and phosphorus (KH_2PO_4) sources (Table 1), along with other essential elements (Table S1). The chemical oxygen demand (COD) of the influent was set to 300 mg/L, in line with concentrations commonly found in WWTPs in China [26]. The

nitrogen and phosphorus concentrations adhered to a COD:N:P ratio of 100:5:1. The food-to-microorganism (F/M) ratio was maintained above 0.2 gCOD/(gSS·d) to support normal activated sludge metabolism [27] (Equation (1)).

$$F/M = \frac{COD \times V_F}{MLSS \times V_R} \quad (1)$$

where the V_F is the daily feeding volume set as 8 L, and V_R is the reacting volume of SBRs set as 3 L; the concentration of mixed liquor suspended solids (MLSS) was maintained around 3 g/L.

Table 1. The composition and concentration of simulated influent and contaminants.

Reactors	PS (mg/L)	Size of PS (μm)	NaAc (mg/L)	NH ₄ Cl (mg/L)	KH ₂ PO ₄ (mg/L)
Control	0	0	385	85	13
PS-1	50	50–100	385	85	13
PS-2	200	50–100	385	85	13
PS-Nano	50	0.08–0.1	385	85	13

2.3. SBRs Operating State and Sludge Characteristic Analysis

The operational status of the SBRs was monitored through several indicators, including COD, NH₄⁺-N, total nitrogen (TN), and total phosphorus (TP), measured in both influent and effluent every two days according to standard methods [28]. Additionally, mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), and extracellular polymeric substances (EPS) were measured every eight days to track changes in activated sludge characteristics. These parameters are commonly used to assess the mass of activated microorganisms and sludge settling properties [29,30]. MLSS and MLVSS were determined following standard protocols [28], while EPS was extracted using a cation exchange resin (CER) method [31]. The contents of polysaccharides, humic acids, and proteins in the EPS were quantified using the anthrone colorimetric method [32] and the modified Lowry method [33], respectively. Further details are provided in the Supplementary Information.

2.4. Physiological Characterization of the Microorganism

To assess the impacts of PS MPs/NPs on activated sludge, reactive oxygen species (ROS), adenosine triphosphate (ATP), ammonia monooxygenase (AMO), and lactate dehydrogenase (LDH) were analyzed using double antibody sandwich enzyme-linked immunosorbent assays (ELISA), with kits from SenBeijia Biological Technology Co., Ltd (Nanjing, China). This method is based on specific antibody–antigen interactions, and detailed protocols can be found in the Supplementary Information.

2.5. Microbial Community and ARGs Analysis

Microbial community composition in the four SBRs was determined using Illumina MiSeq sequencing technology after 40 days of exposure. DNA was extracted from activated sludge samples using the E.Z.N.A.[®] Soil DNA Kit (Omega Biotek, Norcross, GA, USA), purified, and prepared for sequencing on the Illumina MiSeq platform (Biozonon, Shanghai, China). Methodological details are described in prior studies [34]. The abundance of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) in the activated sludge was quantified using a fluorescence-based quantitative polymerase chain reaction (qPCR) (TIB 8600, China) by Lingen Biotechnology Co., Ltd. (Shanghai, China). Five common ARGs and one MGE (sulfonamide-resistance genes *sulI* and *sulII*, tetracycline-resistance genes *tetG*, *tetQ*, and *tetW*, and integron *intI1*) [35] were analyzed to assess the

impacts of PS MPs/NPs on microbial resistance. Detailed procedures are provided in the Supplementary Information.

2.6. Distribution of PS NPs in Microbial Cells

To elucidate the effects of PS NPs on microbial cells, the distribution and internalization of PS NPs were examined using LSCM and TEM. The green fluorescence of PS NPs was visualized via LSCM (LEICA SP8, Mannheim, Germany), while detailed distribution patterns were captured using TEM (HITACHI HT7800, Tokyo, Japan), which offers a resolution of 0.1–0.2 nm. Prior to TEM analysis, microbial samples were fixed in 2.5% glutaraldehyde solution at 4 °C for 12 h. Further detection details are outlined in the Supplementary Information.

2.7. Statistical Analysis

All the tests were conducted in triplicate, and the significance of results was determined by analysis of variance. The visual Heatmap analysis was conducted in the TBtools (v1.098765) analysis module [36].

