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Effects of silver sulfide nanoparticles on the microbial community structure and biological activity of freshwater biofilms†

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Ensuring the safety of silver sulfide nanoparticles (Ag_2S NPs) is essential for their application in various fields. Here, we explored changes in the microbial community composition and biological activity of freshwater biofilms treated with Ag_2S NPs (0.2, 1, and 5 mg L^{-1}) for 5 days. Our results indicated that the Ag_2S NPs significantly changed the physiological properties of the biofilms to different extents, *e.g.*, increasing the amount of extracellular polymeric substances, enhancing the activity of antioxidant systems, and decreasing algal biomass. The microbial community structure was obviously changed; the predominant bacteria *Phormidium* dramatically decreased while *Flavobacterium* became the dominant genus, exhibiting different levels of resistance and tolerance for Ag_2S NPs. Moreover, changes in the functional capacities of biofilms, including photosynthesis potential and functional extracellular enzymes related to nutrient cycling, varied after Ag_2S NP exposure in a concentration-dependent manner. Our findings suggested that the harmful effects of Ag_2S NPs on aquatic ecosystems should be considered carefully.

Environmental significance

Due to their low solubility, silver sulfide nanoparticles (Ag_2S NPs) have been shown to be less toxic than Ag NPs and Ag^+. However, several studies indicated that the safety of Ag_2S NPs could be overestimated in the environment and have attracted renewed attention. Unfortunately, the potential risks of Ag_2S NPs to freshwater microbial communities are still unknown. Biofilms are essential for primary production and nutrient cycling and are very sensitive to the changes in the external environment. Accordingly, Ag_2S NPs may have greater effects than expected when they are exposed to biofilms. Here, we examined changes in the microbial community composition and biological activity of freshwater biofilms treated with Ag_2S NPs. Our results indicated that Ag_2S NPs significantly altered the physiological properties of biofilms to different extents, which may be due to their nanotoxicity rather than ionic toxicity. Moreover, the microbial community structure was obviously changed, as were the functional capacities of the biofilms. Different microorganisms in biofilms have varying sensitivity and tolerance for Ag_2S NPs, and the amount of the predominant bacteria of genus *Phormidium* was dramatically inhibited. Overall, our findings suggested that the harmful effects of Ag_2S NPs on aquatic ecosystems should be considered carefully.

Introduction

Biofilms are an important microbial community in aquatic ecosystems and include various bacteria, microalgae, fungi, and protozoa.[1] Because of their complex microbial community structure, biofilms are essential for primary production and nutrient cycling.[2] Moreover, biofilms are sensitive to contaminants and have been widely used as useful indicators in aquatic ecosystems.[3,4] Hence, the potential toxicants released into bodies of water may have negative effects on the physiological properties and metabolism of biofilms, further affecting the function of aquatic ecosystems.[5]

Silver sulfide nanoparticles (Ag_2S NPs) are engineered nanoparticles with promising applications in industrial processes and have been used in infrared sensors, solar cells, optical filters, and biomedical applications.[6] The concentrations of Ag_2S NPs in different environmental systems have been estimated to be in ng L^{-1} in water and mg kg^{-1} in soil and active sludge.[7,8] However, with the development of NP applications, the release of NPs was predicted to increase exponentially.[9] Considering the above, the increased release into or accumulation in freshwater systems of Ag_2S NPs will result in potential risks to natural bodies of water, impacting microbial ecosystems.

In addition to being directly released in their NP form, sulfidation of silver nanoparticles (Ag NPs) is an important way to form Ag_2S NPs in the environment. Notably, Ag_2S is the main form of silver in the environment.[10] Many studies

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have indicated that Ag NPs can spontaneously transform into Ag_2S NPs (referred to as "transformed" Ag_2S NPs) in freshwater, soil, and sewage sludge within a short time.[11–13] Moreover, the "transformed" Ag_2S NPs were demonstrated to have different morphologies, and one of the typical structures was a core (Ag NPs)–shell (Ag_2S) type structure,[14,15] which was from an uncompleted transformation and different from the pristine Ag_2S NPs. However, studies also indicated that the residual Ag NPs in a typical core (Ag NPs)–shell (Ag_2S) structure could convert completely into individual Ag_2S NPs during the prolonged reaction, and the increase of the S/Ag ratio would be helpful to fully transform the intact Ag_2S NPs.[15,16]

Due to their low solubility, pristine Ag_2S NPs have been shown to be less toxic than Ag NPs and Ag^+.[17] Similar results were obtained for the "transformed" Ag_2S NPs, and that even a low degree of sulfidation could obviously decrease the toxicity of Ag NPs.[18] However, several studies have indicated that the bioavailability of pristine Ag_2S NPs is improved when the particles are released into complex systems, such as natural aquatic systems and soil, and the negative effects of pristine Ag_2S NPs on microbes, plants, and fishes have been observed due to the decreased stability of the Ag_2S NPs or the interactions between the Ag_2S NPs and other compounds.[17,19,20] Besides, the risks of fully transformed Ag_2S NPs to organisms have been raised considering the specific toxicity of the Ag_2S NPs.[21] Hence, the safety of Ag_2S NPs could be overestimated in the environment.[22,23] Additionally, several physicochemical properties in water systems (*e.g.*, pH, dissolved oxygen, ionic strength, and irradiation) could alter the transformation of metal-based NPs, thereby affecting the bioavailability,[24–26] safety, and toxicity of the NPs.[12,27]

As important aggregates of microbes and algae in freshwater, biofilms often have various compositions and living conditions, and factors that can affect the bioavailability of Ag_2S NPs are common in biofilms found in water environments.[8] Accordingly, Ag_2S NPs may have greater effects than expected when they are exposed to biofilms, and the safety of Ag_2S NPs in aquatic ecosystems should be considered carefully.

