JOURNAL OF
EMERGING INVESTIGATORS

Article

Investigating Lemna minor and microorganisms for the phytoremediation of nanosilver and microplastics

Anagha Iyer[1], Juliana Caulkins[1], Leya Joykutty[1]
[1] American Heritage School, Plantation, Florida

SUMMARY

Due to improper disposal of materials, the amount of pollution that enters waterways has greatly increased. Pollutants that can escape into waterways include microplastics and nanosilver - which are used in protective equipment and disinfectants, respectively. Phytoremediation, a method of using plants to remove pollutants from the environment, has shown great potential for remediating such contaminated aquatic environments. In addition, bacteria that promote plant growth have been shown to alleviate stress from plants and make biofilms that can capture small particles. Here, we employed *Lemna minor*, an aquatic floating plant, in combination with three plant growth-promoting bacteria to see which combination could effectively remove more pollutants from a simulated environment. We measured the pollutant-removal efficiency of *L. minor*, chlorophyll content, biofilm efficiency, and water toxicity. The ability of *L. minor* to accumulate nanosilver and microplastics increased significantly with the addition of bacteria. The bacteria with the highest biofilm production, measured through crystal violet staining, was associated with an increase in the amount of pollutants removed. When *L. minor* was exposed to just the pollutants, chlorophyll content significantly decreased from 4.17mg/mL to as low as 2.86 mg/mL; however, with the addition of the bacteria, chlorophyll increased to a point where there was not significant damage. When exposed to the initial and post-remediation concentrations of nanosilver particles, the negative impacts on zebrafish embryos were reduced. These forms of bacteria-assisted phytoremediation provide insight into how treatment plants can effectively remove pollutants from wastewater with the addition of bacteria.

INTRODUCTION

As a result of the COVID-19 pandemic, the demand for single-use plastics and disinfectants to fight the virus has greatly increased (1). Improper disposal of these materials has contributed to a pollution crisis involving many different materials. Microplastics and nanosilver are both commonly found medical wastes present in wastewater and can have detrimental effects on human health (1).

Microplastics are small plastic particles that are less than 5mm in diameter, created by humans. The most immediate problem is that humans and other animals are being exposed to these tiny particles through air, water, and even food (2). Specifically, personal protective equipment (PPE) including face masks, gloves, and other safety products have become ubiquitous in many households due to the pandemic. When these items are disposed improperly, they can enter the environment, break down into microplastics, and cause the accumulation of plastics in oceans, rivers, and soils (3). Microplastics have the potential to escape wastewater treatment facilities and enter waterways, where they linger on surface waters where our drinking water comes from. A study that analyzed drinking water samples found that 80% of their samples had plastic particles (4). Inhaling or ingesting microplastics can lead to detrimental health effects, including respiratory illnesses and tissue inflammation (5).

Silver nanotechnology uses particles between 1 nm and 100 nm in size in the production of a variety of commonly found products today including electronics, ceramics, clothing, glass, papers (6). They are cost effective and also have antibacterial properties, which made them more popular during the pandemic. Nanosilver was found in sewer drainage, which confirms their ability to enter water systems (7). These nanoparticles can move through sediment, groundwater, freshwater, and ultimately end up coming into contact with humans (8). Humans often inhale microplastics in work-related conditions or commercial products unknowingly. They have been shown to enter the brain and cause DNA damage (9).

Current ways of treating polluted water include: chemical precipitation, which converts a substance to its insolvable form; ion exchange, which removes unwanted ionic substances and exchanges them with less harmful ionic compounds; membrane technology, which removes contaminants based on size filtration; and electrochemical treatment, which uses electricity to remove dissolved compounds (10). These methods all suffer major limitations as they are costly, produce secondary pollutants, and have low efficiency (10).

Thus, phytoremediation has become a novel way to remove pollutants from waters and soils. Phytoremediation is a widely encompassing term for the removal of pollutants from the environment using plants (11). There are many forms of phytoremediation, however, this study focuses specifically on how plants can accumulate these pollutants into their biomass. Compared to current techniques, phytoremediation offers greater cost efficiency, is lower maintenance, and is more environmentally-friendly (11). In addition, phytoremediation is less energy intensive and can remove pollutants over a long period of time. Specifically, phytoremediation has been shown to work most effectively with heavy metals (12). The plants can absorb the metals into their roots and store them, making it feasible to filter environmental contaminants (12). Compared to traditional methods, phytoremediation has

https://doi.org/10.59720/23-057

shown promising results in removing pollutants at a steady pace (12). However, phytoremediation is often much slower than mechanical or chemical removal methods and takes up large amounts of land. Therefore, it is vital to improve the efficiency of these plants (13).

Aquatic plants play a crucial role when it comes to filtering chemicals from waste waters. *Lemna minor*, also known as duckweed, is one of the smallest aquatic flowering plants (14). *L. minor* reproduces by vegetative propagation, meaning it can spread quickly and efficiently (14). *L. minor* has also been shown to be a hyperaccumulator of many metals including sodium, lead, copper, zinc, iron, nickel, and aluminum (15). Its ability to reproduce quickly, its relatively large biomass when in large quantities, and its ability to retain chemicals without being harmed make it a potential wide-scale phytoremediator for microplastics and nanosilver.