3. Results and Discussion

3.1. The Effluent and Sludge Characterization of the SBRs

Throughout the 40-day operation, SBR effluent quality remained stable with high removal efficiencies for COD, $\text{NH}_4^+\text{-N}$, TN, and TP (>90%), indicating that PS MPs/NPs did not immediately compromise baseline metabolic activities (Table 2). This resilience aligns with previous observations that nutrient removal is often maintained under plastic stress [37].

Table 2. The average concentration and removal rate of COD, $\text{NH}_4^+\text{-N}$, TN and TP in influent and effluent of different SBRs.

Category	Influent	Effluent			
		Control	PS-1	PS-2	PS-Nano
COD (mg O_2 /L)	321.21 ± 26.49	22.73 ± 2.92	21.72 ± 3.95	19.65 ± 5.63	20.02 ± 1.68
Removal efficiency (%)		92.85 ± 1.39	93.27 ± 0.78	93.83 ± 1.89	93.75 ± 0.59
$\text{NH}_4\text{-N}$ (mg/L)	25.72 ± 2.22	0.19 ± 0.05	0.15 ± 0.05	0.21 ± 0.06	0.14 ± 0.11
Removal efficiency (%)		99.25 ± 0.26	99.41 ± 0.22	99.17 ± 0.26	99.45 ± 0.43
TP (mg/L)	1.96 ± 0.06	0.01 ± 0.003	0.05 ± 0.002	0.06 ± 0.01	0.02 ± 0.008
Removal efficiency (%)		99.04 ± 0.51	97.64 ± 3.94	99.71 ± 0.15	99.01 ± 0.99
TN (mg/L)	24.61 ± 1.41	10.77 ± 0.82	10.19 ± 1.06	10.4 ± 1.17	10.98 ± 0.91
Removal efficiency (%)		56.26 ± 1.16	58.63 ± 2.05	57.83 ± 2.29	55.35 ± 3.4

In contrast to the stable effluent quality, the physical and biological integrity of the activated sludge exhibited significant sensitivity to PS particles (Figure 2). Initially maintained at 2500 ± 100 mg/L, the MLSS concentrations in the PS-1, PS-2, and PS-Nano groups declined significantly by 15%, 24%, and 23%, respectively, by the end of the experiment. Concurrently, MLVSS concentrations followed a similar downward trend; however, the MLVSS/MLSS ratios remained remarkably stable within the range of 75–80% across all groups. This consistency indicates that while PS exposure inhibits overall biomass accumulation and suppresses sludge production [38], the microbial community underwent a degree of acclimatization, maintaining a steady proportion of active biomass relative to the total suspended solids [39]. More critically, the sludge volume index (SVI) in the PS-2 and PS-Nano groups increased significantly (reaching 162 and 155 mL/g, respectively), signaling a deterioration in settleability. Notably, PS NPs (50 mg/L) induced comparable

toxicity to higher-dose MPs (200 mg/L). This enhanced impact of NPs is attributed to their larger specific surface area and smaller size, which facilitate closer adherence to microbial cells and more efficient penetration of biological barriers, ultimately disrupting microbial floc structures [15,40,41].