Studies have demonstrated that several NPs, including TiO_2 NPs, Ag NPs, and CeO_2 NPs, affect the biological functions and community structure of biofilms.[28,29] However, no studies have examined the effects of Ag_2S NPs on biofilms, which play important roles in forming the foundation of food webs in aquatic systems. Our previous research indicated that pristine Ag_2S NPs influenced the denitrification microbial structure and decreased the production of N_2O in sediments due to their nanotoxicity.[17] However, the effects of pristine Ag_2S NPs on freshwater biofilms and the safety of these NPs are still unknown. Additionally, the responses of extracellular polymeric substances (EPSs), the main barrier protecting biofilms, to protect against the harmful effects of pristine Ag_2S NPs are not clear. Importantly, changes in the activity and structure of biofilms in response to pristine Ag_2S NPs have also not been examined.

Accordingly, in this study, we performed a 5 day exposure experiment to explore the effects of pristine Ag_2S NPs on the physiological properties, microbial community structure, and functional capacities of freshwater biofilms. It should be noted that the study did not include "transformed" Ag_2S NPs, so in the following study, we directly used Ag_2S NPs to represent pristine Ag_2S NPs. The specific aims of this study were as follows: (1) to investigate the effects of Ag_2S NPs on the physiological properties of biofilms, including the composition of EPSs, the amount of algae biomass, and the total antioxidant capacity and lactate dehydrogenase (LDH) release; (ii) to examine the effects of Ag_2S NPs on the microbial community structure of biofilms; and (iii) to study the effects of Ag_2S NPs on the ecosystem functional capacities of biofilms, including photosynthesis potential and nutrient cycling enzymes, β-glucosidase (GLU), L-leucine aminopeptidase (LAP), and alkaline phosphatase (AP). This is the first study examining the effects of Ag_2S NPs on freshwater biofilms under environmentally relevant conditions, and the results are expected to improve our understanding of the harmful effects of Ag_2S NPs on ecological environments.

Materials and methods

Preparation and characterization of Ag_2S NPs

Ag_2S NPs were synthesized according to our previous study.[17] Briefly, sulfur (7.1 mg) was dissolved in ethanol (10 mL) at 60 °C, and the obtained solution was added dropwise to a mixture of polyvinyl pyrrolidone-40 (PVP-40) (10 mg) and aqueous $AgNO_3$ (50 mL, 170 mg L^{-1}) in the dark. After reacting for 5 h at 95 °C, the aqueous solution was sonicated for 30 min to avoid agglomeration. Next, the NP solution was washed five times by centrifugation at 9384 × *g* for 30 min and resuspended in ultrapure deionized (DI) water (Milli-Q; Millipore). The obtained NP solution was sonicated for 30 min to get a uniform distribution of Ag_2S NPs, and Ag_2S NP powders were afforded by vacuum lyophilization for further analysis.

The average diameter of the original Ag_2S NPs was confirmed by scanning electron microscopy (S4800; Hitachi, Japan). The results indicated that more than 70% of the NPs were distributed between 57 nm and 72 nm (n = 181), and the average size of the Ag_2S NPs was 64.81 ± 7.03 nm (Fig. S1a and b†). The crystal structures of the Ag_2S NPs were determined by X-ray diffraction (XRD; Philips-X-Pert-MPD-Pro, the Netherlands), with XRD patterns recorded for 2θ = 20–80°, confirming the identity and structure (acanthite) of the Ag_2S NPs (Fig. S1c†). Stock solutions (200 mg L^{-1}) of Ag_2S NPs were prepared in ultrapure DI water by sonication for 30 min at 400 W.[30] The zeta potential and size of the Ag_2S NPs in the stock solution were measured using a Malvern Zetasizer Nano ZSP (Malvern Instruments, UK).

Culture and exposure of biofilms

Biofilm formation was conducted in the laboratory. To better simulate natural conditions, a dynamic ecological water tank made with polymethyl methacrylate (Fig. S2†) was used as previously described.[31] The water used for the biofilm incubation was collected from Xuanwu Lake, Nanjing, China

(32.0403 N, 118.4754 E), and the water parameters are described in Table S1.† The incubator tank was maintained at a temperature of 25 ± 0.5 °C, and light was provided by halogen lamps (90–110 μmol m^{-2} s^{-1}, light : dark = 12 : 12 h). Additionally, WC medium (Table S2†) was added every 5 days to maintain normal levels of nutrition. The biofilms were incubated for 2 months to form stabilized and mature biofilms on the surface of the polymethyl methacrylate. More detailed information about the water tank and incubation methods are available in Test S1.†