Microbe-assisted phytoremediation is the process by which bacteria or fungi are inoculated with plants to improve the phytoremediation process and may improve the removal of microplastics and nanosilver (16). These methods are used to enhance plant growth, alleviate stress, and promote degradation of contaminants (17). Specifically, plant growth-promoting organisms have been shown to maintain and even increase chlorophyll production of plants. These microbes aggregate on the surfaces of plants in communities called biofilms and transfer filtered chemicals into the rizopheric section of the plant, the main source of root growth and activity, which then absorbs the chemicals (17). Certain bacteria can induce plant growth and protect the plant from pathogens (16).

Pseudomonas aeruginosa is a gram-negative, rod-shaped bacteria that is commonly found in soil and water (18). It has been shown to increase the accumulation of zinc in soil-living plants, indicating its tolerance to the heavy metal (19). *Bacillus subtilis*, is a gram-positive bacterium found in soil and marine sponges (20). Its high resistance to cadmium and manganese suggests a potential application in the phytoremediation of heavy metals, such as nanosilver (21). *Pseudomonas putida* is a type of bacteria that has been shown to decompose Polyethersulfone (PES), a type of plastic, and tolerate organic compounds and contaminant induced stress (22). These microorganisms are beneficial to land plants, so we wanted to investigate if they had similar benefits for an aquatic plant like *L. minor.*

We hypothesized that if *L. minor* was inoculated with *P. aeruginosa, B. subtilis,* and *P. putida*, it would be able to filter out larger quantities of pollutants and have increased levels of chlorophyll. Since such microbes and plants are known to work synergistically in land systems, it is possible for *L. minor* to also benefit from such bacteria. In addition, we predicted that the bacteria that create the thickest and most cohesive biofilm will also have the largest impact on accumulation of microplastics and nanosilver within *L. minor* biomass and adhesion of particles onto the surface. Then, because nanosilver shares many similar properties to metals that are known to be taken up by *L. minor*, and microplastics have shown to adhere to roots, we investigated those pollutants to see if they could be removed more efficiently from a simulated polluted water containing microplastics and nanosilver (12).

RESULTS

First, we determined how bacterial inoculation impacts the accumulation of nanosilver and microplastics in *L. minor.* We inoculated *L. minor* with *P. aeruginosa, B. subtilis, or P. putida* and exposed the plants to nanosilver or microplastics. After seven days of exposure, we measured the accumulation of nanosilver or microplastics within *L. minor* biomass via inductively coupled plasma spectrometry analysis and excitation of fluorescence. *L. minor* with no bacterial treatment, but exposed to nanosilver accumulated 328 ug/g of nanosilver. When inoculated with *B. subtilis* or *P. putida*, *L. minor* accumulated significantly higher concentrations of 402 ug/g and 425 ug/g of silver, respectively, at 7 days post-exposure (one-way ANOVA, $p < 0.05$). The accumulation of nanosilver was highest when *L. minor* was inoculated with *P. aeruginosa,* with an accumulation of 440 ug/g (one-way ANOVA, $p < 0.05$) **(Figure 1a)**.

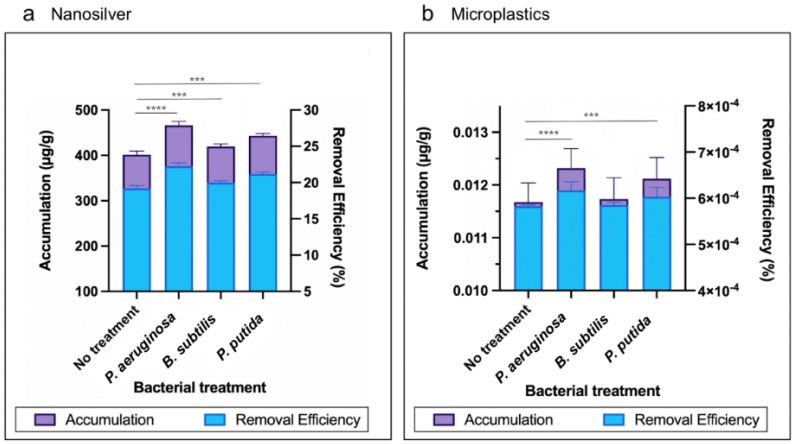

Figure 1. Accumulation and removal efficiency of pollutants in *L. minor.* Amount of pollutants accumulated within *L. minor*'s biomass. ICP-MS spectrometry was used to determine nanosilver concentration and fluorescence was used to determine microplastic concentration. Removal efficiency (%) and the accumulation (ug/g) of **a)** nanosilver and **b)** in combination with different bacterial treatments at seven days post exposure. The no treatment group was not inoculated with bacteria, and the treatment groups included L. minor exposed to 2 uL of 3 bacterial treatments. Removal efficiency was calculated using accumulation data, so significance statements are applicable using either metric. Data shown as mean ± standard error (n = 4). One-way ANOVA with Tukey's HSD; ***$p < 0.0005$, ****$p < 0.00005$.

https://doi.org/10.59720/23-057

L. minor exposed to no bacteria but microplastics accumulated 0.0116 ug/g. Similar to nanosilver accumulation, inoculation with *B. subtilis* and *P. putida* also resulted in minor increases in accumulation at 0.0117 ug/g and 0.01206 ug/g respectively at 7 days post exposure. The removal efficiency with *B. subtilis* was 0.000585%, and *P. putida* was 0.000603%, which indicates the percent of pollutants removed from the initial concentration placed in the simulated water. When exposed to microplastics, we found the highest concentration of microplastic accumulation when *L. minor* was inoculated with *P. aeruginosa* at 0.0123 µg/g (one-way ANOVA, $p < 0.05$) **(Figure 1b)**.