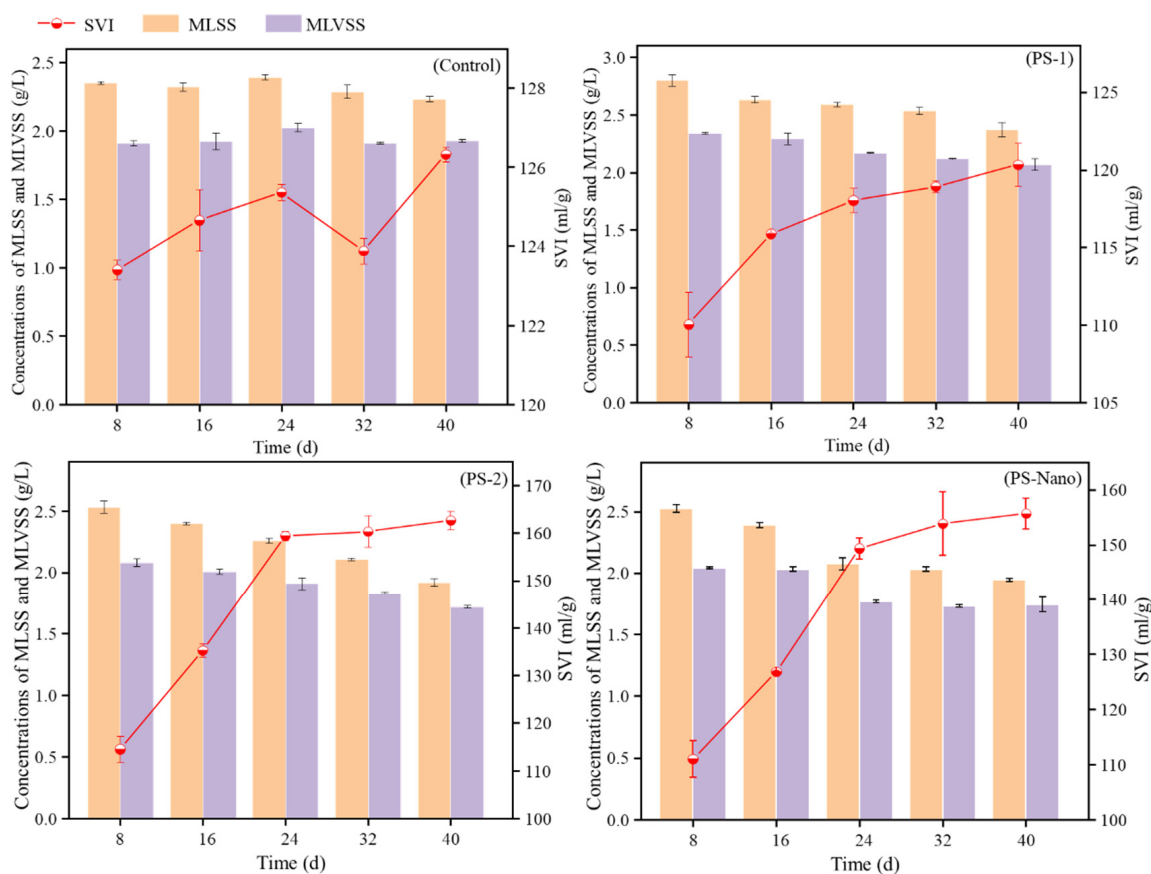


Figure 2. The variation in MLSS, MLVSS and SVI of activated sludge in different reactors (Control, PS-1, PS-2, and PS-Nano). Error bars are presented in the Figures ($n = 3$).

3.2. Cellular Distribution of PS NPs and Microbial Physiological Responses

While the macro-scale data (Section 3.1) suggest that NPs exert more severe physiological stress than MPs, the underlying interaction between these particles and microbial cells requires direct visual evidence. NPs are hypothesized to traverse cell membranes via endocytosis or direct translocation within microseconds, leading to structural disruption [42,43]. However, high-resolution imagery capturing this distribution within the complex activated sludge matrix is relatively scarce. In this study, LSCM and TEM were employed to elucidate the spatial and subcellular localization of PS NPs. LSCM analysis (Figure 3) revealed that green fluorescent PS NPs were uniformly dispersed throughout the sludge flocs, confirming their extensive integration into the microbial matrix. To further resolve the interaction at the single-cell level, TEM was utilized with a resolution of 0.2 μm (Figure 4). The micrographs provide compelling evidence that PS NPs associate with the extracellular polymeric substances (EPS) and successfully penetrate the microbial cell membranes. Once internalized, these NPs are positioned to directly interfere with intracellular organelles and metabolic pathways. This direct visualization confirms that the heightened toxicity of NPs observed in our SBRs, characterized by impaired membrane integrity, is a consequence of their physical entry into the cytoplasm [15].

