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Impacts of Nanoplastics on the Viability and Riboflavin Secretion in the Model Bacteria *Shewanella oneidensis*

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Characterizing the impact of nanoplastics to organism health is important to understand the consequences of the environmental plastic waste problem. This article examines the impact of nano-polystyrene (nano-PS; 159 ± 0.9 nm diameter) to ecologically relevant bacteria *Shewanella oneidensis*. Bacterial viability was evaluated using a growth-based assay. Riboflavin secretion is a critical cell function of *S. oneidensis*, serving as an electron mediator in anaerobic respiration and/or as a signaling molecule when the bacteria are under stress. Thus, changes in cellular function were monitored through riboflavin secretion in order to evaluate toxic responses that may not result in cell death. Under aerobic and anaerobic exposures (4, 8, or 12 h), the viability of the *S. oneidensis* was minimally changed as compared to the control, while the concentration of riboflavin secreted varied with exposure dose. In order to determine if this was a specific response to nanoplastic particles, opposed to a response to either particles or plastic more generally, we exposed the system to colloidal TiO_2 nanoparticles and polystyrene and polyethylene thin films. We confirmed that riboflavin secretion trends were specific to nano-PS and not to these other materials, which showed no significant changes. We investigated the association of the nano-PS with ICP-MS using Pd that was chemically incorporated into the model nanoplastics. While 59.2% of the nano-PS were found in the non-cellular culture media, 7.0 and 6.6% was found associated with the loosely and tightly bound extracellular polymeric substance, respectively. There was significantly more nano-PS (10.9%) strongly associated with the cells. Taken together, we found that nano-PS had minimal impacts to viability but caused a significant change in the function of *S. oneidensis* that can be related to the nano-PS attached or in proximity to the bacterium. These trends are consistent between aerobic and anaerobic cultures, signifying that the stress response of *S. oneidensis* can be generalized between different environmental compartments. This work highlights that the association of nanoplastic materials with microorganisms may modify the cellular function that could ultimately be an impact to ecosystem health.

Keywords: nanoplastic, viability, functional response, polystyrene, association

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INTRODUCTION

Plastic-particle distributions in marine and freshwater systems are an active area of research, with most current work centered on the size distribution, polymer type, and number of particles above 1 micron (Wagner et al., 2014; Zbyszewski et al., 2014; Driedger et al., 2015; Hendrickson et al., 2018; Allen et al., 2019), and estimates suggest there could be up to 125 trillion microplastics in ocean water (Lindeque et al., 2020). Particulate plastics (nano- and microplastic particles, fragments and fibers) pose a concern for organisms in our environment because they can cause a myriad of health outcomes, such as impacting the gill cavity of crab *Carcinus maenas* causing altered O_2 consumption (Watts et al., 2016) or causing inflammation of the liver tissue of zebrafish (Lu et al., 2016). Exposure to microplastics is often through the ingestion pathway because the size range overlaps with that of phytoplankton in both fresh and salt waters, which has resulted in the ingestion of microplastic particles by invertebrates and fish (Imhof et al., 2016). Ingestion is thought to pose challenges to various organisms such as false satiation, leading to nutritional deficits, (Welden and Cowie, 2016; Yin et al., 2018) tissue inflammation (Lu et al., 2016) or toxicity of additives leaching from plastic into tissue (Rochman et al., 2012; Tanaka et al., 2015). While environmental microplastic hazards are concerning, there is growing worry for the impact of nanoplastics in the environment as laboratory studies have shown that they are likely prevalent within these natural systems (Koelmans et al., 2015; Gangadoo et al., 2020; Hebner and Maurer-Jones, 2020). Nanoplastics are particularly concerning with regard to ecosystem health because they have the potential to permeate cell membranes and disrupt cellular behavior (Rossi et al., 2014) while also having a larger surface area-to-volume ratio, increasing the additive leaching potential (Bouwmeester et al., 2015; Hermabessiere et al., 2017).