The biofilms were peeled off of the polymethyl methacylate carefully using a sterile brush and then transferred to 250 mL Erlenmeyer flasks (Fig. S3†).[4] Every flask contained 3 g of fresh biofilm and 150 mL of water collected from the incubation tank. Next, a stock solution of Ag_2S NPs was added into the flasks to achieve final concentrations of 0.2, 1, and 5 mg L^{-1}, and the flasks were incubated under a light/dark cycle (12/12 h) for 5 days in a rotary shaker (at 150 rpm) at 25 °C. The control group had no Ag^+ or Ag_2S NPs, and all treatments were performed in triplicate. Besides, the less influence of PVP over a wide range of organisms has been widely demonstrated, so the toxicity of PVP to biofilms could be ruled out in the study.[32,33] The used concentrations of the NPs may be significantly greater than the environmental level, but a high concentration was often used to explore the possible toxicity mechanisms of the NPs, and observations of no effect or minimal effects at high concentrations will also be applicable to low environmentally relevant concentrations.[17] Moreover, worse cases such as incidental or accidental release of high concentrations of NPs into natural systems should also be considered. Moreover, to better understand the behaviors of the Ag_2S NPs and the potential mechanisms mediating biofilm formation, we performed aggregation and dissolution of Ag_2S NPs under the same incubation conditions. Different stock solutions of Ag_2S NPs were spiked into filtered incubation water to form 0.2, 1, and 5 mg L^{-1} solutions, briefly homogenized by manual shaking, and placed in a rotary shaker (150 rpm, 25 °C). The hydrodynamic diameters (HDDs) of the NPs were determined by dynamic light scattering (Malvern Zetasizer Nano ZSP, UK), and analysis of the dissolved Ag^+ was performed by centrifugation at 20 000 × *g* for 30 min prior to inductively coupled plasma mass spectrometry (ICP-MS) analysis.

Extraction of EPSs from the biofilms

Soluble EPSs (sEPSs), loosely bound EPSs (LB-EPSs), and tightly bound EPSs (TB-EPSs) in the biofilms were extracted using centrifugation, sonication, and thermal extraction (Test S2†), as previously described.[34] The total organic carbon (TOC) content of the three types of EPSs was analyzed using a TOC analyzer (Liqui TOC II; Elementar, Germany). The polysaccharide and protein contents were measured by the modified Lowry method[35] and the anthrone–sulfuric acid method[36] using bovine serum albumin and glucose as the standards, respectively.

Determination of enzyme activity

To analyze the functional enzyme activities of biofilms, 0.5 g of biofilm was ground with phosphate buffer saline (PBS, pH 7.2–7.4) and centrifuged at 2500 × *g* for 15 min. The liquid supernatant was collected for the following measurements. LDH release was used to represent the cell membrane integrity of cells. Total antioxidant capacity (T-AOC) was used to evaluate the toxicity of the NPs on the biofilms. The levels of LDH and T-AOC in the biofilms were detected using an LDH kit and a T-AOC Kit (Jiancheng Bioengineering Co. Ltd., Nanning, China) in accordance with the manufacturer's instructions.

The activities of three extracellular enzymes (GLU, LAP, and AP) were analyzed by double antibody sandwich-enzyme-linked immunosorbent assays (ELISAs), based on the specific interaction between an antibody and the corresponding antigen, using a microbiological ELISA kit (FEIYA BIOTECHNOLOGY, Jiangsu, China), according to the manufacturer's protocol. As an example, for GLU, 50 μL of the GLU standard at different concentrations or 50 μL of the sample was added to a pre-coated 96-well plate, followed by 50 μL of anti-GLU antibodies. The plate was then incubated at 34 °C for 30 min and washed carefully five times. Next, 50 μL of the secondary antibody was added to each well. After incubation, horseradish peroxidase was added to each well, and the plates were incubated under the same conditions. The plates were then washed five times, and 100 μL of the 3,3′,5,5′-tetramethylbenzidine substrate solution was added as the chromogenic agent, followed by 50 μL of the stop solution. The plate was incubated in the dark, and the absorbance was read at 450 nm on a microplate reader. The activity of GLU in the biofilms was calculated based on the standard curve.

Photosynthesis potential and algal biomass

The maximum quantum yield of algal photosynthesis was determined using PHYTO-PAM (Heinz Walz Gmbh, Effeltrich, Germany), a useful tool for monitoring the effects of NPs on photosynthetic organisms based on the measurement of *in vivo* chlorophyll a (Chl a) fluorescence. Briefly, biofilm suspensions in 3 mL of DI water were measured after 30 min of dark adaptation, and the maximum quantum yield of the photosystem IIo (PSosy) was calculated as yield = $(F_m - F_0)/F_m$, where F_0 is the minimal fluorescence determined after the emission of a weak far red modulated light, and F_m is the maximum level of fluorescence measured during a saturating white light pulse.[37] Additionally, the main algal structures were measured based on four different wavelengths (470, 520, 645, and 665 nm) for PAM, yielding the total Chl a and a rough estimate of three algal populations: cyanobacteria, green algae, and diatoms.[38]

High-throughput sequencing

After exposure for 5 days, 0.5 g of wet biofilm samples were collected for DNA extraction and high-throughput sequencing analysis, as previously described.[17] Briefly, DNA was extracted

using an E.Z.N.A. Tissue DNA kit (Omega Bio-tek, Norcross, GA, USA), according to the manufacturer's instructions. Subsequently, primers 515F and 907R were used for quantitative real-time polymerase chain reaction (PCR; TIB-8600, Canada) to determine the copy number of the 16S rRNA gene for all bacteria in the biofilms. Next, Illumina MiSeq high-throughput sequencing was performed at MAGIGENE Biotech Co., Ltd (Guangzhou, China). Amplicons with sequences shorter than 200 bp and of low quality (quality score < 25) were removed, yielding raw sequence data. Then, the normalized samples were individually classified and analyzed with the Ribosomal Database Project database (http://rdp.cme.msu.edu/). More specific methods and data analysis are presented in the ESI.†

Statistical analyses

One-way analysis of variance with Tukey's multiple range test was performed to determine the statistical significance of the results (SPSS 17.0). Differences with p values of less than 0.05 were considered statistically significant. All data are represented as means ± standard deviations.