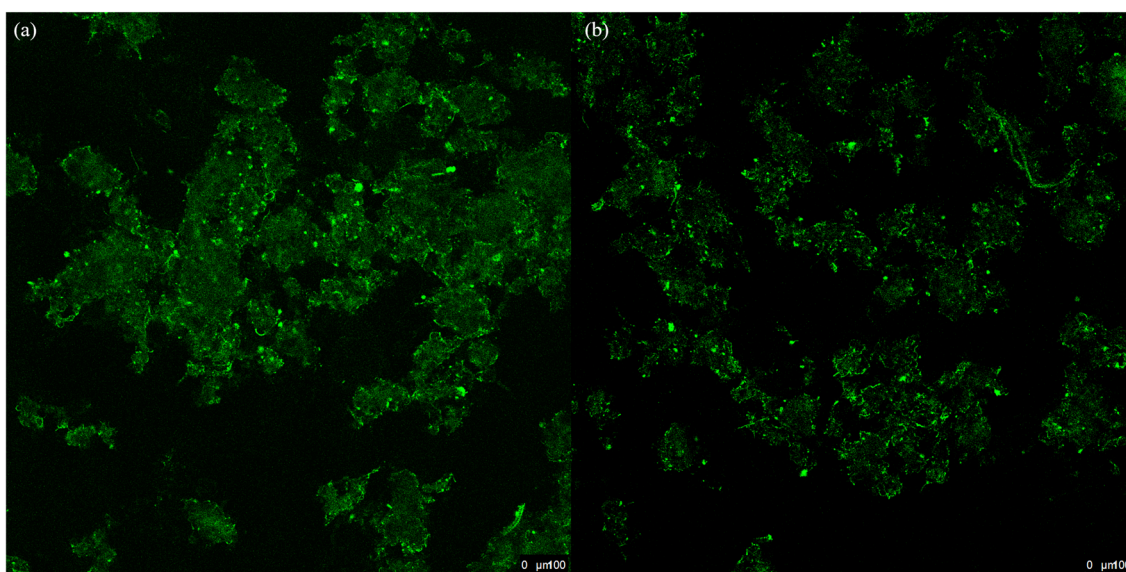


Figure 3. (a,b) Distribution of the PS NPs with green fluorescence after 40 days of exposure in the activated sludge presented by the LSCM.

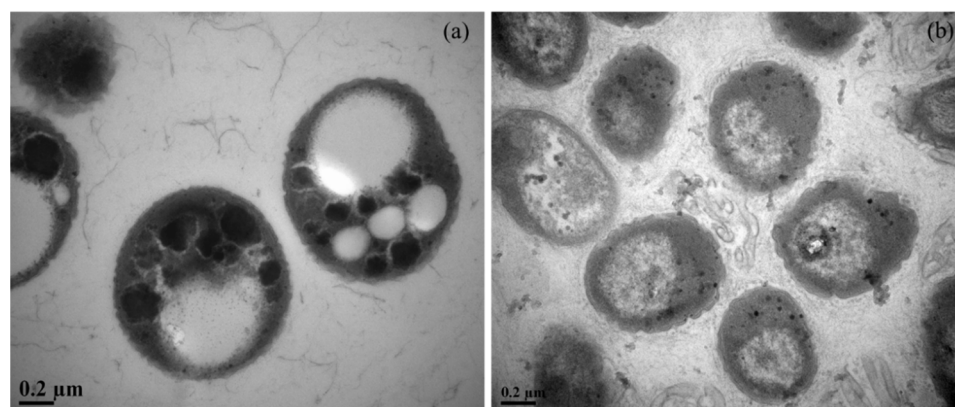


Figure 4. The microbial cells characteristic in (a) Control and (b) PS-Nano after 40 days of exposure presented by the TEM.

As discussed in previous sections, PS particles, notably their nanoparticle fraction, cause physical intrusion that elicits microbial defense mechanisms. EPS serve as the primary biochemical barrier, and their composition is a critical indicator of microbial aggregation and community stability [30,44]. Throughout the 40-day exposure, the total EPS mass in the PS-2 and PS-Nano reactors exhibited a sustained increase (Figure 5), contrasting with the decline observed in the control group. A more granular analysis of EPS components revealed that this increase was primarily driven by the overproduction of proteins (PN) and humic-like substances. By day 40, protein and humic acid levels in the PS-Nano group had surged by 24% and 159%, respectively, while those in the PS-2 group increased by 14% and 61%. In contrast, polysaccharide (PS) content remained relatively stable across all reactors. This shift in EPS composition reflects an adaptive metabolic strategy to mitigate plastic-induced toxicity. The stimulation of protein and humic acid synthesis serves a dual purpose: first, these hydrophobic components can effectively adsorb and sequester PS MPs/NPs, shielding the cell envelope from direct contact; and second, they facilitate the potential biodegradation of plastic fragments by creating a stabilized microenvironment [45,46]. Consistent with our microscopic observations, PS NPs exerted a more pronounced impact than MPs, inducing significantly higher EPS secretion. However, while this enhanced EPS production maintains cellular integrity, the resulting increase in

sludge viscosity and the altered protein-to-polysaccharide ratio likely contributed to the deterioration in SVI.

