Protection Mechanisms of Periphytic Biofilm to Photocatalytic Nanoparticle Exposure

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ABSTRACT: Researchers are devoting great effort to combine photocatalytic nanoparticles (PNPs) with biological processes to create efficient environmental purification technologies (i.e., intimately coupled photobiocatalysis). However, little information is available to illuminate the responses of multispecies microbial aggregates against PNP exposure. Periphytic biofilm, as a model multispecies microbial aggregate, was exposed to three different PNP s (CdS, TiO2, and Fe2O3) under xenon lamp irradiation. There were no obvious toxic effects of PNP exposure on periphytic biofilm as biomass, chlorophyll content, and ATPase activity were not negatively impacted. Enhanced production of extracellular polymeric substances (EPS) is the most important protection mechanism of periphytic biofilm against PNP exposure. Although PNP exposure produced extracellular superoxide radicals and caused intracellular reactive oxygen species (ROS) accumulation in periphytic biofilm, the interaction between EPS and PNP s could mitigate production of ROS while superoxide dismutase could alleviate biotic ROS accumulation in periphytic biofilm. The periphytic biofilms changed their community composition in the presence of PNP s by increasing the relative abundance of phototrophic and high nutrient metabolic microorganisms (families Chlamydomonadaceae, Cyanobacteriacea, Sphingobacteriales, and Xanthomonadaceae). This study provides insight into the protection mechanisms of microbial aggregates against simultaneous photogenerated and nanoparticle toxicity from PNP s.

INTRODUCTION

Processes based on photocatalyst nanoparticles (PNPs) are playing critical roles in several fields including disinfection of bacteria,1 energy harvesting,2 electrochemical reactions,3 and environmental remediation via advanced oxidation/reduction processes (AO/RP).4 Researchers also exploit strategies to combine PNP based technology with biological processes (i.e., intimately coupled photobiocatalysis) to improve contaminant removal efficiency.5 However, the biological compatibility of PNP s must be taken into consideration to avoid microorganisms being attacked or killed.5–7 CdS, TiO2, and Fe2O3 PNP s have shown great application potential in combination with various biological processes,8,9 such as ammonia synthesis from nitrogen,10 removal of organic pollutants in wastewater,5 and microbial photoelectrochemical system.5 Due to large-scale uses, it is necessary to investigate their potential environmental impacts and possible effects on biological systems under sunlight irradiation.
Traditionally, PNPs have been investigated in disinfection of single species microorganisms or biofilm including bacteria (E. coli\textsuperscript{11}), fungi (Fusarium sp.\textsuperscript{12}) and algae (Microcystis aeruginosa\textsuperscript{13}) under sunlight illumination. These studies confirmed the antimicrobial mechanisms of PNPs on single species microorganisms or simple microbial communities, including DNA damage, oxidative stress by reactive oxygen species (such as superoxide radicals and hydroxyl radicals), or membrane destruction. Recently, researchers have started to investigate the toxic effects of nanoparticles (including PNPs) on multispecies microbial aggregates that are the form of most microorganisms in nature.\textsuperscript{14–16}

Periphytic biofilm, a typical microbial aggregate in aquatic ecosystem (such waterways in paddy fields and wetland), has a complex community composition, a complete food chain, and abundant EPS.\textsuperscript{17} Periphytic biofilms have been demonstrated to play a major role in the environmental behaviors of nanoparticles (NPs) in environmental systems, including NP accumulation,\textsuperscript{18} biotransformation,\textsuperscript{19} and transfer through food chain.\textsuperscript{20} The characteristics of microbial aggregates, such as complex community composition and abundant EPS, could also result in complex responses to NPs for periphytic biofilm which is different from the study on single species populations.\textsuperscript{15,21–23} Periphytic biofilm display versatile responses (including enzyme activity, production of extracellular polymeric substances (EPS), photosynthesis) to NP (Ag, Fe\textsubscript{2}O\textsubscript{3}, and CeO\textsubscript{2}) or heavy metal ion (Cd\textsuperscript{2+}, As\textsuperscript{3+}, and Pb\textsuperscript{2+}) intrusion.\textsuperscript{15,24} Multispecies microbial aggregates constantly adapt their population fitness to increase their tolerance to NPs through their complex population structure and corresponding interspecies interactions.\textsuperscript{15,24,26} When exposed to TiO\textsubscript{2}, Ag, and CeO\textsubscript{2} NPs, multispecies microbial aggregates showed significant increases in the α diversity of bacterial communities.\textsuperscript{16,22,27} Our previous studies focused on the responses of periphytic biofilm to NP exposure demonstrating the important role of EPS in defending against nanotoxicity.