We next examined particle adsorption on the surface of *L. minor* using a scanning electron microscope. *L. minor* exposed to nanosilver with no bacterial treatment had only an average of eight or nine visible nanosilver particles **(Figure 2a)**. When inoculated with *B. subtilis,* this increased to 34 particles, inoculation with *P. putida* resulted in an average of 53 particles, and *P. aeruginosa* resulted in an average of over 70 particles adhered to the surface **(Figure 2b-d)**.

L. minor exposed to microplastics without bacteria had zero visible microplastic particles on the surface **(Figure 3a).** Similarly, for *B. subtilis,* there were no visible particles **(Figure 3c).** *L. minor* inoculated with *P. putida* had 13 particles on average, and inoculation with *P. aeruginosa* had 22 particles adhered **(Figure 3b and d).** These patterns show that inoculation with *P. aeruginosa* had the most visible adhered particles on the *L. minor* surface, followed by *B. subtilis* and *P. putida*.

To determine the effect of bacterial inoculation on *L. minor* plant growth, we measured chlorophyll content in *L. minor* by spectrophotometry. The chlorophyll content of *L. minor* exposed to no pollutants was 4.17 mg/mL **(Figure 4a).** When put into an environment only with silver, this decreased to 3.03 mg/mL. This large decrease in chlorophyll production can be detrimental to the plant and make it ineffective for long term phytoremediation. With the inoculation of *L. minor* with *P. aeruginosa, B. subtilis,* and *P. putida*, chlorophyll increased back to 3.36 mg/mL, 3.26 mg/mL, 3.78 mg/mL respectively.

L. minor exposed to only microplastics experienced significant damage, shown by decreased chlorophyll content, compared to the control (one-way ANOVA, $p < 0.05$) at 2.86 mg/mL **(Figure 4b).** Similar to when the plants were exposed to nanosilver, these numbers rebounded to normal chlorophyll levels when *L. minor* was inoculated with any of the three bacterial species. With the addition of *P. aeruginosa, B. subtilis,* of *P. putida*, chlorophyll content rose to 3.76 mg/mL, 3.96 mg/mL, and 4.15 mg/mL respectively. Bacterial inoculation increased chlorophyll content when exposed to pollutants, suggesting the ability of the microorganisms to increase *L. minor's* tolerance to microplastics and nanosilver.

Bacterial biofilms have been shown to play a major role in the microbe-assisted phytoremediation process, so biofilm production of the bacteria was quantified to compare to previous accumulation, adhesion, and chlorophyll content results. The bacterial samples that read at the highest absorbency created the strongest and most active biofilm. *P. aeruginosa* had the highest biofilm production with an absorbance of 1.73 au. *P. putida* had an absorbance of 0.56 au, and *B. subtilis* 0.16 au **(Figure 5).** *P. aeruginosa* had a significantly stronger biofilm compared to *P. putida* and *B. subtilis* (one-way ANOVA, $p < 0.05$).

Zebrafish embryos were exposed to the initial and post-remediation concentrations of pollutants to determine whether the amount of pollutants removed could significantly decrease the toxicity of water. The initial concentration of pollutants was 1 mg/mL for both nanosilver and microplastics, while the final concentration, post-phytoremediation, for nanosilver was 0.8 mg/mL, and microplastics was 0.99 mg/mL, which was determined using the averages of removal efficiencies between the 3 bacterial treatments. The group of fish exposed to no chemicals developed with almost no deformities, meaning regular heartbeats, hatching, and anatomy **(Figure 6a).** However, when exposed to nanosilver-containing water at 1 mg/mL, this increased to an average of a 37% abnormality rate **(Figure 7a)**. When exposed to nanosilver-containing

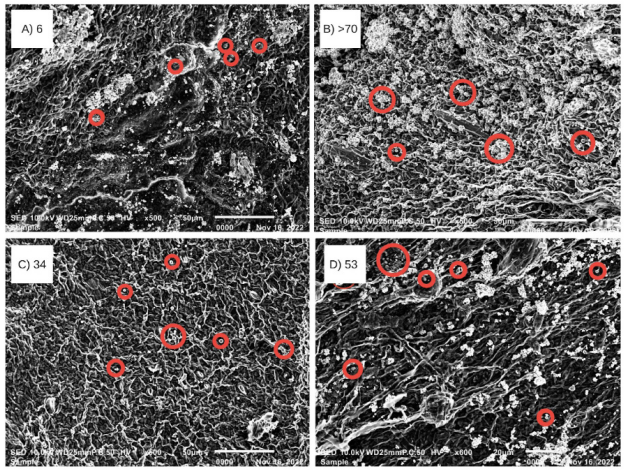

Figure 2. Scanning electron microscopy images at 500x of adhered nanosilver on *L. minor* surface. *L. minor* exposed to **a)** no bacterial treatment, **b)** *P. aeruginosa,* **c)** *B. subtilis,* and **d)** *P. putida.* Numbers on each panel indicate the number of particles on the surface, based on three replicates. Red circles indicate nanosilver particle presence.