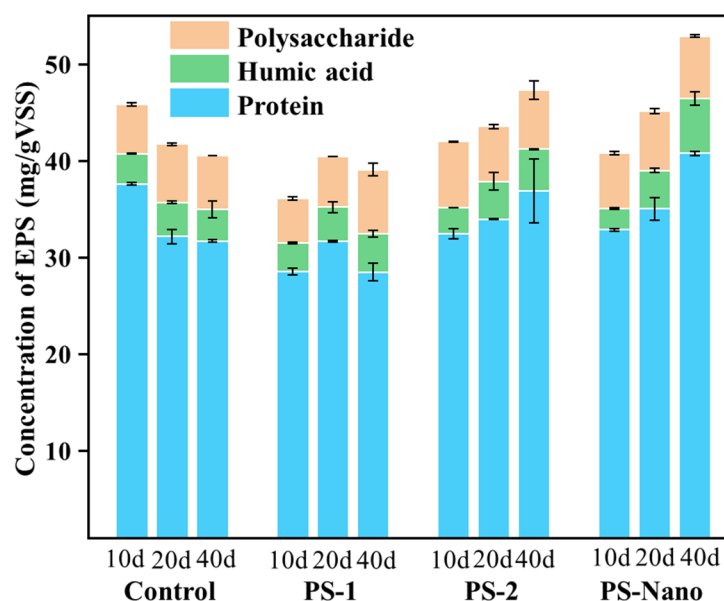


Figure 5. The variation in EPS of activated sludge in different reactors (Control, PS-1, PS-2, and PS-Nano). Error bars are presented in the Figures ($n = 3$).

3.3. Physiological and Metabolic Responses to PS-Induced Stress

The internalization of PS NPs into the microbial cytoplasm inevitably triggers a cascade of physiological and metabolic responses, which were quantified by evaluating key indicators of microbial activity and structural integrity. The cellular activity of microorganisms and structural integrity in the SBRs were evaluated through physiological indicators, including ROS, ATP, AMO, and LDH [47–49] (Table 3).

Table 3. The average abundance of AMO, LDH, ATP and ROS in activated sludge detected at the initiation and end of the exposure experiment.

Time	SBRs	Microbial Activity Index			
		AMO (U/L·gVSS)	LDH (U/L·gVSS)	ATP (nmol/L·gVSS)	ROS (ng/L·gVSS)
1 day	Control	12.66 ± 0.046	9.39 ± 0.023	24.786 ± 0.67	985.11 ± 24.81
	PS-1	11.54 ± 0.11	8.55 ± 0.41	21.56 ± 0.06	856.14 ± 22.72
	PS-2	12.99 ± 0.41	10.06 ± 0.047	26.34 ± 0.13	1007.56 ± 2.07
	PS-Nano	12.89 ± 0.27	9.95 ± 0.31	25.87 ± 0.33	977.93 ± 0.001
40 days	Control	14.32 ± 0.58	9.41 ± 0.23	27.69 ± 0.62	958.67 ± 26.92
	PS-1	12.13 ± 0.11	8.87 ± 0.36	24.46 ± 0.41	855.47 ± 31.06
	PS-2	14.54 ± 0.47	10.39 ± 0.17	27.30 ± 0.14	1010.13 ± 0.035
	PS-Nano	15.18 ± 0.074	10.64 ± 0.38	29.35 ± 0.14	1016.44 ± 1.55

The intrusion of PS particles, particularly NPs, initiated a distinct oxidative stress response. After 40 days of exposure, ROS levels in the PS-Nano and PS-2 groups increased by 4% and 0.3%, respectively, while the control group exhibited a decline. This elevation in ROS is a direct consequence of the enhanced reactivity and membrane permeability of PS NPs, which disrupt the balance between oxidative and antioxidative processes [38]. Such excessive ROS generation typically leads to the oxidation of cellular lipids and proteins, resulting in membrane damage [50,51]. This was corroborated by the LDH results, a sensitive marker of membrane compromise, which reached their peak in the PS-Nano

reactor. The elevated LDH release confirms that the physical penetration of NPs leads to the dissociation or breakage of microbial cell membranes [52].

Interestingly, despite the localized membrane damage, the overall metabolic activity remained resilient. ATP concentrations and AMO activities increased across all SBRs, suggesting that the microbial community successfully maintained, or even enhanced, its energy production and nitrification capacity through acclimatization. This explains the stable COD and $\text{NH}_4^+\text{-N}$ removal efficiencies. However, the observed membrane permeability and oxidative stress likely served as a metabolic trigger. To counteract these detrimental effects and prevent further cellular infiltration, the microbial community was compelled to alter its extracellular matrix, transitioning to the protective role of EPS, as discussed in the previous section.