Although these studies have demonstrated the responses of multispecies microbial aggregates (e.g., periphytic biofilm) to NP exposure from a physiological and ecological view, there is little information about the relationship between biological responses and protection mechanisms against exposure to NPs (e.g., PNPs). Compared with other NPs, PNPs can induce more oxidative stress to microorganisms due to their photoresponses under suitable wavelengths of light irradiation.\textsuperscript{28} Numerous studies have correlated the band gaps of metal oxide NPs to their capacity to generate oxidative stress\textsuperscript{29,30} and thus toxicity to cells.\textsuperscript{31,32} However, little information is available to evaluate the influence of the band gaps of PNPs on their toxicities to multispecies microbial aggregates (periphytic biofilm) under light irradiation. Most importantly, how the multispecies microbial aggregate (periphytic biofilm) protects itself from the stress of PNP exposure remains unclear.

In this study, TiO\textsubscript{2}, CdS, and Fe\textsubscript{2}O\textsubscript{3} PNPs were chosen to evaluate their toxic effect on periphytic biofilm. The objectives of this study were to (i) explore the distribution of PNPs in the periphytic biofilm matrix, (ii) evaluate the influence of PNPs on periphytic biofilm, and (iii) explore the protection mechanisms of periphytic biofilm in defending against PNPs exposure. This study is expected to provide new insight into the protection mechanisms of multispecies microbial aggregates against PNPs.

### MATERIALS AND METHODS

#### Preparation of Periphytic Biofilm and PNP Suspensions

Periphytic biofilm, originated from Xuanwu Lake, Nanjing, China, was inoculated into our biofilm culture systems with WC medium (see Supporting Information).\textsuperscript{21} CdS, TiO\textsubscript{2}, and Fe\textsubscript{2}O\textsubscript{3} PNPs (uncoated) used in this study were purchased from Aladdin, China. The primary particle sizes based on TEM images of CdS and TiO\textsubscript{2} PNPs were 36.7 ± 3.5 nm and 42.6 ± 5.8 nm, while the width and length of Fe\textsubscript{2}O\textsubscript{3} PNPs were 9.9 ± 0.6 nm and 72.4 ± 7.5 nm, respectively. The stock solutions of individual PNPs were prepared by adding 100 mg of respective PNPs to 100 mL deionized water, shaking vigorously under ultrasonic treatment (300 W, 100 Hz) for 30 min, and then diluting to 1000 mL in a volumetric flask. Before preparing the stock solution of PNPs, the PNPs were sterilized using UV irradiation (low-pressure UV lamp, UVC15W/T8, CREATOR, China) for 1 h in clean benches. The distilled water for solution preparation was sterilized by autoclave sterilization. All the experimental operations for solution preparation before sealing were carried out under sterilized conditions.

#### Periphytic Biofilm Exposure to PNPs

The 14 day old periphytic biofilms were exposed to PNP suspensions for 7 days (for exposure concentration of PNPs, see Supporting Information, Table S1). The 7 day period was based on a 21 day periphytic biofilm lifecycle according to our previous study.\textsuperscript{33} Periphytic biofilm was collected from our biofilm culture systems by a sterilized silicone spatula for the following experiments. All the periphytic biofilms were washed three times using sterilized 0.9% NaCl solution and centrifuged before each experiment. Then, 20, 50, and 500 mL of PNP stock solution and 1 mL WC media stock solution (1000 times concentration of the culture medium) were put in 1000 mL volumetric flasks and diluted by distilled water to obtain the final required concentration of PNPs (see Supporting Information, Table S1). Then the required concentration PNP solutions containing WC medium were obtained, and 50 mL of the PNP solutions were added into 150 mL flasks. Then 1.0 g of periphytic biofilm (after centrifugation) was added into every flask. The control flask contained no PNPs. All flasks were then sealed with sterilized parafilm to allow for gas exchange and placed in an incubation room with a standard light–dark cycle of 12 h/12 h (xenon lamp, 150 W) at 28 ± 1°C. Both the control and each treatment consisted of six replicates. After a 7 day exposure, the biomass, concentration of chlorophyll, and EPS productivity were measured to evaluate the influence of the PNPs on periphytic biofilm growth. The analytical methods to measure biomass, concentration of total chlorophyll content, and EPS were detailed in Supporting Information.