Nanoparticle toxicity, largely focused on engineered inorganic materials, has been widely studied with the growing demand of novel nanomaterials along with their incorporation into commercially available products. Efforts in this arena have established the framework for studying the interaction between nanoparticles and organisms at various trophic levels (Maurer-Jones et al., 2013b; Zhang et al., 2018) and there are substantial efforts to have the toxicity response to inorganic nanomaterials inform nanoparticle design (Buchman et al., 2019a). Yet, there is much less understood about the toxicity of nanoplastics. The most studied nanoplastic in terms of toxicity is nanoparticle polystyrene (nano-PS) because of its commercial availability with a variety of controlled size ranges and surface chemistries. Many nano-PS toxicity studies have focused on a range of ecologically relevant, multi-cellular model organisms (Besseling et al., 2014; Della Torre et al., 2014; Greven et al., 2016; Chen et al., 2017; Pitt et al., 2018) but there is a gap in knowledge in the toxicity of nano-PS to single-celled organisms.

Toxicity evaluation of single-celled microorganisms is critical because these organisms provide a bedrock of the ecosystems. To date, studies of nano-PS toxicity has primarily focused on algal species. For example, algae exposed to nano-PS have demonstrated a decrease in photosynthetic activity due to their adsorption onto the surface of the algae, and thus screening sunlight (Bhattacharya et al., 2010). Beyond the toxic implications for the algae itself, sorption to the surface can also be the first step within the food web for bioaccumulation of nanoplastic, moving from algae to water fleas to carp (Cedervall et al., 2012). These studies of photosynthetic activity changes and trophic transfer highlight the important role that microorganisms play within the context of nanoplastic hazards to larger organisms. It is expected, due to the close algal-bacterial relationship (Ramanan et al., 2016), that the influence of nano-PS to bacteria systems could be similar, including changes to the primary productivity from these organisms, the transfer of nano-PS to larger organisms through ingestion, or changes in health to other organisms that rely on the cellular function of the microorganisms (Azam and Malfatti, 2007). Yet, there is not a wide understanding of the impact of nano-PS to ecologically relevant bacteria. In bacteria models, Fu et al. (2018) showed nano-PS causes a decrease in growth and metabolism of *Acetobacteroides hydrogenigenes*, a species relevant to anaerobic digestion. Qu and coworkers studied the impact of micro- and nano-PS to marine bacteria *Halomonas alkaliphilia* and showed both sizes of particles inhibited growth at high concentrations but that nano-PS also negatively impacted the ammonia conversion efficiencies of the bacteria (Sun et al., 2018). There is still a lot to be understood about the impact of nano-PS to other ecologically relevant bacteria, including where the nanoplastics are in relationship to the bacteria (i.e., associated to or localized within the bacteria) and/or the response to nano-PS from a bacterial species in a range of growing conditions such as aerobic and anaerobic settings.

Shewanella oneidensis is an ideal model bacterium for nanoplastic studies because they are a facultative anaerobe (i.e., they can switch between anaerobic and aerobic respiration), are distributed worldwide and live in varied environmental conditions (e.g., variable salt concentrations and/or temperatures) (Hau and Gralnick, 2007). The fact that these bacteria live in such diverse conditions means it is likely that the organism will encounter nano-PS in a natural system. Additionally, their adaptability may aid in ecosystem modeling as their response may inform our understanding of the impact of nano-PS to similar organisms. In anaerobic conditions, *Shewanellae* have a unique ability to respire on compounds found in the environment, such as reducing iron to its bioavailable state [Fe(III) to Fe(II)] (Valls and De Lorenzo, 2002; Hau and Gralnick, 2007; Shi et al., 2007), which contribute to the process of metal cycling that other organisms within the environment rely on for their survival (Sheng and Fein, 2014; Wang et al., 2016). The metal reduction function is facilitated through the secretion of flavin mediators, including riboflavin and flavin mononucleotide. Riboflavin secretion is a critical cell function of *S. oneidensis*, serving as both an electron mediator in anaerobic respiration and as a signaling molecule when the bacteria are under stress in both aerobic and anaerobic environments (Brutinel et al., 2013; Oram and Jeuken, 2019). Finally, this model organism has been used in previous nanoparticle toxicity assessments and thus provides precedent benchmarks for toxicity responses that go beyond only live versus dead cell counts, with

evaluation of how substances induce changes of cellular function and gene expression (Maurer-Jones et al., 2013a,c; Zhi et al., 2019; Buchman et al., 2020; Clement et al., 2020). Taken together, these advantages provide an improved understanding as to what cellular processes are most impacted and how this will in turn influence the surrounding environment.