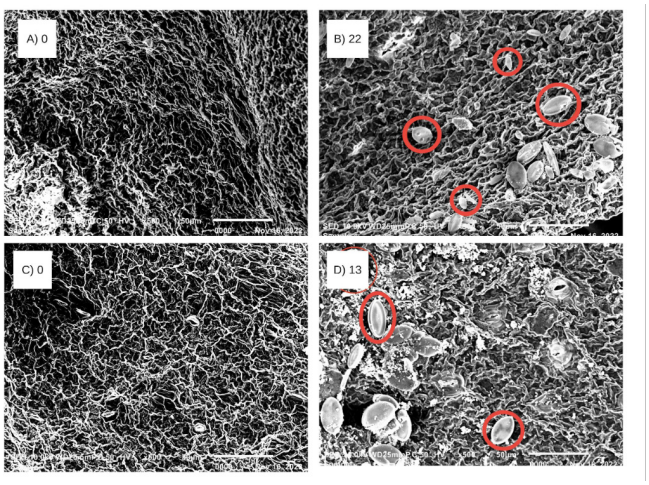

Figure 3. Scanning electron microscopy images at 500x of adhered microplastics on L. minor **surface.** *L. minor* **e**xposed to **a)** no bacterial treatment, **b)** *P. aeruginosa,* **c)** *B. subtilis***, and d)** *P. putida.* Numbers on each panel indicate the number of particles adhered on the surface, based on three replicates. Red circles indicate microplastic presence.

https://doi.org/10.59720/23-057

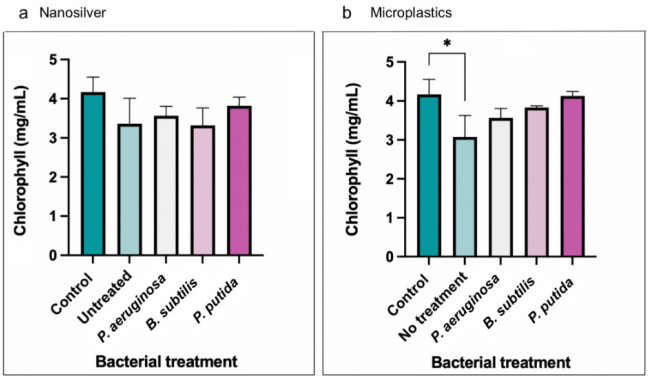

Figure 4. Chlorophyll content of *L. minor* exposed to pollutants. After being exposed to the pollutants with and without bacterial inoculation, chlorophyll content was recorded to show toxicity. Chlorophyll content (mg/mL) within *L. minor* biomass of plants in standard SHS media after being exposed to a) nanosilver or b) microplastics. The no treatment group was not inoculated with any bacteria, and the treatment groups included *L. minor* exposed to 2 uL of 3 bacterial treatments. Data shown as mean ± standard error (n = 4). One-way ANOVA, *p < 0.05.

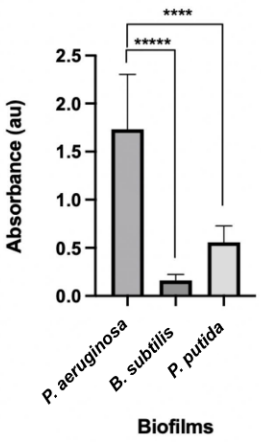

Figure 5. Biofilm formation by different species of bacteria. Crystal violet absorption at 550 nm of different biofilms created with *P. aeruginosa, B. subtilis*, and *P. putida*. Biofilms were created through incubation, stained with crystal violet stain, and the absorbance was read to determine biofilm formation. Data shown as mean ± standard error (n = 8). One-way ANOVA, ****p < 0.00005, and ******p < 0.000005.

water after pollutant accumulation by *L. minor* (0.8 mg/mL), this significantly decreased to a 27% deformity rate, including a noticeable decrease in the severity of deformities (t-test, p < 0.05) **(Figure 6b-c, Figure 7a)**. When exposed to 1 mg/mL of microplastic-containing water, the deformity rate was 33% and stayed the same when exposed to the final concentration **(Figure 6d-e, Figure 7b)**. This was expected because the amount of microplastics removed (0.01mg/mL) is likely not great enough to significantly decrease the toxicity of water.

DISCUSSION

In the present study, we sought to investigate the plant growth-promoting properties of three bacteria to enhance efficacy of *L. minor* to remove nanosilver and microplastics through pollutant-accumulation and adhesion. Then, these results were confirmed by testing chlorophyll content, biofilm formation, and investigating changes in water toxicity through in vivo testing.

First, we observed accumulation of pollutants by using ICP-MS spectrometry and fluorescence quantification. When *L. minor* was inoculated with *P. aeruginosa*, it significantly increased the accumulation of nanosilver and microplastics. This correlates with research conducted by Shi et al., where land plants grown with *P. aeruginosa* could accumulate more pollutants compared to just the land plant individually (19). This was similar for inoculation with *B. subtilis* and *P. putida*. *L. minor* could remove nanosilver at a 20% efficiency and microplastics at a 0.0005% efficiency at baseline where, in both cases, these removal percentages increased with bacterial inoculation. This all supports the idea that *L. minor* inoculation with any of the three tested bacteria results in more efficient pollutant removal compared to the plants alone. *L. minor* was most likely able to accumulate these amounts because of biofilm production. It is important to note that the amount of microplastics accumulated was most likely due to their adherence to the surface of *L. minor*, as their large size makes them unable to enter the plant. This is why the removal efficiency for microplastics was significantly lower compared to silver. Thus, *L. minor* was not able to remediate through phytoaccumulation, but rather through interactions between the surface of the plant and microplastics.

As stated before, bacterial biofilms are vital for plant growth promotion. The bacterial biofilm assay measured the amount of crystal violet absorbed by the biofilms to assess bacterial attachment as aggregates. *P. aeruginosa* created the most — biofilm, which was significantly stronger compared to *P. putida and B. subtilis*. This directly correlates with the data showing that *P. aeruginosa* inoculation led to an increase in *L. minor* pollutant accumulation. *P. aeruginosa* was shown to be beneficial for both accumulation and protection against harmful pollutants. This supports the hypothesis that the bacteria that make the best biofilm would correlate with plant growth promotion. While these results do not directly show that the bacteria created these biofilms on *L. minor* fronds, we have shown that bacterial inoculation impacts plant pollutant uptake and growth.