3.4. Impact on Microbial Community Diversity and Structure

Microbial community structure is a vital indicator for evaluating the metabolic activity and stability of SBR systems. To thoroughly investigate the impact of PS particles on microbial ecology, high-throughput sequencing was employed to analyze the microbial communities across different reactors (Table 4). The results revealed that after the 40-day exposure period, the number of Operational Taxonomic Units (OTUs) in the PS-Nano group reached its minimum at 988. Furthermore, diversity index analysis indicated that community structures underwent significant changes as PS concentration increased and particle size decreased. Specifically, the Shannon index in the PS-2 and PS-Nano reactors exhibited a downward trend, while the Simpson index increased accordingly. These data clearly demonstrate that the presence of high-concentration microplastics (200 mg/L) and nanoplastics (50 mg/L) reduced both the species richness and diversity of the activated sludge microbial community.

Table 4. Richness and diversity estimators of the microorganisms in SBRs.

Reactors		Similarity 0.97					
		Reads	OTUs	Chao	Coverage	Shannon	Simpson
SBR 40 d	Control	26,925	1000	1145	0.9928	5.41	0.0141
	PS-1	44,594	1115	1191	0.9969	5.57	0.0113
	PS-2	45,003	1082	1160	0.9968	5.32	0.0159
	PS-Nano	27,255	988	1170	0.9927	5.34	0.0159

At the phylum level (Figure 6a), the microbial compositions across all reactors displayed a degree of similarity. *Proteobacteria* (48.9–57.2%) and *Bacteroidota* (23.0–29.3%) were the absolute dominant phyla in all reactors. Other major phyla included *Myxococcota* (5.2–6.2%), *Chloroflexi* (1.8–2.5%), *Acidobacteriota* (1.6–3.7%), and *Patescibacteria* (2.7–3.2%). Despite minor variations in the composition of major phyla, significant differences in community succession were observed at the genus level (Figure 6b). Notably, core functional genera associated with nutrient removal, such as *Dechloromonas*, *Haliangium*, *Prostheobacter*, *Thauera*, and *Nitrospira*, maintained relatively stable abundances across all reactors. This finding is highly consistent with the high $\text{NH}_4^+\text{-N}$ and TP removal efficiencies observed previously.

However, the experiment observed a significant enrichment of *Acinetobacter* in the PS-2 and PS-Nano sludge. As a well-recognized typical antibiotic-resistant bacterium (ARB), the proliferation of *Acinetobacter* may be linked to the survival pressure induced by PS particles or the specific microenvironment they provide. This alteration in community structure not only reflects the microbial response to plastic pollution but also establishes a potential host foundation for the subsequent enrichment of ARGs.