To address the gaps in knowledge of nanoplastic toxicity to single-celled organisms, we evaluated the impacts of nano-PS on the model bacteria *S. oneidensis* MR-1. The toxicity evaluation included assessments of the viability and riboflavin secretion and iron reduction upon exposure to varied concentrations of nano-PS under both aerobic and anaerobic culture conditions. These sublethal endpoints are critical to more completely evaluate the environmental risk of nanoplastics. While previous work discussed above had characterized inorganic nanoparticle toxicity in aerobic conditions, this is the first study of toxicity to *S. oneidensis* under anaerobic conditions. We hypothesized that the markers of respiration (i.e., riboflavin secretion) would be varied between the two culture conditions, thus suggesting that nano-PS interrupts the signaling and iron reduction capabilities. To better understand the changes observed in cellular function, we evaluated the association of the nano-PS with the cells using ICP-MS, taking advantage of a Pd-label incorporated into the particle. While increasing understanding of nano-PS impacts to bacteria, this work provides an example of toxicity evaluation that considers diverse culture conditions (aerobic and anaerobic) that could enable generalization of the nano-PS toxicity response.

MATERIALS AND METHODS

A suite of analyses was used to assess the impact of nano-PS to *S. oneidensis*. The approach of our analyses is shown in **Figure 1**.

Exposure Materials

Nano-PS was synthesized and characterized as previously described in Mitrano et al. (2019). Briefly, a polyacrylonitrile material containing a chemically entrapped Pd-tracer was capped with crosslinked polystyrene shell, ultimately resulting in a core/shell nanoplastic, which was approximately 160 nm in diameter. On average, the nano-PS contained 0.49% Pd, which equated to 5.1×10^{-9} ng Pd per nano-PS particle. The nanoplastic particle demonstrated a high stability, with no leaching of the Pd tracer in complex matrices. Therefore, we are confident the interaction of the bacteria is with the polystyrene shell material and all impacts to bacterial cell viability and function is due to the plastic, and not the metal tracer. *S. oneidensis* were exposed to other materials including TiO_2 nanoparticles (25 nm, anatase; Aldrich, Burlington, MA, United States) and polystyrene (30 μm thick) and polyethylene (25 μm thick) thin films (Goodfellow, Huntington England). Thin films were pre-processed by soaking in n-hexane, methanol, and water, each for 24 h prior to bacteria experiments to remove potential contaminants from the commercially acquired films that could cause bacterial toxicity. A single 1 × 1 cm piece (∼3 mg) of pre-soaked and dried film was placed directly into the bacterial culture.

Bacterial Cell Culture

S. oneidensis MR-1(ATCC BAA-1096, Manassas VA, United States) was inoculated in Luria-Burtani (LB, Fisher Scientific) growth media overnight at 30°C with shaking at 200 rpm under aerobic conditions. After inoculation, cells were washed in triplicate with M4 growth media via centrifugation at 1500 rpm for 15 min followed by resuspension in fresh media. After washing, cells were diluted with M4 to a specific optical density by monitoring the absorbance at 600 nm (OD_{600}). M4 broth is a media with little bioavailable carbon and is optimized to sustain bacteria, though does not encourage growth (detailed preparation of M4 in **Supplementary Information**). M4 was chosen to prevent artificially nutrient rich conditions of the LB and also encourage an interaction of *S. oneidensis* with the nano-PS because there it has minimal bioavailable carbon.

Growth Based Viability Assay

Using an adapted growth-based viability assay developed by Qiu et al. (2017) the viability of *S. oneidensis* was quantified after exposure to nano-PS. Briefly, the assay relates the onset of exponential growth of *S. oneidensis* to calibrate viability, using a mathematical model to fit the exponential growth phase. Bacteria exposure was performed under aerobic and anaerobic environments with nano-PS suspended in MQ water at concentrations ranging from 18.75 to 300 mg/L for periods of 4, 8, or 12 h. These dosages were chosen as they cover the exposure ranges previously studied in other nanoplastic toxicity evaluations (Sun et al., 2018; Shen et al., 2019). The exposure times were chosen because the bacteria were able to be sustained for this period of time in the M4 broth without displaying stress caused from lack of bioavailable carbon. For aerobic growth studies, cells were diluted to an OD_{600} of 0.5 in M4 broth, which corresponds to approximately 5×10^8 cells/mL. For anaerobic growth, where the doubling rate of *S. oneidensis* is significantly slowed, the M4 media was supplemented with 15 mM lactate to allow for cell survival during the prolonged experiments, while the LB was supplemented with 20 mM lactate and 20 mM fumarate to allow for anaerobic growth. Anaerobic conditions were achieved by purging samples with an $N_2/CO_2/H_2$ [85:10:5] gas mixture in an anaerobic chamber (Coy Lab Products, Grass Lake MI, United States) and cells were equilibrated for 4 h prior to exposure. Growth was monitored by optical density readings at 600 nm. A Biotek Synergy two (Winooski, VT, United States) plate reader was used to take optical density measurements every 20 min for 13 h under aerobic environments. Alternatively, anaerobic growth measurements were observed approximately every 3 h for 48 h with a SpectraMax Plus 384 Microplate Reader (Molecular Devices, San Jose CA, United States). Anaerobic measurements required the transfer of plates from the anaerobic chamber to aerobic conditions and a subsequent transfer back to the anaerobic chamber. Anaerobic conditions in the 96-well plates were maintained by sealing the plates with an adhesive film cover.