#### Characteristics of EPS and PNPs

Two EPS extraction methods were adopted to evaluate productivity of EPS and content of metals in the EPS and prepare periphytic biofilm samples for the ROS accumulation experiments (details provided in Supporting Information). To determine the distribution of PNPs in periphytic biofilm system, metal content in different fractions (metals in solution, loosely bound metals, and irreversibly bound metals) were detected by ICP-MS (7700x-JP1250221S, Agilent Technologies, USA) (details provided in Supporting Information). Electron spin resonance (ESR) analysis (EMX 10/12, Bruker, Germany) was performed to detect the ROS spectra in the PNPs/EPS system.
under xenon lamp irradiation (see Supporting Information). The morphologies of PNPs were investigated using transmission electron microscope (TEM, Zeiss 900, Carl Zeiss, Germany) operating at 200 kV after exposure to WC medium and EPS. Hydrodynamic diameters of PNPs in WC medium or EPS extracted from periphytic biofilm were measured using a Zetasizer (90PLUS PALS, NanoBrook, USA). The components of PNPs were determined using X-ray diffraction (XRD) (LabX, XRD-6100, Japan) with Cu Kα radiation (λ = 1.54056 nm) at a power of 40 keV × 30 mA. The XRD data were recorded with 2θ varying from 20° to 60° at counting time of 10 s and a scanning mode with a step size of 0.02°.

**Characteristics of Periphytic Biofilm.** The production of reactive oxygen species (ROS) by periphytic biofilm caused by PNPs in the presence and absence of EPS was also detected. The superoxide dismutase (SOD) activity and adenosine triphosphatase (ATPase) activity were measured to evaluate the response of the periphytic biofilm to PNPs exposure. The distribution of PNPs in the periphytic biofilm matrix was also studied using scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM, JEOL Co, Ltd., Japan; EDX, Oxford Instruments, UK) and TEM (Zeiss 900, Carl Zeiss, Germany). To evaluate the influence of PNPs on community composition and diversity of periphytic biofilm, high-throughput sequencing of the 16S rDNA gene of EPS extracted from periphytic biofilm was also performed. All the detailed methods are provided in Supporting Information.

**Statistics.** Principal component analysis (PCA) was used to analyze the genus-level OTU-table from high-throughput sequencing to investigate variation of community composition of periphytic biofilm in the presence of PNPs. The detected biological and phycological parameters of periphytic biofilm (productivity of EPS, activities of ATPase and SOD, concentrations of ROSs and chlorophyll, and distribution of PNPs in solution or in EPS) were used as the matrix for canonical correspondence analysis (CCA) to distinguish their relationships with community composition of periphytic biofilm (represented by the genus-level OTU-table). PCA and CCA were calculated in the vegan package by R software. The t-tests were used to evaluate whether the differences between the treatments and control were statistically significant, unless otherwise specified. The probability p value was set at 0.05 level for all analyses. All figures were prepared using Origin 9.0 software.

**RESULTS**

**Characteristics of Photocatalytic Nanoparticles.** The XRD patterns showed the existence of CdS with hexagonal phase which was demonstrated by diffraction peaks at 2θ = 26.74, 43.84, and 52.38 degrees (Figure S1A). TiO₂ crystals with the anatase phase were proved by the diffraction peaks at 25.40, 37.96, 48.16, 54.10, and 55.23 (Figure S1B). The α-Fe₂O₃ crystal was confirmed by the diffraction peaks at 33.24, 35.77, 40.89, and 54.16 for Fe₂O₃ PNPs (Figure S1 C).

The TEM images and the corresponding primary particle sizes of PNPs in WC medium and EPS are shown in Figure S2. Results showed that no statistically significant change (p > 0.05) was observed in primary particle size between PNPs in WC medium and EPS. If the primary particle size of PNPs is consistent regardless of the media used, the change of hydrodynamic diameters (AHD) could reflect the aggregation tendency of PNPs. The AHD of PNPs were 53.5 ± 9.8, 61.4 ± 8.1, and 76.3 ± 10.5 nm for CdS, TiO₂, and Fe₂O₃ in WC medium, respectively (Table S2). In the presence of EPS extracted from periphytic biofilm, the AHD of PNPs were 46.7 ± 8.5, 52.2 ± 7.7, and 68.1 ± 12.4 nm for CdS, TiO₂, and Fe₂O₃, respectively. There was also no significant difference (p > 0.05) in the hydrodynamic diameters between PNPs in WC medium and EPS. Therefore, combining with EPS did not show a significant influence on the aggregation of the PNPs.

Photocatalytic materials can produce reactive oxygen species (*OH and O₂⁻*) under suitable light irradiation which may pose oxidative stress on microorganisms. ESR tests demonstrated the presence of O₂⁻* represented by the three-line signal remaining the same proportionately in the CdS/EPS and TiO₂/EPS systems under Xe-lamp irradiation (Figure 1).