Then, we quantified the number of particles adhered to the surface of *L. minor* to observe if bacterial inoculation can assist in the removal of pollutants through an alternative mechanism of adherence. This was done using scanning electron microscopy and observing the area of the plant with the most particles attached. In general, *L. minor* exposed to bacteria had many more nanosilver particles adsorbed to the surface. Although there were no microplastics adhered to the surface of *L. minor* inoculated with *B. subtilis*, plants exposed to *P. aeruginosa* and *P. putida* did have significantly more particles adhered compared to plants without any bacterial inoculation. The results of both groups show that when *L. minor* was grown with bacteria, more particles adhered to the plants' surface. This is vital in removing pollutants, especially if they are too large to enter plant biomass.

We measured the chlorophyll content of *L. minor* to ensure that the bacteria were ultimately promoting plant and decreasing contaminant related stress. When *L. minor* was exposed to both the pollutants, even with the bacteria, chlorophyll content went down relative to the no pollutant

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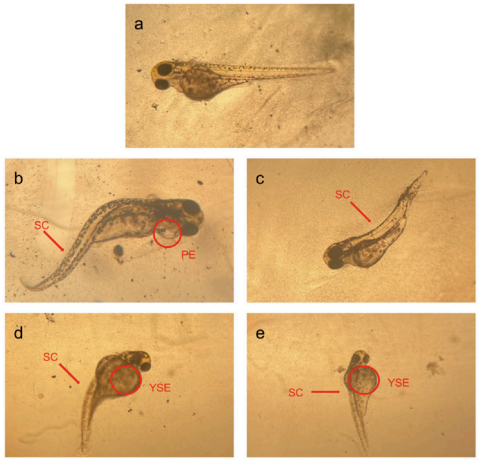

Figure 6. Deformity observations in zebrafish embryos exposed to pollutants. Red circles indicate deformities. SC = spinal curving, PE = pericardial edema, and YSE = yolk sac edema. **a)** Negative control embryo exposed to no pollutants; there are no deformities present. **b)** Embryo exposed to nanosilver at the starting concentration of 1 mg/mL where there are multiple deformities present. **c)** Embryo exposed to the after concentration of nanosilver; there are some deformities present. **d)** Embryo exposed to the before concentration of microplastics where there are many deformities present. **e)** Embryo exposed to the after concentration of microplastics where there still appear to be many deformities. In addition to deformities, heartbeat, hatching, and deformity rates were measured.

control, suggesting that the pollutants still caused damage. This can be a result of high concentrations of pollutants during testing. However, the addition of bacteria resulted in higher chlorophyll content compared to no bacterial treatment. These results support the hypothesis that bacteria would limit damage done to the plant. This suggests that these bacteria do indeed have plant growth-promoting properties that protect and build resilience against harmful pollutants.

Zebrafish embryos are commonly used model organisms because they share many of the same major organs and are genetically similar (23). Embryos were used to determine whether the amount of pollutants removed truly had an effect on the development of embryos. The amount of nanosilversilver removed did indeed have a significant effect in how zebrafish embryos developed. However, when exposed to microplastics, hatching rates and heart rate stayed low and deformities remained at high rates. This indicates that the amount of microplastics removed was not enough to have a significant benefit to the development of zebrafish embryos. The reason why there was such a drastic decrease in zebrafish ambormatilues when comparing the initial and final concentrations of nanosilver may be due to the large quantity of pollutants that were removed due to hyperaccumulation.

Although this research is a step in improving the efficacy of removing harmful pollutants from wastewater, there are multiple limitations that challenge its validity. First, the experimental setup of the accumulation assay lacked a negative control showing what would occur if there was no plant or bacteria. This is relevant since the amount of pollutants retained by *L. minor* will never be 100% of the starting pollutant amount. The bacteria in this research can also be considered pollutants. This means this technology could not be applied in natural aquatic ecosystems since these bacteria could produce unintended consequences such as the spread of these bacteria to humans or marine organisms. Also, while these results do not directly show that the bacteria created biofilms on *L. minor* fronds, it can be assumed that they still did have an impact on plant uptake and growth. In addition, it can be difficult to find a viable area to dispose of these plants. Current methods include incineration or composting, which can create secondary pollutants.

There are many future prospects in the field of phytoremediation. Less harmful and more natural bacteria can be investigated for their biofilm forming properties. Such bacteria could allow this technology to be applied in aquatic environments where animals may come into contact with pollutants. Different concentrations of bacteria are also important to consider since there may be a point where *L. minor* and the microorganisms may no longer work effectively.