Growth curves were modeled using R coding (RStudio, Boston, MA, United States). Under aerobic conditions the exponential growth phase of bacterial growth was modeled to

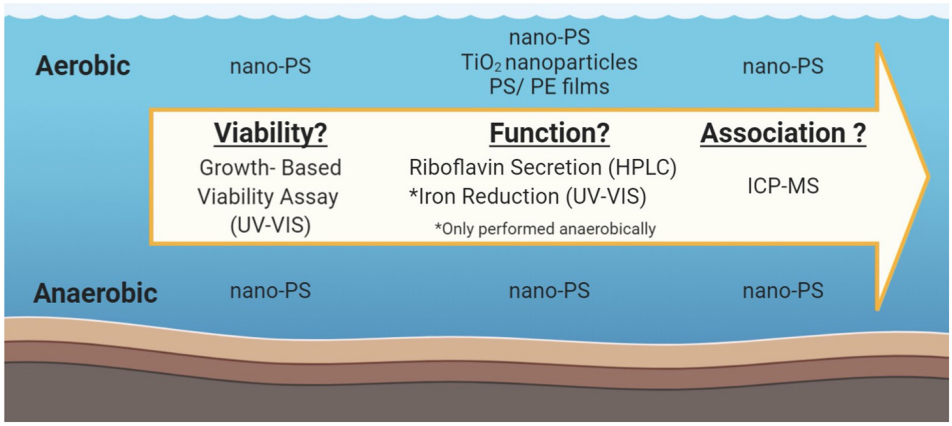

FIGURE 1 | Schematic of experimental workflow, including the exposure materials studied in the various assays.

evaluate viability through the onset of exponential growth (Qiu et al., 2017). Anaerobic growth lacked a noticeable lag phase, requiring an alternative model. A Gompertz model was chosen and viability was evaluated using the end of exponential growth (Zwietering et al., 1990). A detailed data analysis code is provided in the **Supplementary Information** along with example growth curves for 12 h exposure conditions (**Supplementary Figure S1**). Results of nano-PS exposure were compared to a negative control where the nano-PS exposure volume was replaced by an equivalent volume of MQ water without nano-PS, as the nano-PS stock was suspended in MQ water. A serial dilution of the negative control was performed after the exposure period for the construction of a calibration curve.

Cellular Function

The impact of nano-PS to bacterial cell function was evaluated by secretion of the external electron mediator, riboflavin. Suspended cells were diluted to an OD_{600} of 0.1 in media containing 1 mM iron hydroxide to a final volume of 3.2 mL. Aerobic studies were performed with LB broth, while anaerobic studies were performed with M4 broth supplement 9 mM lactate. After a 5-day exposure to nano-PS with concentrations ranging from 18.75 to 300 mg/L, riboflavin concentration in the supernatant was determined by isolating the non-cellular media by centrifugation for 5 min at 13000 rpm (Eppendorf Minispin Microcentrifuge Z606235) and quantifying using a Dionex UltiMate 3000 Series UHPLC (LPG-3400SD) with a 50 × 4.6 mm ID, Phenomenex Gemini C18 (3 μm particles) column. The mobile phase consisted of 85% Milli-Q water/15% acetonitrile v/v (Millipore Sigma; Sigma-Aldrich, Darmstadt Germany) with a flow rate of 0.5 mL/min. Riboflavin eluted after approximately 10 min and detected using fluorescence with excitation and emission wavelengths of 450 and 525 nm, respectively. A comparative analysis of riboflavin secretion after exposure to the same mass-based concentrations (18.75–300 mg/L) of TiO_2 nanoparticles was performed under aerobic conditions. The cell density of the bacteria after exposure was measured through changes in the OD_{600} in comparison to the control after exposure and used as an estimation of viability.