![Figure 1. ESR spectra of (A) TEMP O₂⁻* adducts in CdS–EPS systems irradiated for 1 min. (B) TEMP O₂⁻* adducts in TiO₂–EPS systems irradiated for 1 min.](image)

There was no *OH detected in any PNPs/EPS systems. Therefore, O₂⁻* might be the main ROS produced in the PNPs/EPS system. It should be noted that neither *OH nor O₂⁻* was detected in Fe₂O₃/EPS system (data not shown). However, Fe₂O₃ PNPs have been well demonstrated as photoreponse materials under suitable light irradiation (i.e., natural sunlight), which could pose oxidative stress on microorganisms. The low response to the stimulated sunlight irradiation or the low position of the conduction band may be...
the reason for the undetectable ROS in the Fe2O3/EPS system.39

**Biological and Physiological Effects on Periphytic Biofilm.** The biomass of periphytic biofilms were weighed after a 7 day exposure to evaluate the growth of periphytic biofilm in the presence of PNPs (Table 1). The average biomass in the control was 1.45 g after a 7 day cultivation. Biomasses were not statistically different (p > 0.05) in the presence of TiO2 (ranging from 1.55 ± 0.14 to 1.3 ± 0.06 g, p > 0.05) or Fe2O3 (ranging from 1.56 ± 0.09 to 1.37 ± 0.05 g, p > 0.05) and 2 mg L−1 of CdS (1.42 ± 0.13, p > 0.05). The presence of 5 mg L−1 CdS was beneficial to the growth of periphytic biofilm as the biomass increased to 1.86 g which was significantly higher (p < 0.05) than that in the control. However, the presence 50 mg L−1 of CdS was unfavorable to periphytic biofilm growth as the biomass significantly decreased to 0.96 g (p < 0.05).

The concentration of chlorophyll content was calculated to reflect the growing condition of the photosynthetic microorganisms (cornerstone species in periphytic biofilm) in the periphytic biofilm. Only high concentrations of CdS (>5 mg L−1) significantly decreased the chlorophyll content in comparison to the control (p < 0.05) while TiO2 and Fe2O3 and low CdS concentration did not produce a statistical difference (p > 0.05) in the concentration of chlorophyll content of periphytic biofilm (Table 1). The total production of EPS in the presence of the different PNPs is presented in Table 1. The presence of CdS (low concentration), TiO2, and Fe2O3 facilitated production of EPS. In particular, TiO2 and Fe2O3 (>2 mg L−1) and low concentration of CdS (2 and 5 mg L−1) significantly increased the productivity of EPS (p < 0.05).

The intracellular protein content of periphytic biofilm between control and PNPs treatments was not statistically different (p > 0.05), indicating that the increased EPS productivity was unlikely to be attributed to the increasing of cell numbers in periphytic biofilm (Figure S3).40 This result was consistent with previous studies, which also observed excessive production of EPS as a main response of microbial aggregates exposed to metal contaminations, especially for nanoparticles.15,17,24 However, the total amount of EPS significantly decreased in the presence of high concentrations of CdS (>5 mg L−1) compared to the control (p < 0.01).

The presence of PNPs often causes accumulation of reactive oxygen species (ROS) in microorganisms because PNPs have been proven to produce radical species, such as hydroxyl radicals (·OH) and superoxide radicals (O2·−), which cause strong oxidative stress to microorganisms.41 The presence of CdS, TiO2, and Fe2O3 resulted in significantly increased ROS accumulation of 160.3% (p < 0.01), 145.7% (p < 0.01), and 125.0% (p < 0.05), respectively, compared to the control in the presence of EPS (Figure 2A). The ROS accumulation was then detected after EPS removal from periphytic biofilm using heat treatment. The ROS accumulation significantly (p < 0.01) increased by 374.1%, 336.5%, and 194.3% for CdS, TiO2, and Fe2O3 respectively, compared to the control in the absence of EPS. Results also showed that heat treatment did not cause any significant difference in ROS accumulation in periphytic

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**Table 1. Biomass (g), Chlorophyll (mg g−1), and Total EPS (mg L−1) Productivity of Periphytic Biofilm in the Control and after Exposure to PNPs (CdS, TiO2, and Fe2O3).**