Results and discussion

Aggregation and dissolution of Ag_2S NPs

Studies have shown that the behaviors of NPs are important for the determination of their bioavailability and toxicity when being released into aquatic systems. Hence, NP aggregation and dissolution experiments were conducted before the exposure experiment. In our study, the HDDs of Ag_2S NPs in the three groups were dramatically increased within the first 50 min in the order of $5 > 1 > 0.2$ mg L^{-1} (Fig. S4a†). After 24 h, the HDDs in incubated water have remained stable (Fig. S4b†). The average sizes of the Ag_2S NPs at the end of the experiment was 196.55 ± 10.4, 264.50 ± 11.00, and 443.44 ± 18.9 nm for the 0.2, 1, and 5 mg L^{-1} groups, respectively. However, the dissolution of the Ag_2S NPs in incubated water was quite limited, and the concentrations of the released Ag^+ were lower than the detection limit of ICP-MS (<0.007 ng mL^{-1}) in all three groups during the entire exposure time, indicating that the incubation conditions did not affect the stability of the Ag_2S NPs. However, some previous studies have demonstrated that Ag_2S NPs can release more Ag^+ in some oxidizing systems, suggesting that different environmental conditions have varying effects on the stability of Ag_2S NPs and may alter the bioavailability of Ag_2S NPs for the organism.[27]

Influence of Ag_2S NPs on the physiological properties of biofilms

Responses of resistance systems of biofilms after Ag_2S NP exposure. Biofilms are widespread aggregated communities in aquatic systems and have been shown to be highly sensitive to changes in external conditions; thus, their physiological characteristics may be affected by toxicants. EPSs are metabolic products that are normally attached to the cell surface and are the main protective barrier against adverse foreign substances and functions by trapping toxicants and preventing their diffusion into bacteria.[39,40] In this study, three types of EPSs were separated from biofilms, *i.e.*, TB-EPSs, LB-EPSs, and sEPSs. The obtained results showed that sEPSs were the major type of EPSs, followed by TB-EPSs and LB-EPSs (Table 1), similar to the findings of other studies.[29] Additionally, the amounts of all three types of EPSs were increased as the concentration of Ag_2S NPs increased, concurrent with the increased total protein content and stabilized total polysaccharides. Specifically, the 5 mg L^{-1} Ag_2S NPs increased the protein contents in sEPSs, LB-EPSs, and TB-EPSs from 37.48 ± 2.81 to 50.38 ± 3.20, 13.36 ± 1.64 to 27.98 ± 2.65, and 15.99 ± 2.18 to 48.01 ± 3.58 mg per g dry weight, respectively. Notably, the polysaccharide content decreased in the sEPSs but increased in the TB-EPSs for all three groups, resulting in stabilization of the total concentrations of polysaccharides between the control and Ag_2S NP-treated groups (Table 1). Moreover, higher concentrations of Ag_2S NPs led to higher ratios of protein to polysaccharides and increased protein production, thereby improving the ability of biofilms to bind NPs and metal ions.[41] Hence, more EPSs were produced due to the stimulation of NPs, and the resistance of the biofilms against Ag_2S NPs was increased.

As another important indicator of the resistance of biofilms to NP exposure, the levels of T-AOC in ecological systems reveal changes in the oxidant stress status of the biofilms. In general, the T-AOC in biofilms was enhanced as the Ag_2S NP concentration increased, with a significant difference observed between the control and 5 mg L^{-1} Ag_2S NP groups ($p < 0.05$; Fig. 1). Studies have shown that an effective antioxidant defense system can remove excess oxygen free radicals and their metabolites, thereby maintaining normal oxidant stress in the cells. The unique properties of NPs (*e.g.*, small size effect, surface and boundary effects) could destroy this balance and increase oxidant stress in cells, potentially leading to cell death in severe cases. The degree of the bioresponse depends on the type, concentration, and exposure time of the NPs and on the aquatic species.[42] Oxidative stress can be divided into three processes: antioxidant response, oxidation inhibition, and antioxidant inactivation.[43] NPs can cause a dramatic decrease in the T-AOC, resulting in loss of the antioxidant capacity of cells and leading to serious toxicity. However, in this study, the T-AOC was increased after exposure to the Ag_2S NPs, indicating that the Ag_2S NPs did not destroy the oxidant system in cells but increased the resistance of biofilms to the oxidant system. Moreover, the release of LDH was also slightly increased, but there were no significant differences, even in the presence of 5 mg L^{-1} Ag_2S NPs (Fig. 1), consistent with the low toxicity of Ag_2S NPs.

Altered biomass of algae in the biofilms after Ag_2S NP exposure. The amount of Chl a is widely used to represent the biomass of algae in biofilms. In this study, the total amount of Chl a decreased following exposure to the NPs in the order of $5 > 0.2 > 1$ mg L^{-1} Ag_2S NPs (Fig. 2), indicating that the

Table 1 Different amounts and compositions of EPSs of biofilms with and without exposure to Ag_2S NPs after 5 days. All data are represented as means ± standard deviations (n = 3). The different lower-case letters represent significant differences