Beyond quantification of the riboflavin, the anaerobic cultures described above were also used to evaluate the reduction of iron. That is, while 1 mL sample of each exposure was taken for riboflavin and optical density analysis, the remainder of the culture was used to evaluate the reduction of iron. Iron reduction was halted before analysis by the addition of 0.56 mL of 12 M HCl. Reduced iron concentrations in the supernatants of lysed cells were quantified by the ferrozine (Sigma-Aldrich, Darmstadt Germany) assay, which used UV–Visible spectroscopy to selectively monitor the formation of a Fe(II)-ferrozine complex at 562 nm (Lies et al., 2005).

Sample Preparation and Digestion for ICP-MS Analysis

Various components of the cellular culture were evaluated for the presence of nano-PS. Specifically, nano-PS was quantified in the non-cellular portions of the growth media, lightly bound extracellular polymeric substance (LB-EPS), tightly bound extracellular polymeric substance (TB-EPS), and in the cell biomass, using the Pd-label as means for detection of the nano-PS with ICP-MS analysis. Collection of the ICP-MS samples relied on the physical extraction of the TB-EPS maintained intact cells, as opposed to chemical extractions that increase the risk of lysing the cells. Initially, the non-cellular growth broth solution was collected via centrifugation at 5000 × *g* for 10 min. The cell-containing pellet was then washed twice with 0.9% NaCl at 5000 × *g* for 10 min each, with the supernatant of each NaCl wash being collected and analyzed as the LB-EPS. The cell-containing pellet was resuspended in 0.9% NaCl and incubated at 30°C for 30 min to dislodge the TB-EPS from the cells. Samples were centrifuged at 5000 × *g* for 10 min and the supernatant containing the TB-EPS was collected while the pellet was resuspended in M4 growth media and collected as the cell biomass.

Upon collection of the culture components, the samples were digested to dissolve and homogenize the bacteria and nano-PS.

Specifically, an acid digestion was performed where 8 mL of each component of the cellular culture were mixed with 4.8 mL of a 1:10:1 (H_2O_2 (30%, Fisher): HNO_3(69.2% Fischer): H_2SO_4(95%, Arcos) acid mixture. Samples were added to a glass digestion tube (N9308049, Perkin Elmer) with H_2O_2 and allowed to stand for 30 min to initiate the digestion of the organic matter. HNO_3 was then added, followed by a 30-min standing period to allow for gas formation to slow. Finally, H_2SO_4 was added followed by a final 30-min standing period. H_2SO_4 was added slowly as it reacts with H_2O_2 to produce perania acid, which causes off-gassing and bubbling. The samples were then covered with aluminum foil and placed into the autoclave with the chamber temperature set at 270°C and a pressure of approximately 15 psi for 135 min. During the heating phase, the temperature fluctuated around 250°C. Two heating phases, which were deemed necessary upon visual inspection of the samples for particle presence, were performed on each sample.

A Shimadzu ICP-MS-2030 was used to detect the Pd label of the nano-PS. Pd standards (Inorganic Ventrures, Christiansburg VA, United States) were prepared at a range of 0.5–50 ppb from a 1000 ppb stock to a final volume of 10 mL. Culture components were quantified as a percent recovery of Pd by normalizing results to nano-PS control samples analyzed with ICP-MS that were of the same nano-PS concentration as the exposure. For some samples, a conversion factor of 5.1×10^{-9} ng Pd/nano-PS particle was used to quantify the number of nano-PS particles within each sample.

Statistical Analysis

Numerical representations of the data are presented as mean ± standard error. Single factor ANOVA analyses (alpha = 0.95) were used to determine the overall statistical significance of the varied concentrations for the different exposure conditions. ANOVA analyses that were statistically different (i.e., $p < 0.05$) were subsequently evaluated with Tukey posthoc to determine the pairwise p-values between the different exposure levels.