<table>
<thead>
<tr>
<th>treatments</th>
<th>biomass (g)</th>
<th>chlorophyll (mg g−1)</th>
<th>EPS (mg L−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>1.45 ± 0.07</td>
<td>2.7 ± 0.2</td>
<td>70 ± 6</td>
</tr>
<tr>
<td>CdS 2</td>
<td>1.42 ± 0.13</td>
<td>2.6 ± 0.1</td>
<td>81 ± 4</td>
</tr>
<tr>
<td>CdS 5</td>
<td>1.86 ± 0.08</td>
<td>2.4 ± 0.3</td>
<td>116 ± 7</td>
</tr>
<tr>
<td>CdS 50</td>
<td>0.96 ± 0.12</td>
<td>1.2 ± 0.1</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>TiO2 2</td>
<td>1.55 ± 0.14</td>
<td>2.8 ± 0.4</td>
<td>77 ± 6</td>
</tr>
<tr>
<td>TiO2 5</td>
<td>1.47 ± 0.11</td>
<td>2.6 ± 0.2</td>
<td>103 ± 9</td>
</tr>
<tr>
<td>TiO2 50</td>
<td>1.30 ± 0.06</td>
<td>2.5 ± 0.2</td>
<td>102 ± 8</td>
</tr>
<tr>
<td>Fe2O3 2</td>
<td>1.51 ± 0.15</td>
<td>2.5 ± 0.2</td>
<td>75 ± 7</td>
</tr>
<tr>
<td>Fe2O3 5</td>
<td>1.56 ± 0.09</td>
<td>2.8 ± 0.3</td>
<td>94 ± 5</td>
</tr>
<tr>
<td>Fe2O3 50</td>
<td>1.37 ± 0.05</td>
<td>2.3 ± 0.3</td>
<td>107 ± 8</td>
</tr>
</tbody>
</table>

*Mean value ± SD; n = 3. All the statistical significance between each treatment and control were calculated by t-test. The a represents nonsignificance between treatment and control (p > 0.05) while the b represents statistical significance between treatment and control (p < 0.05).
biofilm (Figure S4) and the presence of PNPs did not likely affect fluorescence measurement (Figure S4). Therefore, EPS may help mitigate ROS attacks of PNPs to protect microorganisms in periphytic biofilm matrices.

SOD is the key enzyme to mitigate attacks from ROS and is produced because of the oxidative stress caused by adverse environmental conditions.\(^{15-17}\) The SOD activity increased significantly in the CdS (429.5% higher than the control, \(p < 0.01\)), TiO\(_2\) (385.4% higher than the control, \(p < 0.01\)), and Fe\(_2\)O\(_3\) (263.6% higher than the control \(p < 0.01\)) treatments (Figure 2B). The metabolic activity of microorganisms (represented by activity of ATPase) in periphytic biofilm was then evaluated.\(^{15}\) The activity of ATPase was significantly enhanced in the presence of CdS, TiO\(_2\), and Fe\(_2\)O\(_3\) (increased by 141.0% (\(p < 0.01\)), 127.7% (\(p < 0.05\)), and 116.9% (\(p < 0.05\)), respectively) (Figure 2B). According to biological and phycological parameters, periphytic biofilm could still maintain its growth with CdS (2 and 5 mg L\(^{-1}\)), TiO\(_2\), and Fe\(_2\)O\(_3\) PNPs.

### Diversity and Community Composition of Periphytic Biofilm in the Presence of PNPs

The diversity of microorganisms in periphytic biofilm was analyzed by MiSeq sequencing technology. Results showed that exposure to TiO\(_2\) and CdS had negative impacts on all \(\alpha\) diversity (Shannon, Simpson indices) and richness indices (observed species, Chao 1) of periphytic biofilm (\(p < 0.05\)) compared to the control (Table 2). Thus, TiO\(_2\) and CdS PNPs had a significant negative effect on the microbial diversity of periphytic biofilm. Interestingly, the presence of Fe\(_2\)O\(_3\) significantly decreased the richness (observed species and Chao 1 indices, \(p < 0.05\)) of periphytic biofilm but did not significantly affect the \(\alpha\) diversity (Shannon and Simpson indices, \(p > 0.05\)). Therefore, Fe\(_2\)O\(_3\) PNPs showed a relatively small inhibition on the diversity of community in periphytic biofilm compared to TiO\(_2\) and CdS NPs. The Simpson index is more sensitive to evenness, and the Shannon index is more sensitive to richness. For all the three PNPs, Shannon indices showed a greater decline than Simpson indices, indicating that PNP exposure induced a more negative effect on the richness of community in periphytic biofilm while having a lower influence on the evenness of community.

Changing the community composition of different populations in multispecies microbial aggregates is an effective strategy to protect a microbial community against unfavorable environmental stress and enable adaptation to new environments. In the presence of PNPs, the composition of the periphytic biofilms changed dramatically (Figure 3A). Periphytic biofilms in the control and treatments were mainly composed of phyla Proteobacteria, Cyanobacteria, Bacteroidetes, Armatimonadetes, and Planctomycetes. The detected main community of periphytic biofilm in phyla level was consistent with former studies.\(^{15,22}\) When CdS was present, the relative abundance of Cyanobacteria and Armatimonadetes decreased significantly while Proteobacteria was almost two times more abundant than that in the control. The presence of TiO\(_2\) and Fe\(_2\)O\(_3\) promoted an increase in Cyanobacteria but caused a decrease in Proteobacteria. PCA was used to investigate community composition of the periphytic biofilm in the presence of the different PNPs. PCA results showed significant variation in community composition of periphytic biofilm between the control and PNP treatments (represented by the spatial distribution of samples in Figure 3B). Therefore, periphytic biofilm possesses the ability to change its community composition to protect microorganisms against PNPs (i.e., CdS, TiO\(_2\), and Fe\(_2\)O\(_3\)).