		Control	0.2 mg L^{-1} Ag_2S NPs	1 mg L^{-1} Ag_2S NPs	5 mg L^{-1} Ag_2S NPs
EPS (mg TOC per g dry weight)	TB-EPS	53.87 ± 3.93^{c}	64.29 ± 2.66^{b}	$68.57 \pm 3.78^{a,b}$	73.03 ± 5.21^{a}
	LB-EPS	22.33 ± 2.29^{d}	28.68 ± 2.87^{c}	35.24 ± 2.84^{b}	42.93 ± 3.00^{a}
	S-EPS	104.09 ± 4.59^{c}	$108.58 \pm 7.94^{b,c}$	119.15 ± 4.70^{b}	131.64 ± 7.67^{a}
Total EPS		182.36 ± 6.65^{d}	201.94 ± 8.09^{c}	224.92 ± 0.68^{b}	250.56 ± 4.55^{a}
Protein (mg per g dry weight)	TB-EPS	15.99 ± 2.18^{c}	20.39 ± 1.90^{c}	32.15 ± 2.44^{b}	48.01 ± 3.58^{a}
	LB-EPS	13.36 ± 1.64^{c}	17.87 ± 1.90^{c}	21.37 ± 2.78^{b}	27.98 ± 2.65^{a}
	S-EPS	37.48 ± 2.81^{c}	40.89 ± 3.17^{c}	44.00 ± 2.50^{b}	50.38 ± 3.20^{a}
Total protein		66.49 ± 0.85^{d}	79.20 ± 4.74^{c}	97.69 ± 3.20^{b}	126.83 ± 8.74^{a}
Polysaccharides (mg per g dry weight)	TB-EPS	10.67 ± 1.22^{c}	$15.09 \pm 2.23^{b,c}$	$15.34 \pm 1.73^{a,b}$	18.55 ± 1.41^{a}
	LB-EPS	7.49 ± 1.31^{a}	7.75 ± 1.26^{a}	7.95 ± 0.79^{a}	8.30 ± 0.97^{a}
	S-EPS	27.14 ± 2.83^{a}	15.03 ± 1.43^{b}	18.34 ± 1.58^{b}	16.21 ± 1.07^{b}
Total polysaccharides		45.75 ± 3.23^{a}	37.96 ± 3.32^{b}	42.34 ± 4.03^{a}	43.45 ± 1.86^{a}
Protein/polysaccharides	TB-EPS	1.52 ± 0.29^{b}	1.39 ± 0.33^{b}	2.07 ± 0.34^{a}	2.55 ± 0.10^{a}
	LB-EPS	1.83 ± 0.48^{c}	$2.35 \pm 0.53^{b,c}$	$2.70 \pm 0.09^{a,b}$	3.40 ± 0.30^{a}
	S-EPS	1.35 ± 0.03^{c}	2.71 ± 0.10^{b}	2.36 ± 0.15^{b}	3.12 ± 0.24^{a}

biomass of algae in the biofilms was obviously inhibited by the Ag_2S NPs. Moreover, a rough estimate of different algal populations was obtained from PAM, and the results showed that the amount of green algae were dramatically increased in all exposed groups ($p < 0.05$), with a particularly large increase following treatment with a high concentration of Ag_2S NPs (Fig. 2). Conversely, the amounts of cyanobacteria and diatoms dramatically decreased after exposure to Ag_2S NPs, with the 5 mg L^{-1} Ag_2S NP group showing the strongest inhibition ($p < 0.05$). Similar results have been obtained when exploring the effects of pharmaceuticals on algae populations in biofilms, and these differences between the groups may be caused by differences in the morphological and metabolic properties.[44] In contrast, the 1 mg L^{-1} Ag_2S NPs showed weaker inhibition of total Chl a than the 0.2 mg L^{-1} Ag_2S NPs, which was expected to be the least toxic concentration according to a dose–response analysis of the NP toxicity. This may be because the population of green algae was significantly enhanced by treatment with the 1 mg L^{-1} Ag_2S NPs compared with that treated with the 0.2 mg L^{-1} Ag_2S NPs ($p < 0.05$); similar inhibition was observed for cyanobacteria and diatoms. Thus, the negative effects of low concentrations of NPs on the structure and content of algae in the biofilms should be considered. In general, Ag_2S NPs caused higher sensitivity to algae in the biofilms, exhibiting strong toxicity for the algae biomass, and different types of algae had different responses to Ag_2S NPs. Further studies are required to examine this effect in greater detail.

Changes in the microbial community structure in the biofilms. Biofilms have abundant autotrophic and heterotopic bacteria[45] and can alter their community structure in response to toxicants.[4] Our results showed that the microbial

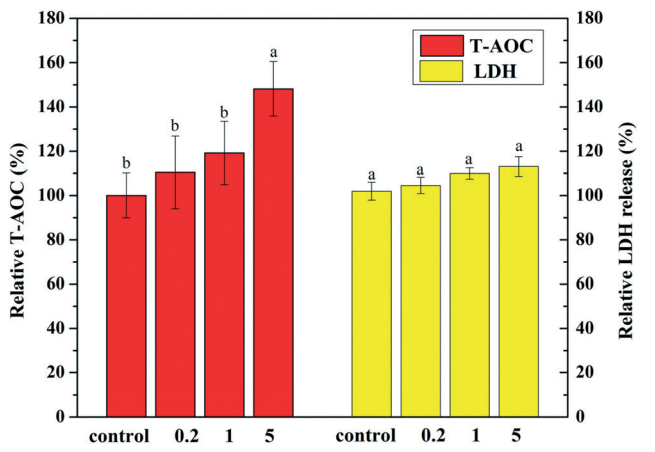

Fig. 1 Changes in total antioxidant capacity (T-AOC) and lactate dehydrogenase (LDH) release from biofilms with and without exposure to 0.2, 1, and 5 mg L^{-1} Ag_2S NPs for 5 days. The values for each sample are presented as averages with standard deviations (n = 3). The different letters shown above the error bars represent significant differences.