RESULTS AND DISCUSSION

Viability of *S. oneidensis*

The impact to *S. oneidensis* viability was evaluated with a growth-based viability assay (Qiu et al., 2017). The viability of the suspended bacteria was determined by calibrating to a point on the bacterial growth curve, either at the onset or at the end of exponential phase for aerobic and anaerobic growth, respectively. The viability of *S. oneidensis* exposed to increasing doses of nano-PS for varying exposure times showed minimal changes compared to the control (**Figure 2**), where values were normalized to the calibration samples for the 100% viability. In aerobic conditions (**Figure 2A**), there are slight, but significant ($p < 0.05$), decreases in viability between control viability (dashed line) and the viability of *S. oneidensis* aerobically exposed to 75, 150, and 300 mg/L nano-PS for 8 h. However, this decrease in viability was not observed with the other aerobic exposure times, so it was concluded the impact of nano-PS on aerobically grown *S. oneidensis* is small. For anaerobic conditions, there were no significant changes to the cell viability as compared to the control (**Figure 2B**). There is larger error associated with the viabilities determined under anaerobic conditions that could be the result of small, but variable amounts of O_2 entering the sealed samples over the course of the experiment. Considering both aerobic and anaerobic cultures, nano-PS caused minimal changes to cell survival under either growth environment. Previously, bacterial viability has shown a significant negative correlation with the presence of nano-PS. Both marine bacterium *Halomonas alkaliphila*, after 2 h of exposure (Sun et al., 2018), and fermentation bacterium *Acetobacteroides hydrogenigenes*, after over 3 days of exposure (Fu et al., 2018), experienced significant decreases in growth or viability upon exposure to 55 nm nano-PS. Our findings are not consistent with these observations as there is not a significant decrease in the viability observed consistently for the all exposure conditions. Due to the lack of single-species literature studies, we cannot conclude if our results vary from literature because of experimental parameters such as nanoparticle size, exposure length, or species differences.

Riboflavin Secretion

While the viability of *S. oneidensis* was minimally affected by the presence of nano-PS in our exposure conditions, we sought to also evaluate changes in cellular function. The exuded riboflavin concentration upon exposure to various concentrations of nano-PS in aerobic and anaerobic growth conditions is shown in **Figure 3A**. Aerobic exposure conditions (diagonal lines) showed significant increases in riboflavin secretion of cells with doses up to and including 150 mg/L nano-PS, but there was a significant ($p < 0.05$) decrease at the highest (300 mg/L) exposure concentration as compared to the control. It should be noted that 300 mg/L would be an unexpectedly high dose in ecologically relevant conditions. This increase in riboflavin secretion with a decrease at 300 mg/L nano-PS exposure was also observed in anaerobic conditions (solid gray bars), although the effect size was much smaller overall when compared to aerobic conditions. It is interesting to note that while the absolute value of riboflavin per cell was different, secretion trends were similar for aerobic and anaerobic conditions. *S. oneidensis* exudes riboflavin and other flavin mediators for a number of bacterial functions (Marsili et al., 2008; Mcanulty and Wood, 2014; Kim et al., 2016). Primarily, these flavin mediators act as part of the anaerobic respiratory pathway resulting in the reduction of a variety of metals. However, upon quantifying the bacterially reduced iron in anaerobic conditions, we observed no significant change in iron reduction in the presence of the nano-PS (**Figure 3B**). This signifies that there is not a correlation between riboflavin secretion and iron reduction after exposure to nano-PS. Therefore, we conclude that riboflavin is being secreted by *S. oneidensis* for a purpose beyond acting as an electron mediator itself. There is evidence that *S. oneidensis* also uses riboflavin as a signaling molecule, where riboflavin activates the cells for a number of different cellular behaviors, including repulsion of foreign bodies (Moriyama et al., 2008; Brutinel et al., 2013) or chemotaxis of other *S. oneidensis* bacteria (Li et al., 2012; Kim et al., 2016; Oram and Jeuken, 2019). To elucidate the purpose of