### Table 2. \(\alpha\) Diversity Parameters from the Results of the MiSeq Sequencing

<table>
<thead>
<tr>
<th>treatment</th>
<th>observed species</th>
<th>Chao1</th>
<th>Shannon</th>
<th>Simpson</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>959 ± 42</td>
<td>1495.40 ± 55.55</td>
<td>6.17 ± 0.13</td>
<td>0.94 ± 0.01</td>
</tr>
<tr>
<td>CdS</td>
<td>686 ± 29</td>
<td>1097.28 ± 29.62</td>
<td>5.15 ± 0.09</td>
<td>0.88 ± 0.01</td>
</tr>
<tr>
<td>TiO(_2)</td>
<td>868 ± 42</td>
<td>1352.47 ± 158.50</td>
<td>5.49 ± 0.54</td>
<td>0.90 ± 0.06</td>
</tr>
<tr>
<td>Fe(_2)O(_3)</td>
<td>708 ± 48</td>
<td>1036.6 ± 77.7</td>
<td>5.72 ± 0.25</td>
<td>0.94 ± 0.02</td>
</tr>
</tbody>
</table>

*Mean value ± SD; n = 3. Chao 1 is an index that was reported by Chao first which was used for nonparametric estimation of the number of classes in a population.*
A phylogenetic tree of 88 types of bacteria in periphytic biofilm was constructed using MEGA 5 (Figure 4). Results show the relative abundance of families Sphingobacteriales, Xanthomonadaceae, and Hyphomonadaceae (phylum Proteobacteria), and families Cytophagaceae and Chitinophagaceae (phylum Bacteroidetes) increased in the CdS treatment compared to the control. The relative abundance of families Chlamydomonadaceae and Cyanobacteriaceae (phylum Cyanobacteria) increased significantly in the TiO₂ or Fe₂O₃ treatment (p < 0.05) compared to the control. Although the presence of PNPs had no statistically different influence (p > 0.05) compared to the control. Although the presence of PNPs had no statistically different influence (p > 0.05) on periphytic biofilm diversity, the relative abundance of beneficial populations increased in periphytic biofilm (phototrophic and high nutrient metabolic microorganisms such as families Chlamydomonadaceae, Cyanobacteriaceae, Sphingobacteriales, and Xanthomonadaceae). Furthermore, these microorganisms may use the photogenerated electrons from PNPs to accelerate their N-metabolism or have a high primary productivity which is also beneficial for a higher productivity of EPS, and improvement in the activity of related enzymes such as CAT and SOD. The higher productivity of EPS and enzyme activity was helpful for periphytic biofilm exposed to PNPs.

**Interaction between EPS and PNPs.** The distribution forms of PNPs in biological systems are closely related to toxic responses of microorganisms to PNPs. To further distinguish the distribution forms of PNPs in periphytic biofilm matrices, the concentrations of PNPs in ion or particle form in solution or embedded in EPS were determined after cultivation experiments. There are few ionic forms of Cd, Ti, and Fe (6, 2, and 3 μg L⁻¹, respectively) present in solution (Figure S5). The loosely bound and irreversibly bound forms of PNPs were present in EPS. There were few loosely bound forms of PNPs (70, 40, and 110 μg L⁻¹ for CdS, TiO₂, and Fe₂O₃, respectively). Thus, TiO₂, CdS, and Fe₂O₃ were mainly distributed in EPS in the irreversibly bound forms.

SEM-EDS images showed that the presence of PNPs affected the morphology of the periphytic biofilm surface (Figure S6). A loose and porous biofilm structure was observed in the control. The morphology then changed to rough with some particle distribution in the presence of TiO₂ and Fe₂O₃. The periphytic biofilm exhibited a compact morphology with particle distribution in the presence of CdS. Further EDS spectra demonstrated that Ti (1.53%, wt %), Fe (2.16%, wt %), and Cd (1.02%, wt %) were distributed in the periphytic biofilm after exposure to TiO₂, CdS, and Fe₂O₃, respectively (Figure S6). An SEM-EDS test was also used to scan the cross-section of periphytic biofilm (Figure S7). Results showed that no Cd, Ti, or Fe were observed in the cross-sections of periphytic biofilm in PNPs treatments. These results indicated that PNPs affected the morphology of the periphytic biofilm; TiO₂, CdS, and Fe₂O₃ were mainly distributed on the surface of the periphytic biofilm matrix.