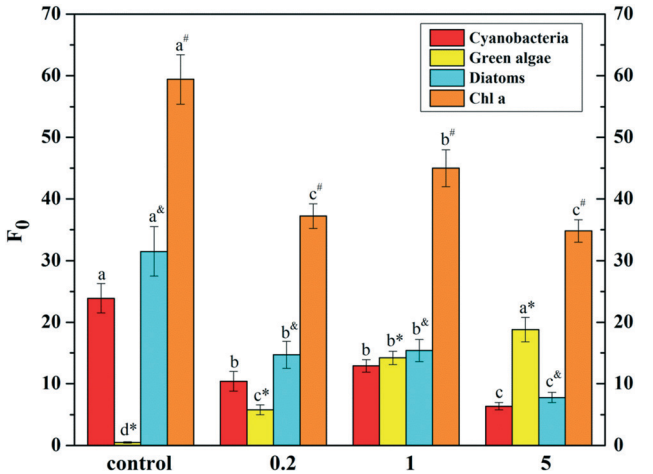

Fig. 2 Chlorophyll fluorescence (F_0) of Chl a, cyanobacteria, green algae, and diatoms in biofilms with and without exposure to 0.2, 1, and 5 mg L^{-1} Ag_2S NPs for 5 days. The values for each sample are presented as averages with standard deviations (n = 3). The different letters shown above the error bars represent significant differences, and superscripted *, &, and # represent differences in green algae, diatoms, and Chl a, respectively.

community structure changed after Ag_2S NP exposure. Fig. 3 shows the classification of the bacteria at the phylum level based on high throughput sequencing. Compared with the control, Ag_2S NPs decreased the abundance of *Cyanobacteria*, but increased the amount of *Gemmatimonadetes*, *Acidobacteria*, and *Planctomycetes* regardless of the concentration. The reduced *Cyanobacteria* amount was consistent with the result obtained by PAM. These results indicated that *Cyanobacteria* were more sensitive to Ag_2S NPs and easily inhibited. A similar conclusion was obtained for other foreign substances exposed in biofilms such as antibiotics and metal Cu.[44,46]

To further explore the effects of Ag_2S NPs on the microbial community structure, the bacteria with a relative sequence abundance of at least 1% were identified at the genus level (Table 2). In the control, *Phormidium* was the predominant bacteria in the microbial community, accounting for up to 19.06% ± 4.52% of all bacteria, but was dramatically inhibited after exposure to Ag_2S NPs, directly leading to the obvious decline in the *Cyanoobacteria* phylum. The lack of significant differences between the 0.2, 1, and 5 mg L^{-1} Ag_2S NPs demonstrated that *Phormidium* was highly sensitive to Ag_2S NPs, and a low concentration could dramatically inhibit the relative abundance of this organism. Additionally, *Opitutus* was also decreased, whereas *Flavobacterium*, *Gemmata*, *Verrucomicrobium*, *Haliscomenobacter*, and *Siphonobacter* were enhanced by the three treatments, and *Flavobacterium* became the new predominant bacteria. Notably, the responses of different bacteria to different concentrations of Ag_2S NPs varied. For example, *Emticicia* exhibited strong tolerance for Ag_2S NPs, regardless of the concentration, and *Flavobacterium* was dramatically increased, without significant differences between the three exposure groups. *Opitutus* showed different tolerance levels based on the concentration of the Ag_2S NPs. Hence, we concluded that the bacteria in the biofilms had different levels of tolerance and resistance to Ag_2S NPs, which were highly related to the exposure concentration and microbial type. The results will be helpful to determine the environmental risk of Ag_2S NPs to different freshwater biological systems. Biofilms are an important microbial community in aquatic ecosystems, and play a fundamental role in primary production and nutrient cycling. Therefore, the activity and function of biofilms, which contain the high abundance of these less resistant microorganisms, will be more easily affected by Ag_2S NPs than those of biofilms that include higher tolerant and resistant bacteria. Besides, the higher tolerant bacteria such as *Flavobacterium* could be used in wastewater treatment to improve the removal of Ag_2S NPs from wastewater.

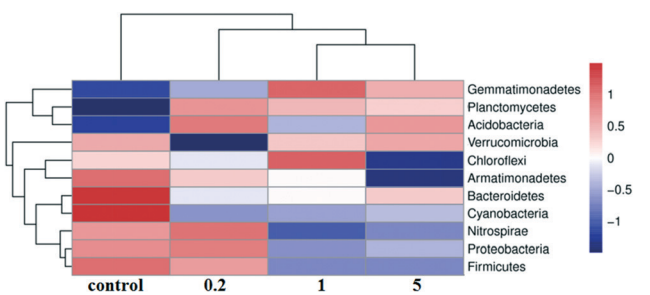

Fig. 3 Color-scale mean-heat map of biofilms showing the top representative predominant 16S rRNA gene-based microbial sequences (at the phylum level).

Functional responses of biofilms to Ag_2S NPs

Photosynthetic yields of biofilms. As the typical microbial community in water systems, biofilms have very important effects on the primary production, nutrient transformation, and energy interchange.[47] In this study, the 5 mg L^{-1} Ag_2S NPs dramatically increased the photosynthetic yield ($p < 0.05$), whereas the 0.2 and 1 mg L^{-1} Ag_2S NPs had no effect (Fig. 4d). Although the content of Chl a dramatically decreased ($p < 0.05$; Fig. 2, orange column), the photosynthetic yield of the biofilms still remained at a normal level (in the 0.2 and 1 mg L^{-1} groups) and even increased (in the 5 mg L^{-1} group) (Fig. 4d). The possible reason for this increase was that the amount of green algae, which have been reported to have stronger photosynthetic activity than cyanobacteria and diatoms,[48] was very low in the control, but dramatically increased after exposure to the Ag_2S NPs (Fig. 2, yellow column). The altered structure of the green algae in the biofilms could maintain the normal photosynthetic yield after the Ag_2S NP exposure, and the dramatically increased amount of green algae led to improved photosynthetic yields in the presence of 5 mg L^{-1} Ag_2S NPs. Moreover, the decreased content of cyanobacteria and diatoms, which could reduce the competition for nutrient substances and light among the algae, increased the activity of green algae and led to improved photosynthesis in the 5 mg L^{-1} Ag_2S NP group.