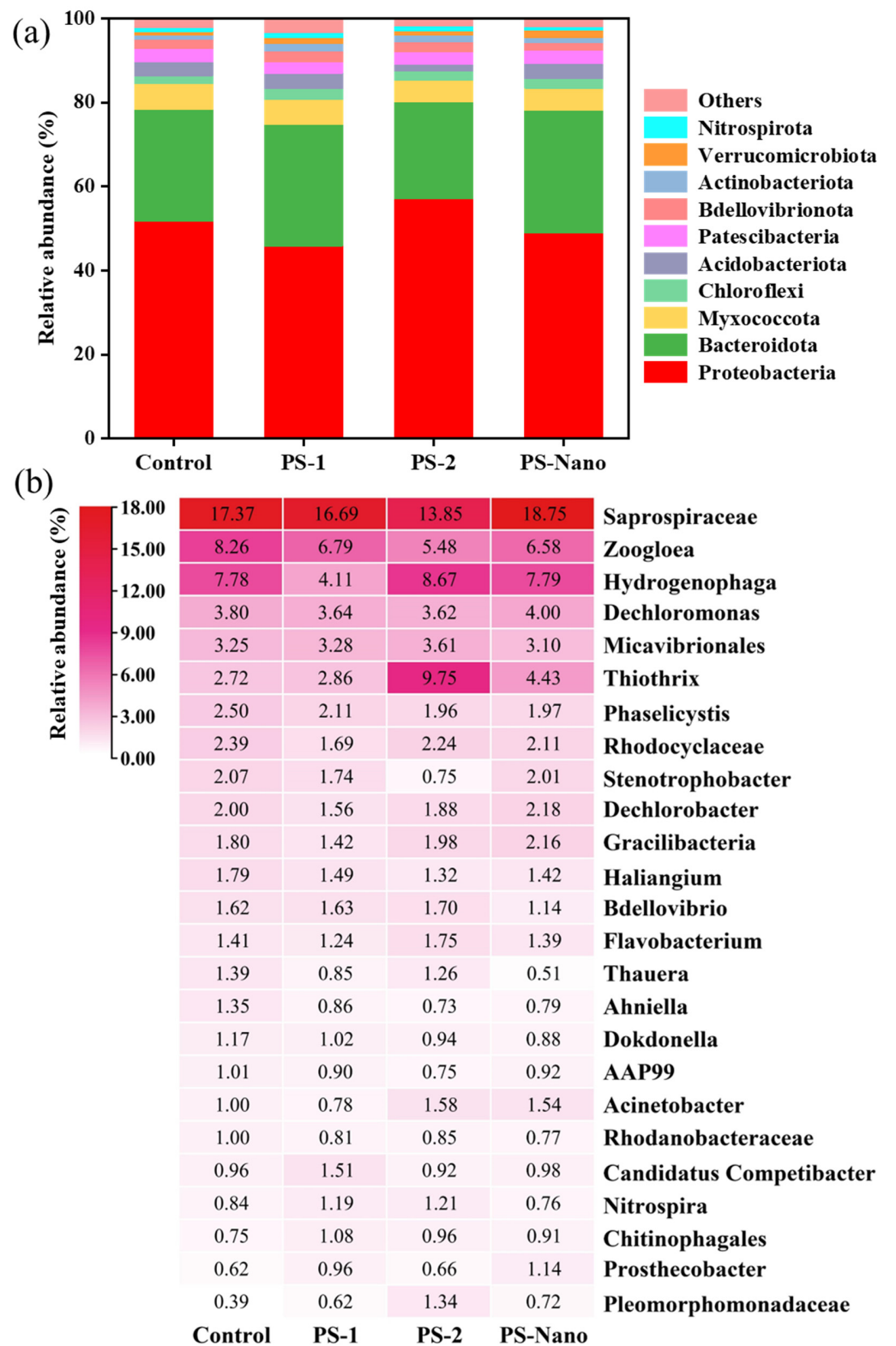


Figure 6. (a) Bacterial community composition at the phylum level and (b) genus level (taxa with an abundance of >1% in at least one sample).

3.5. Quantification and Dissemination Mechanisms of ARGs and MGEs

The presence of common sulfonamide-resistance genes (*sulI* and *sulIII*), tetracycline-resistance genes (*tetG*, *tetQ*, and *tetW*), and mobile genetic elements (MGEs, specifically *intI1*) was quantified using qPCR (Figure 7). The results demonstrated that these ARGs and MGEs were significantly enriched in the PS-2 and PS-Nano reactors. Specifically, the

average abundance of *sulII* in the PS-Nano sludge reached 1.21×10^8 copies/g, which was approximately 1.95 times higher than that in the control. Similarly, *tetG* abundance in the PS-Nano group exceeded that of all other SBRs. In the PS-2 group, *tetQ* and *tetW* were notably enriched, with *tetW* levels 1.22 times higher than the control. The simultaneous elevation of *intI1* abundance, particularly in the PS-Nano sludge, suggests that higher MGE levels act as a catalyst for horizontal gene transfer (HGT) among the bacterial community. This supports the hypothesis that PS particles serve as vectors that facilitate the recruitment and dissemination of ARGs. Overall, PS NPs induced a more pronounced enrichment of ARGs and greater genomic risk compared to PS MPs. This phenomenon can be explained by a synergistic mechanism: first, the enrichment of *Acinetobacter* provides a robust genetic reservoir of ARGs; second, as demonstrated in Section 3.3, PS NPs induce higher ROS generation and LDH release. This resulting membrane damage increases cell permeability and lowers the physical barriers for gene translocation. Therefore, by penetrating microbial cells and accelerating the transport of genetic material, PS NPs significantly aggravate the dissemination of antibiotic resistance in SBR systems, even at relatively low mass concentrations. The recent research also reported that compared with pristine PS NPs, carboxyl- and amino-modified PS NPs significantly facilitate the ARG transformation frequency and efficiency [53], and the PS NPs stimulated a marked proliferation of key pathogenic ARG-carrying taxa in soil [54]. Thus, in the future, investigating the surface-modified NPs environmental impacts with chronic exposure and studying ecological hazards of NPs in different environmental media, which should be further studied as the research hotspots.