**DISCUSSION**

Many researchers have endeavored to evaluate the nanotoxicity of engineered nanoparticles (including PNPs) to individual cells and single/multispecies aggregates. The hypotheses were related to how nanoparticles result in toxicity to the organisms involved in the transportation of nanoparticles in biofilm matrices (result in the release of heavy metal ions, intact with cells or endocytosis potential by cells). PNPs...
could also pose threats to microorganisms due to ROS attack.\textsuperscript{54,55} While traditional studies on nanotoxicity and multispecies microbial aggregates marked a step forward in understanding the response of microorganisms to nanoparticles,\textsuperscript{5,35}\textsuperscript{3,52} the protection mechanisms of multispecies aggregates against intrusion of nanoparticles remained unclear. This study presented a systemic examination of this issue.

Multispecies microbial aggregates can protect cells against unfavorable environments because of the interdependencies and convoluted intraspecific and interspecific networks that are beneficial to detoxification and reduction of ROS.\textsuperscript{56} Periphytic biofilm, as a typical autotropic multispecies microbial aggregate, enhanced productivity of EPS to protect microorganisms against intrusion of PNPs. Unlike the toxicity of nanoparticles to single species microorganisms, the presence of nanoparticles can affect many aspects of multispecies microbial aggregates including biomass,\textsuperscript{55} metabolic activity (e.g., enzyme activity, photosynthesis),\textsuperscript{22} and EPS productivity.\textsuperscript{17} Thus, multispecies microbial aggregates show changes in community composition to adapt to the presence of nanoparticles.\textsuperscript{16} CCA was used to evaluate the influence of seven factors (EPS productivity, ATPase, SOD, ROS, concentration of chlorophyll, and distribution of PNPs in solution or in EPS) on community composition of periphytic biofilm. CCA results showed that EPS productivity played the most important role in community composition of periphytic biofilm (Figure S9). The second most important factor is distribution of PNPs in EPS.

EPS played an important role in protecting cells against adverse environments. EPS not only interacted with nanoparticles but also affected their aggregation tendency and distribution forms.\textsuperscript{12,33} EPS could interact with PNPs through adsorption processes over a short period: PNPs showed a fitted pseudo second kinetic model to adsorption of EPS (Figure S10), which indicated that adsorption of EPS by PNPs was mainly due to chemical adsorption.\textsuperscript{21,57} Previous studies had proved the existence of abundant functional groups in EPS, such as amino, hydroxyl, and carboxyl, which may facilitate PNP adsorption of EPS by formation of hydrogen bonds.\textsuperscript{17,58} The adsorbed EPS inevitably influenced the aggregation tendency and distribution of nanoparticles which is an important factor in toxicity to microorganisms.\textsuperscript{35,58} SEM-EDS (Figure S6 and Figure S7) and TEM (Figure S8) images of periphytic biofilm further proved that the interaction of PNPs with EPS affected the distribution of PNPs in periphytic biofilm. Thus, periphytic biofilm could produce more EPS to maintain PNPs in the surface which enabled biofilm cells to resist PNP further intrusion to the cells.

PNPs could also cause oxidative stress on periphytic biofilm which causes accumulated ROS. ROS generation of photocatalysts was dependent on whether their position of the conduction band (Ec) was located within the range of cellular redox potential (CRP, \(-4.12\) to \(-4.84\) eV vs NHE).\textsuperscript{39} The Ec values of CdS (around \(-4.70\) eV vs NHE)\textsuperscript{60,61} and TiO\textsubscript{2} (around \(-4.16\) eV vs NHE)\textsuperscript{27} were positioned within the CRP which could pose strong oxidative stress through generation of O\textsuperscript{2-} to periphytic biofilm. The Ec of Fe\textsubscript{3}O\textsubscript{4} (around \(-4.99\) eV vs NHE)\textsuperscript{29} was out of CRP, suggesting that O\textsuperscript{2-} was unlikely to originate from photoresponse of Fe\textsubscript{3}O\textsubscript{4}. Interestingly, the biodissolution of nanoparticles in aquatic environments was involved with toxicity to microorganisms.\textsuperscript{19} The Fe ions had been detected (Figure S5) which may cause oxidative stress on periphytic biofilm through Fenton like reactions.\textsuperscript{62} ROS accumulation could also occur due to the disruption of metal homeostasis.\textsuperscript{15} Fortunately, periphytic biofilms also possess the ability to protect microorganisms from ROS attack. On the one hand, periphytic biofilm enhanced SOD activity in the presence of PNPs to alleviate biotic ROS attack. The strong positive correlation between EPS productivity and SOD activity was observed (Figure S11), indicating that the presence of EPS was beneficial to maintain enzymatic activity.\textsuperscript{63,64} On the other hand, the EPS also played an important role in mitigating biotic/abiotic ROS attack which relieved oxidative stress.\textsuperscript{64} EPS is mainly composed of protein, polysaccharose, and humic substances.\textsuperscript{24,25} Interestingly, the proteins and polysaccharose in EPS are reactive with radicals.\textsuperscript{55} The presence of CdS and TiO\textsubscript{2} decreased the polysaccharose content of EPS compared to the control and Fe\textsubscript{3}O\textsubscript{4} treatment (Figure S12). The decreased polysaccharose content may have been due to consumption as hole scavengers of CdS or TiO\textsubscript{2} to alleviate ROS damage.\textsuperscript{72,64}