Extracellular enzymes related to nutrient cycling in the biofilms. The extracellular enzymes GLU, LAP, and AP are important for carbon, nitrogen, and phosphorus cycling by providing the necessary nutrient sources and hydrolysis of substances.[49] Moreover, extracellular enzymes are located outside the cells and could be the first site of interaction with the NPs.[50] In this study, the activity of GLU was increased following the exposure to 0.2 and 1 mg L^{-1} Ag_2S NPs and remained constant in the 5 mg L^{-1} group (Fig. 4a). In contrast, the activities of LAP obviously decreased by 4.05%, 14.03%, and 25.25% in the presence of 0.2, 1, and 5 mg L^{-1} Ag_2S NPs, respectively (Fig. 4b). However, the activity of AP remained constant after exposure to 0.2 and 1 mg L^{-1} Ag_2S NPs and decreased following exposure to 5 mg L^{-1} Ag_2S NPs (Fig. 4c).

The results showed that functional extracellular enzyme activity was influenced by Ag_2S NPs to varying degrees, depending on the concentrations of NPs and the types of enzymes. GLU is essential for the first step in the transformation of complex forms of carbon,[51] which is thought to be more sensitive to some NPs such as Ag NPs and TiO_2 NPs.[45,50] The obtained data showed that only GLU activity

Table 2 Bacterial genera with a relative sequence abundance of at least 1%. All data are represented as means ± standard deviations (n = 3). The different lower-case letters represent significant differences

	Control	0.2 mg L^{-1} Ag_2S NPs	1 mg L^{-1} Ag_2S NPs	5 mg L^{-1} Ag_2S NPs
Phormidium	19.06 ± 4.52^{a}	2.38 ± 0.46^{b}	4.72 ± 1.68^{b}	1.52 ± 0.06^{b}
Emticicia	3.18 ± 1.47^{a}	3.26 ± 1.01^{a}	3.29 ± 0.31^{a}	3.71 ± 0.20^{a}
Flavobacterium	2.14 ± 0.21^{b}	5.12 ± 0.85^{a}	3.72 ± 1.25^{a}	4.73 ± 0.64^{a}
Opitutus	2.85 ± 0.82^{a}	0.67 ± 0.28^{b,c}	1.48 ± 0.73^{b}	0.32 ± 0.08^{c}
Gemmata	0.86 ± 0.15^{b}	2.07 ± 0.82^{a}	2.07 ± 0.39^{a}	1.69 ± 0.17^{a,b}
Verrucomicrobium	0.80 ± 0.14^{b}	1.91 ± 0.45^{a}	1.72 ± 0.63^{a}	1.98 ± 0.12^{a}
Haliscomenobacter	0.65 ± 0.31^{a}	1.17 ± 0.30^{a}	1.17 ± 0.29^{a}	1.06 ± 0.07^{a}
Siphonobacter	0.56 ± 0.16^{a}	0.95 ± 0.33^{a}	0.98 ± 0.20^{a}	0.93 ± 0.18^{a}
Prosthecobacter	0.54 ± 0.19^{b}	1.31 ± 0.20^{a}	0.91 ± 0.28^{a,b}	0.76 ± 0.31^{b}