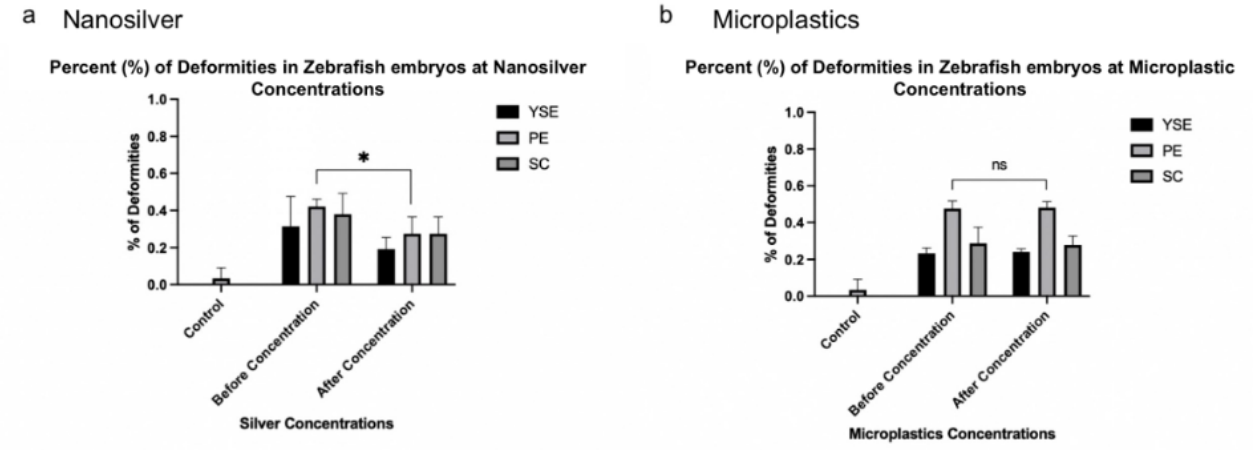

Figure 7. Deformity percentages in zebrafish embryos exposed to the before and after phytoremediation concentration of pollutants. SC = to spinal curving, PE = pericardial edema, and YSE = yolk sac edema. **a)** Percentage of deformities in zebrafish embryos exposed to no nanosilver (control), the concentration of nanosilver before remediation, and after remediation. **b)** Shows the percentage of deformities in zebrafish embryos exposed to no microplastics (control), the concentration of microplastics before remediation, and after remediation. Data shown as mean ± standard error (n = 4). One-way ANOVA, *p < 0.05.

https://doi.org/10.59720/23-057

In addition, there are many more aquatic plants with larger biomasses that can be researched in combination with the bacteria. Also, nanosilver and microplastics are just a few of the many particles that enter water systems and contaminate drinking water. Doing more tests to assess the damage done by pollutants and recovery with bacteria, like histology or COMET assays to assess DNA damage, may provide more insight into whether the changes in chlorophyll reflect changes in plant health.

Phytoremediation is an effective technology that has the potential to remove these waste like microplastics and nanosilver from primary wastewater treatment. The findings of this research suggest the strong possibility of enhancing phytoremediation properties with plant growth promoting bacteria. This technology can be applied in wastewater treatment plants, after sludge separation and before chlorination. This would allow for small particles to be removed efficiently, cost-effectively, and in an environmentally-friendly manner.

MATERIALS AND METHODS

L. minor care and inoculation

L. minor (Carolina Biological) was grown in sterile Schenk-Hildebrandt medium supplemented with 1% w/v sucrose (SHS) (Sigma-Aldrich) in plastic containers under LED grow lights and remained in these environments for reproduction until an appropriate sample size was obtained. Bacterial cultures (*P. aeruginosa, P. putida, and B. subtilis)* (Carolina Biological) were grown in 2 mL of LB Broth (Fisher Scientific) in 15 mL conical tubes for 18 hours at 30°C shaking at 225 rpm. Five grams of *L. minor* were sterilized in 10% bleach for 10 seconds, transferred to 70% ethanol (Sigma-Aldrich) and submerged for 10 seconds, and recovered in SHS. Each well of this 24-well plate was filled with 2mL of SHS and 5 - 7 plant fronds. 5 mL of an overnight bacterial culture was centrifuged (*P. aeruginosa, B. subtitles, and P. putida)* for 5 minutes at 5000 x g, and the cell pellet was resuspended in 1 mL of SHS. 2 µL of the bacterial suspension was placed in each well with the fronds in it. The same bacterial suspension was used for all groups and replicates. 2 mg of fluorescent microplastics (Cospheric) or nanosilver (Sigma-Aldrich) was placed into each well after bacterial inoculation. The plants remained in the created environments for one week, and four samples of the plant fronds and roots were taken at two, four, and seven days post-inoculation.

Determining accumulation of microplastics and nanosilver

0.5 g of plant samples, including frond and root taken at two, four, and seven days after exposure, were washed multiple times with distilled water. Samples were oven dried at 70°C and measured until a constant weight was obtained. Four different samples were collected to provide an adequate sample size. A mortar and pestle were used to grind plant material into a powder. Plant material was digested with 10 mL of HNO_3–$HClO_4$ in the ratio of 2:1 respectively (Sigma-Aldrich, Fisher Scientific) on a hot plate by slowly raising the temperature until it reached 70°C. This was then decanted into a graduated cylinder where the volume was brought up to 50 mL. For quantifying microplastic concentration, a standard curve was created by using increasing amounts of fluorescent microplastics. 200 uL of each sample was placed in a 96-well plate and fluorescence was read in a fluorometer (excitation: 515 nm). For quantifying nanosilver concentration, a standard curve was made by using increasing amounts of nanosilver and reading in the ICP-MS. Part per billion (ppb) concentration was determined by placing 50 mL of each sample in the ICP cuvette. The number of particles in the plant was calculated using the dilution factor in the equation below:

$$Metal\ (ug/g)\ in\ plant\ =\ metal\ reading\ of\ digested\ sample\ (mg/L)\ \times\ dilution\ factor$$

Where the dilution factor is equal to:

$$\frac{total\ volume\ of\ sample\ (mL)}{weight\ of\ plant\ material\ (g)}$$

The percentage efficiency was calculated from initial and remaining concentration of metal according to Tanhan et. al (22):

$$\%\ efficiency\ =\ (C_0\ -\ C_1/C_0)\ \times\ 100$$

Where C_0 is the initial concentration and C_1 is the remaining concentration in the medium (mg/L). The final concentration reported was taken after seven days of exposure.