Furthermore, our experimental results demonstrated significant increases in protein content in the presence of PNPs. The protein contents play an important role in the three-dimensional structure of biofilm matrix,\textsuperscript{25} which is crucial to cell–cell interspecies and intraspecies interactions.\textsuperscript{66} The SEM morphology (the green rectangle in Figure S6) showed a loose and porous structure in the control periphytic biofilm and a rough structure in the presence of PNPs. The latter biofilm structure was more favorable to diffuse public good secretion (including allelochemicals) compared to the loose structure of biofilm in the control.\textsuperscript{66} Thus, increasing protein content may also contribute to the diffusion of allelochemicals which help to optimize community composition of periphytic biofilm.\textsuperscript{15,53}

Although the presence of PNPs had a minor negative influence on periphytic biofilm diversity, the relative abundance of beneficial populations increased in periphytic biofilm (photo- trophic and high nutrient metabolic microorganisms such as families Chlamydomonadaceae, Cyanobacteriacea, Sphingobacteriales, and Xanthomonadaceae) which may be helpful to produce more EPS. Overall, due to the increasing EPS productivity and population changes, periphytic biofilm was competent to defend itself against intrusion of PNPs.

**Prospective Environmental Applications.** Recently, researchers have been able to develop combined photocatalytic–biological systems to enhance contaminant removal efficiency.\textsuperscript{5} These strategies have been successfully demonstrated by fast biodegradation rates of organic pollutants such as trichlorophenols and dyes.\textsuperscript{67,68} Considering the oxidative stress of photocatalysts (especially the PNPs) to organisms, intimately coupled photocatalytic processes need complicated reactor configurations to protect microorganisms from being attacked or killed by PNPs or materials with lower toxicity to microorganisms.\textsuperscript{2–7} Periphytic biofilm, as a model multispecies microbial aggregate, exhibits constant adaptation of its population fitness to protect microorganisms against PNP stress. This study demonstrated that periphytic biofilm possesses the potential to combine with photocatalysis as periphytic biofilm can maintain its growth in the presence of PNPs. Although periphytic biofilm persists in the presence of PNPs, it is equally important to demonstrate whether it would still maintain the biological treatment function in further research in the future.
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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b04923.

Periphytic biofilm culture system; composition of the Woods Hole culture medium; measurement of biomass and total chlorophyll content; EPS extraction methods; determination of PNPs distribution in periphytic biofilm system; determination of ROS by electron paramagnetic resonance spectroscopy; measurement of the ROS accumulation; measurement of SOD and ATPase activity; preparation for morphology study; phylogenetic analysis of periphytic biofilm; adsorption assay experimental procedures; table with exposure of periphytic biofilm to photocatalytic nanoparticles (PNPs) with different concentrations (mg L\(^{-1}\)); table with hydrodynamic diameters (HD) of PNPs in WC medium or in EPS extract; XRD patterns of PNPs used (CdS, TiO\(_2\), and Fe\(_2\)O\(_3\)); TEM images of PNPs in WC medium or in EPS extract; intracellular protein content in periphytic biofilm; effects of heat treatment on reactive oxygen species (ROS) accumulation in periphytic biofilm in the presence (CK1) and absence (CK2) of EPS; distribution of PNPs in periphytic biofilm system (in EPS and solution); SEM images of the surface of periphytic biofilm and the corresponding EDS spectra; SEM images of cross-section of periphytic biofilm and the corresponding EDS spectra; TEM images of periphytic biofilm; canonical correspondence analysis (CCA) of the bacterial communities with responses of periphytic biofilm; kinetic adsorption of EPS by PNPs; correlation between EPS productivity and SOD activity; polysaccharose, protein, and humic acid content in EPS of periphytic biofilm in the control and in the presence of PNPs; and references (PDF)

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Notes

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