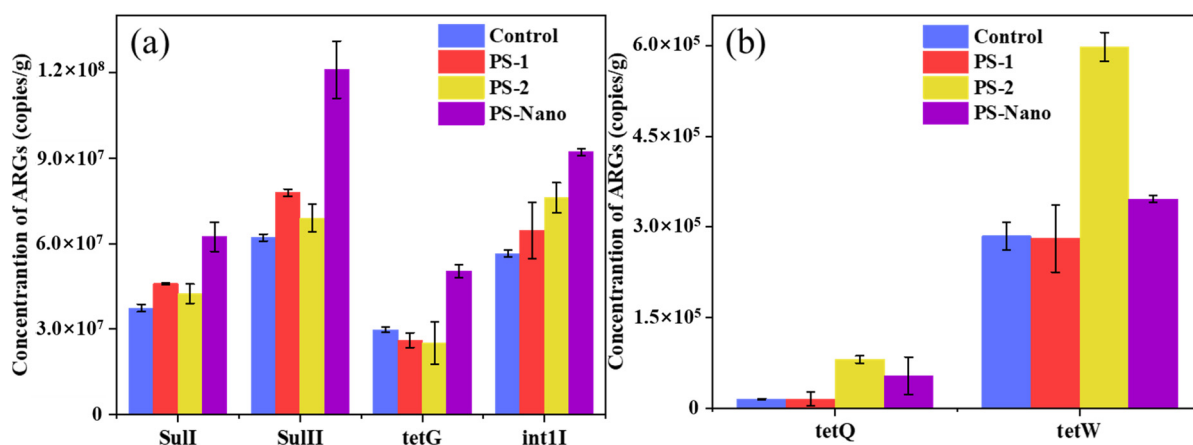


Figure 7. The relative abundance of ARGs and MGE ((a): *sulI*, *sulIII*, *tetG* and *intI1*; (b): *tetQ* and *tetW*) in activated sludge from the SBRs. Error bars are presented in the Figures ($n = 3$).

4. Conclusions

This study provides a comprehensive assessment of the differential impacts of PS MPs and NPs on the performance, physiology, and genetic risks of activated sludge in SBR systems. Regarding macro-stability versus micro-alterations, while the SBRs maintained stable pollutant removal efficiencies, PS particles, especially NPs, inhibited biomass accumulation and led to a notable increase in SVI (up to 42%), indicating a decline in sludge settleability during extended exposure. Direct visual evidence from LSCM and TEM confirmed size-dependent internalization, as PS NPs penetrated microbial cell membranes and accumulated in the cytoplasm, triggering a cascade of physiological responses including elevated ROS generation and LDH release that were more prominent than those induced by micron-sized PS. In response to this plastic-induced stress, the microbial community exhibited an adaptive defense mechanism by altering its EPS composition, specifically

increasing the secretion of proteins and humic-like substances to sequester particles and shield cellular structures. Furthermore, both PS MPs and NPs served as selective stressors that reduced microbial diversity and favored the enrichment of ARB such as *Acinetobacter*, which led to a substantial increase in the absolute abundance of ARGs (*sulI*, *sulIII*, *tetG*, etc.) and mobile genetic elements (*intI1*). PS NPs were identified as more effective catalysts for ARG dissemination than MPs, an effect attributed to a synergistic mechanism where NP-induced membrane damage creates pathways that facilitate horizontal gene transfer (HGT) among enriched ARB hosts. Overall, these results underscore that while nanoplastics may not immediately disrupt the chemical treatment efficiency of WWTPs, they alter the microbial ecosystem and escalate the biosafety risks associated with the environmental dissemination of antibiotic resistance.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/w18172142/s1>. The supplementary materials contain the details of EPS extraction and determination, the physiological characterization of the microorganism, ARG analysis, and the distribution of PS NPs; Table S1: The compositions and concentrations of trace element reserve solutions; Table S2: Primers for common ARGs (*sulI*, *sulIII*, *tetG*, *tetQ* and *tetW*) and MGE (*intI1*); Table S3: Comparing our key findings with recent literature on MP/NP effects on activated sludge systems; Figure S1: Main compositions of the SBRs built in the exposure experiments. Figure S2: The concentrations of COD, $\text{NH}_4^+\text{-N}$, TN and TP in influent and effluent of different SBRs.

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