Determining adhesion of chemicals

Three *L. minor* fronds from each experimental group were observed and analyzed with scanning electron microscopy. The tissue was fixed in 100% methanol for 10 minutes or longer and was vacuum infiltrated with a syringe if it did not sink immediately. These plants were transferred to 100% ethanol and allowed to dry overnight until all liquid was evaporated. These samples were placed under a scanning electron microscope (Model JSM-IT100) and the number of particles adhered to the surface (nanosilver and microplastics) was counted on the most populated space.

Biofilm Assay

Cultures of *P. aeruginosa, B. subtilis, and P. putida* were grown in LB Broth. The overnight culture was diluted at a 1:100 ratio into M63 minimal medium (BioBasic) and 100µL of this dilution was added per well in a 96-well dish. Eight samples were distributed for each bacterial sample. The microtiter plate was incubated for 24 hours at 37°C in a bacterial incubator. The plate was submerged in a small tub of water, and the water was then shaken out. 125 µL of a 0.1% solution of crystal violet (Sigma-Aldrich) was added to each well of the plate. The microtiter plate was incubated at room temperature for 10-15 minutes. 125 µL of 30% acetic acid (Fisher Scientific) in water was pipetted to each well of the microtiter plate. The microtiter plate was incubated at room temperature for 10-15 minutes. 125 µL of the solubilized crystal violet stained sample was pipetted to a new flat bottomed microtiter dish. The absorbance was quantified in a plate reader at 550 nm with 30% acetic acid as the blank.

Chlorophyll Assay

A mass of 0.2 g of fresh weight plant material was weighed on a scale. 2 mL of extraction solution (ammonium hydroxide, Sigma-Aldrich; 80% acetone, Fisher Scientific; distilled water) was added and ground with a pestle while

https://doi.org/10.59720/23-057

being kept chilled over ice for approximately 30 seconds until the tissue was a fine slurry. 3 mL of extraction solution was poured over the pestle into the homogenization tube. This mixture was refrigerated for two hours and reground to extract any remaining chlorophyll. 5 mL of 80% aqueous acetone solution was pipetted into the sample, poured into a centrifuge tube, and centrifuged for 20 minutes at high speed (approximately 500 x g). The supernatant was decanted and the volume was brought to 10 mL with 80% aqueous acetone. The 80% aqueous acetone was used as the blank to zero the instrument. All samples analyzed were read at 645 nm and 663 nm.

Chlorophyll test Calculations

$$Chlorophyll\ a\ (mg/mL) = 12.7\ A663 - 2.69\ A645$$

$$Chlorophyll\ b\ (mg/mL) = 22.9\ A645 - 4.68\ A663$$

where:

$$A645 = absorbance\ at\ a\ wavelength\ of\ 645\ nm;$$

$$A663 = absorbance\ at\ a\ wavelength\ of\ 663\ nm$$

$$Total\ Chlorophyll\ (mg/mL) = Chlorophyll\ a + Chlorophyll\ b$$

Zebrafish Embryo Toxicity

2 mL of zebrafish medium (University of Miami) was pipetted in each well of a 12 well plate. Then, 1 mg/mL of either nanosilver or microplastics was added to each well. In another 12 well plate, pollutant-containing water, using the average concentrations of pollutants after exposure to *L. minor*, was set up. No bacteria were added for this assessment. Also, a control plate was created with only two mL of zebrafish medium. Five zebrafish embryos (University of Miami) were pipetted in each well for each group. At four, five, and six days post-fertilization, hatching rates were observed by counting the number of embryos that hatched out of their protective chorion layer, the thin tissue surrounding the embryo. At seven days post-fertilization, the heartbeat rates were observed by counting the number of heartbeats in ten seconds. This included pumps of zebrafish blood through the pericardium region. Lastly, at seven days post-fertilization deformities, including yolk sac edema, pericardial edema, and spinal curving, of zebrafish were observed, based on reference pictures from Zhou et al. (23).

Statistical Analysis

The number of trials varied with the assay. The accumulation and assays had 3 trails, the chlorophyll and zebrafish embryo toxicity utilized 4 trials, and the biofilm assay has 8 trials. All statistical analyses were performed using Excel's Analysis Toolpak. We used one-way ANOVAs to compare all experimental groups. A p-value threshold of 0.05 was used, where values less than 0.05 were considered statistically significant. Using Tukey's HSD post hoc t-Test, experimental groups were compared against the control group.

ACKNOWLEDGEMENTS

We would like to thank American Heritage School Plantation for the materials and equipment provided, Dr. Ping Jiang for her assistance in using FIU's ICP-MS, and American Heritage School Boca Delray for their help in using their scanning electron microscope.