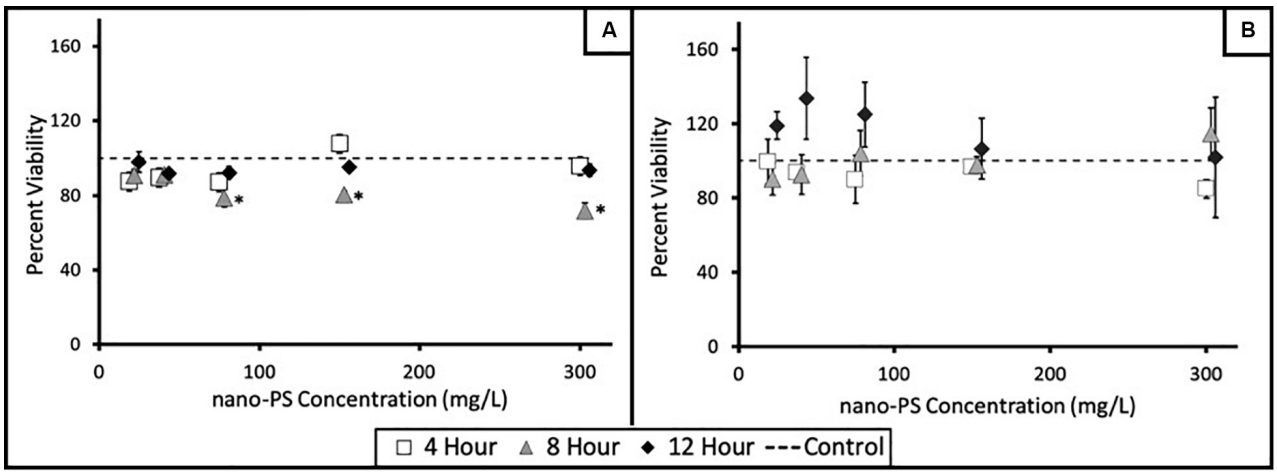

FIGURE 2 | Viability of *S. oneidensis* exposed to nano-PS as measured with a growth-based viability assay. Cells were exposed to varied concentrations of nano-PS (18.75–300 mg mg/L) for varying exposure lengths (4, 8, 12 h) in **(A)** aerobic and **(B)** anaerobic conditions. Markers at each of the nano-PS exposure concentrations are slightly staggered to highlight the viabilities for exposure length. The dashed line represents the control wells, which were normalized to the 100% viable calibration wells (*$p < 0.05$). Biological triplicates were performed for each exposure condition, where one assay was considered a single biological replicate containing four exposure wells for each nano-PS exposure concentration. Error bars represent the SEM of biological triplicates.

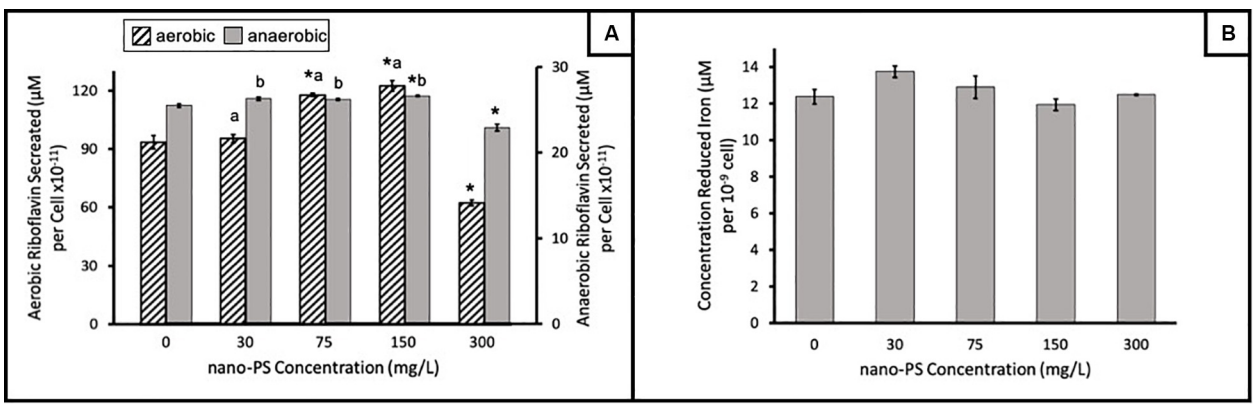

FIGURE 3 | (A) Riboflavin secreted per cell from *S. oneidensis* as a function of exposure concentration of nano-PS in aerobic (diagonal lines) and anaerobic (solid gray) conditions. A significant increase (*$p < 0.05$) was observed as compared to the control (0 mg/L nano-PS) for aerobic 75, and 150 mg/L nano-PS and anaerobic 150 mg/mL. However, 300 mg/mL was significantly decreased riboflavin secreted per cell as compared to the control. The highest exposure concentration, 300 mg/L nano-PS, was significantly different ($^{a \text{ or } b}p < 0.05$) in secreted riboflavin compared to the other exposure concentration. The SEM among triplicate trials is represented by the error bars. **(B)** Iron reduced by *S. oneidensis* under anaerobic conditions exposed to varying concentrations of nano-PS does not show significant differences from the control (i.e., non-exposed) cells (single-factor ANOVA, $p = 0.26$). Error bars present the SEM of triplicate trials.