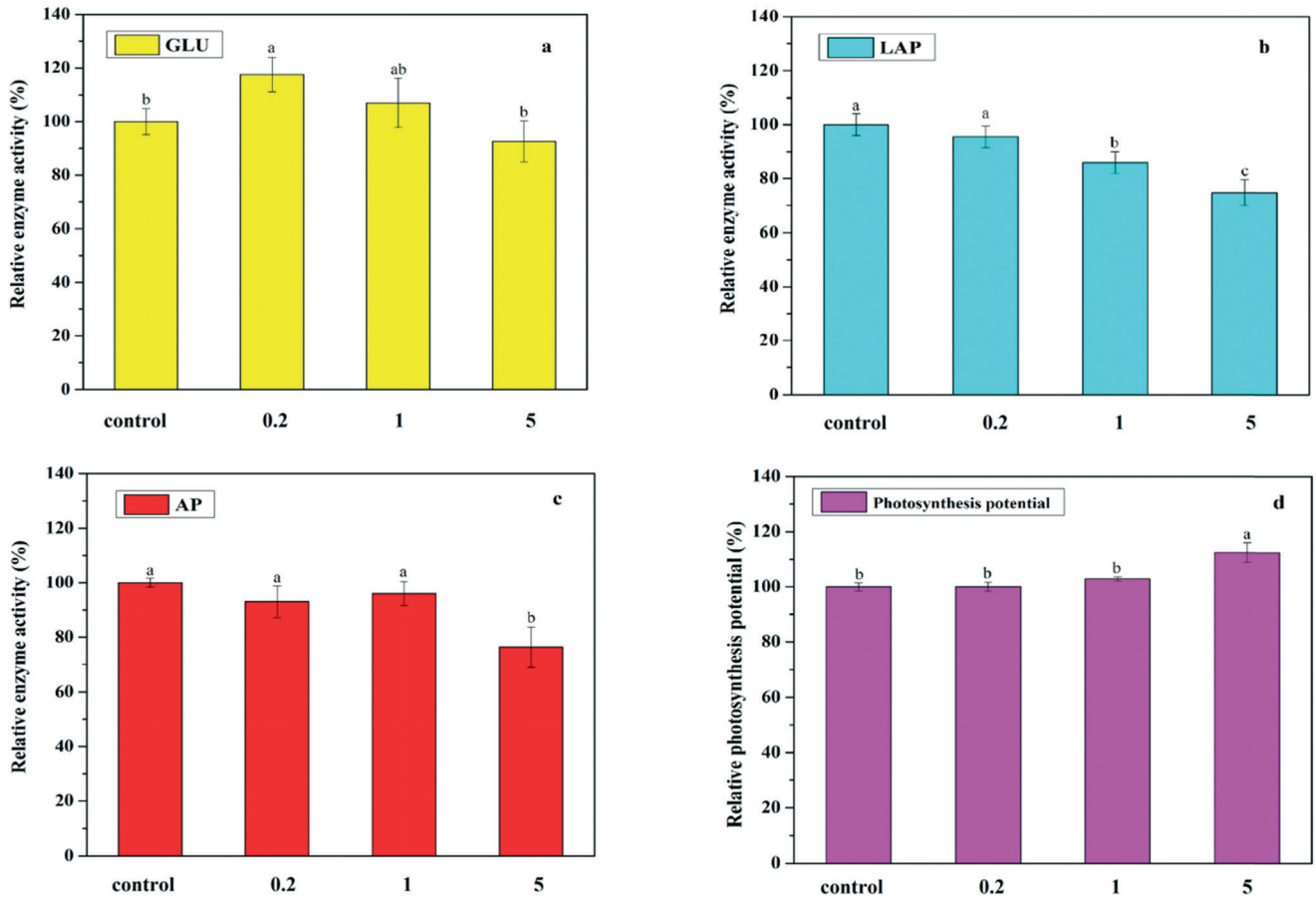

Fig. 4 Changes in the activities of three extracellular functional enzymes related to nutrient cycling (β-glucosidase [GLU], L-leucine aminopeptidase [LAP], and alkaline phosphatase [AP] for a, b, and c, respectively) and photosynthesis potential (d) in biofilms with and without exposure to 0.2, 1, and 5 mg L^{-1} Ag_2S NPs for 5 days. The values for each sample are presented as averages with standard deviations (n = 3). The different letters shown above the error bars represent significant differences.

was increased among the three enzymes. Consistent with this, a previous study showed that GLU immobilized by modified Fe_3O_4 NPs exhibited enhanced thermostability and activity,[52] whereas similar results were not reported for LAP and AP. Moreover, the shapes of the NPs and exposure time also influence the enzyme activity.[53] In contrast with GLU, the activity of LAP was more easily inhibited by Ag_2S NPs, with a clear negative dose–response relationship. However, AP was more tolerant towards Ag_2S NPs than GLU and LAP, because the former was affected only at high concentrations, providing evidence of the different sensitivities of the extracellular enzymes of biofilms for Ag_2S NPs. Previous studies have shown that AP was mostly expressed in microbial cells or organic particles and that the AP present in the insoluble fraction could resist some of the effects of the NPs.[38,50] Similar conclusions were also confirmed by Gil-Allué *et al.*, who indicated that AP maintained normal activity when exposed to Ag NPs, despite obvious inhibition of GLU and LAP.[49] The

altered activities of GLU and LAP indicated the potential effects of Ag_2S NPs on C and N cycling, which should be investigated in future studies.

Possible mechanisms mediating the effects of Ag_2S NPs. The mechanisms through which metal NPs exert their effects are still unclear. Some studies have indicated that the released Ag^+ from Ag-based NPs is a major cause of toxicity, *e.g.*, the released Ag^+ from Ag NPs is responsible for the obvious inhibition of stream periphyton activity.[49] The obtained results showed that Ag_2S NPs increased the antioxidant capacity and photosynthetic yield of the biofilms due to the hormesis effects of the NPs. Hormesis effects have been reported to stimulate cell growth, metabolism, and antibody production due to the resistance of microbes.[54] The dissolution of Ag_2S NPs in incubated water could be neglected because of the undetectable levels of Ag^+ in this study; therefore, the observed influences of Ag_2S NPs on the biofilms were caused by their nanoeffects rather than the released Ag^+, which could explain the strong stability and reduced toxicity of Ag_2S NPs. However, these particle-specific effects of Ag_2S NPs still negatively affected the functional enzyme activity related to phosphorus and carbon cycling. In addition, some bacteria, such as *Phormidium*, which were sensitive to Ag_2S NPs, were dramatically inhibited, resulting in harmful effects on the other biological activities and functions of freshwater biofilms. Hence, the harmful effects of Ag_2S NPs on organisms should be considered carefully, particularly with regard to redox or irradiation systems.

Conclusion

In this study, we demonstrated that Ag_2S NPs had obvious effects on the biological activity and community composition of freshwater biofilms after exposure for 5 days, which may be due to their nanotoxicity rather than their ionic toxicity. The EPS content and the antioxidant capacity of the biofilms were also improved due to the stimulatory effects of the Ag_2S NPs. However, the community structure of the biofilms was altered to adapt to the disruption caused by the NPs, and different species have varying responses to the Ag_2S NPs due to their different sensitivities to Ag_2S NPs. Additionally, the algal biomass was obviously decreased, although the photosynthetic potential of the biofilms was improved, which could be related to the different types of algal biomass. Moreover, Ag_2S NPs decreased the activities of three functional extracellular enzymes (GLU, LAP, and LP) to different extents, depending on the NP concentration and enzyme type. Furthermore, the long-term effects of Ag_2S NPs on biofilms and other microbial aggregations should also be evaluated, especially in more complex systems where the stability of Ag_2S NPs could be less pronounced, increasing the bioavailability and toxicity of Ag_2S NPs to the organism.

Conflicts of interest

There are no conflicts to declare.

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