Received: March 17, 2023
Accepted: October 4, 2023
Published: April 1, 2024

REFERNCES

1. You, Siming. "Covid-19's Unsustainable Waste Management." *Science*, 2020, https://doi.org/10.1126/science.abc7778.
2. Shemitz, Leigh. "Yale Experts Explain Microplastics." 2020, www.sustainability.yale.edu/explainers/yale-experts-explain-microplastics.
3. Chen, Xianchuan, et al. "Used disposable face masks are significant sources of microplastics to environment." *Environmental Pollution*, vol. 285, no. 117485, Sep. 2021, https://doi.org/10.1016/j.envpol.2021.117485.
4. Koelmans, Albert A., et al. "Microplastics in freshwaters and drinking water: Critical review and assessment of data quality." *Water Research,* vol. 155: 410-422. 2019, https://doi.org/10.1016/j.watres.2019.02.054.
5. Campanale, Claudia, et al. "A Detailed Review Study on Potential Effects of Microplastics and Additives of Concern on Human Health." *International Journal of Environmental Research and Public Health*, vol. 17,4 1212. 13 Feb. 2020, https://doi.org/10.3390/ijerph17041212.
6. Ferdous, Zannatul and Abderrahim Nemmar. "Health Impact of Silver Nanoparticles: A Review of the Biodistribution and Toxicity Following Various Routes of Exposure." *International Journal of Molecular Sciences* vol. 21,7 2375. 30 Mar. 2020, https://doi.org/10.3390/ijms21072375.
7. Seltenrich, Nate. "Nanosilver: weighing the risks and benefits." *Environmental Health Perspectives*, vol. 121, 2013, https://doi.org/10.1289/ehp.121-a220.
8. "Nanosilver: Environmental Effects." *Beyond Pesticides*, www.beyondpesticides.org/resources/antibacterials/nanosilver/environmental-effects. Accessed 15 Dec. 2023.
9. Kaur, Leela, and Nupur Kanwar. "Duckweed: A Model for Phytoremediation Technology." *The Holistic Approach to Environment, 12*, 39-58. 2022, https://doi.org/10.33765/thate.12.1.4.
10. LaBlanca, Sophia. In Phytoremediation, Plants Extract Toxins from Soils. 18 Nov. 2020, daily.jstor.org/in-phytoremediation-plants-extract-toxins-from-soils/.
11. Fertig, Walter. "Common Duckweed (*Lemna Minor*)." *U.S. Forest Service*, www.fs.fed.us/wildflowers/plant-of-the-week/lemna_minor.shtml.
12. Sharma, Jitendra Kumar et al. "Phytoremediation technologies and their mechanism for removal of heavy metal from contaminated soil: An approach for a sustainable environment." *Frontiers in Plant Science* vol. 14. 27 Jan. 2023, https://doi.org/10.3389/fpls.2023.1076876.
13. Gałczyńska, Małgorzata. "Possibilities and Limitations of Using *Lemna Minor*, *Hydrocharis Morsus-Ranae* and *Ceratophyllum Demersum* in Removing Metals with Contaminated Water." *Journal of Water and Land Development*, vol. 40. 2019, pp. 161–173., https://doi.org/10.2478/jwld-2019-0018.
14. Bogino, Pablo C et al. "The role of bacterial biofilms and surface components in plant-bacterial associations."

https://doi.org/10.59720/23-057

International Journal of Molecular Sciences, vol. 14,8 15838-59. 30 Jul. 2013, https://doi.org/10.3390/ijms140815838.
15. Chandra, Harish et al. "Plant growth promoting *Pseudomonas aeruginosa* from *Valeriana wallichii* displays antagonistic potential against three phytopathogenic fungi." *Molecular Biology Reports,* vol. 47,8. 2020, 6015-6026. https://doi.org/10.1007/s11033-020-05676-0.
16. He, Chi, and G.E. Tan. "Effect of Zn-tolerant Bacterial Strains on Growth and Zn Accumulation in *Orychophragmus Violaceus*." *Applied Soil Ecology*, Jan. 2010, https://doi.org/10.1016/j.apsoil.2009.07.003.
17. Piggot, P.J. "Bacillus Subtilis." *Encylopdeia of Microbiology (Third Edition)*, 2009, https://doi.org/10.1016/B978-012373944-5.00036-5.
18. Luo, et al. "Analysis and characterization of cultivable heavy metal-resistant bacterial endophytes isolated from Cd-hyperaccumulator *Solanum nigrum* L. and their potential use for phytoremediation." *Chemosphere*. 2011, https://doi.org/10.1016/j.chemosphere.2011.07.053
19. Shi, et al. "Exogenous Pseudomonas Aeruginosa Application Improved the Phytoremediation Efficiency of Lolium Multiflorum Lam on Cu–Cd Co-contaminated Soil." *Environmental Technology and Innovation,* vol 27, August 2022, https://doi.org/10.1016/j.eti.2022.102489.
20. Brandenberg, Schubert, et al. "Towards synthetic PETtrophy: Engineering *Pseudomonas putida* for concurrent polyethylene terephthalate (PET) monomer metabolism and PET hydrolase expression." *Microb Cell Fact*, 2022, https://doi.org/10.1186/s12934-022-01849-7.
21. Teame, Tsegay, et al. "The Use of Zebrafish (*Danio Rerio*) as Biomedical Models". *Animal Frontiers*, vol. 9, no. 3 July 2019, pp. 68–77, https://doi.org/10.1093/af/vfz020.
22. Tanhan, et al. "Uptake and accumulation of cadmium, lead and zinc by Siam weed [*Chromolaena odorata* (L.) King & Robinson]." *Chemosphere* vol. 68, 2007, https://doi.org/10.1016/j.chemosphere.2006.12.064.
23. Zhou, Yimeng, et al. "Toxicity Effects of Captan on Different Life Stages of Zebrafish (*Danio rerio*)." *Environmental Toxicology and Pharmacology*, vol. 69, July 2019, https://doi.org/10.1016/j.etap.2019.04.003.