the flavin response, future transcriptomics work is being pursued. In comparing aerobic and anaerobic exposures, we have shown that the observed stress response to nano-PS is consistent, no matter the culture conditions. Consequently, we hypothesize that the interactions (e.g., association) of the nano-PS with *S. oneidensis* would be similar between culture conditions.

To confirm that these cellular responses were the result of the particulate polymer, and not simply a response to particulate matter in solution more generally, aerobic riboflavin production was quantified in the presence of TiO_2 nanoparticles (**Figure 4A**). The optical density of the bacteria was measured to roughly estimate changes in viability at the end of exposure for all exposure doses. Like the nano-PS, TiO_2 did not induce a change in optical density as compared to the control (see **Supplementary Figure S5**), which is interpreted as minimal changes in viability, but unlike nano-PS, there was no significant difference in the secretion of riboflavin. The difference in chemical makeup and size could potentially contribute to the observed response, but previous work with nickel manganese cobalt oxide(NMC) nanosheets (Mitchell et al., 2019) and iron oxide nanoparticles (Buchman et al., 2019b) were similar, where exposure did not result in a significant changes in the amount of riboflavin secretion from *S. oneidensis*. Considering these inorganic nanomaterials, with varied sizes and shapes, cause a similar secretion behavior from *S. oneidensis,* our results of nano-PS significantly changing the riboflavin secretion is unique. This

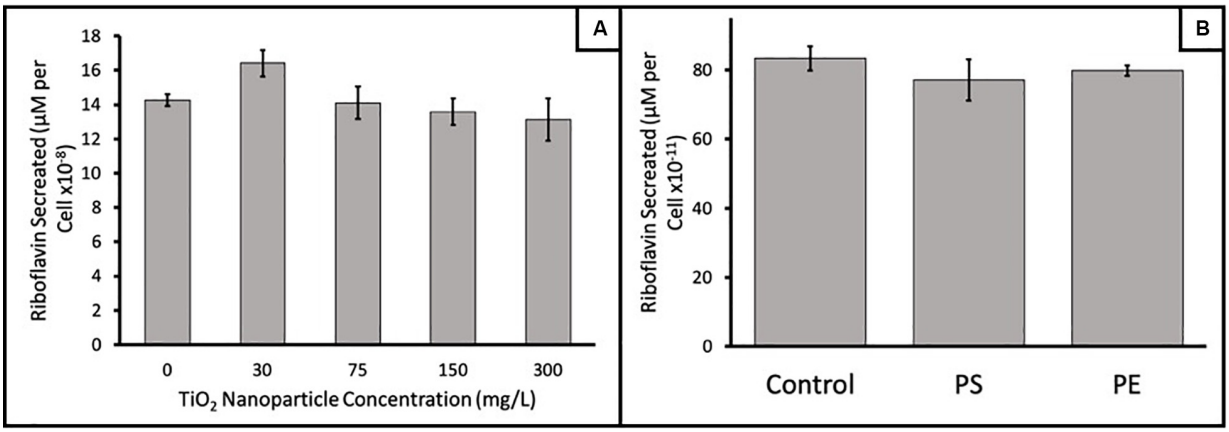

FIGURE 4 | (A) Riboflavin secreted from *S. oneidensis* upon aerobic exposure to varying concentrations of 25 nm TiO_2 nanoparticles were not statistically different than control cultures (single-factor ANOVA, $p = 0.69$). **(B)** Riboflavin secreted by *S. oneidensis* after aerobic exposure to sheets of polystyrene (PS) and polyethylene (PE) thin films. No statistical difference was observed (single-factor ANOVA, $p = 0.58$). Trials were performed in triplicate; error bars represent the SEM in triplicate values. Note the different order of magnitudes on the riboflavin secretion for panels **(A,B)**.

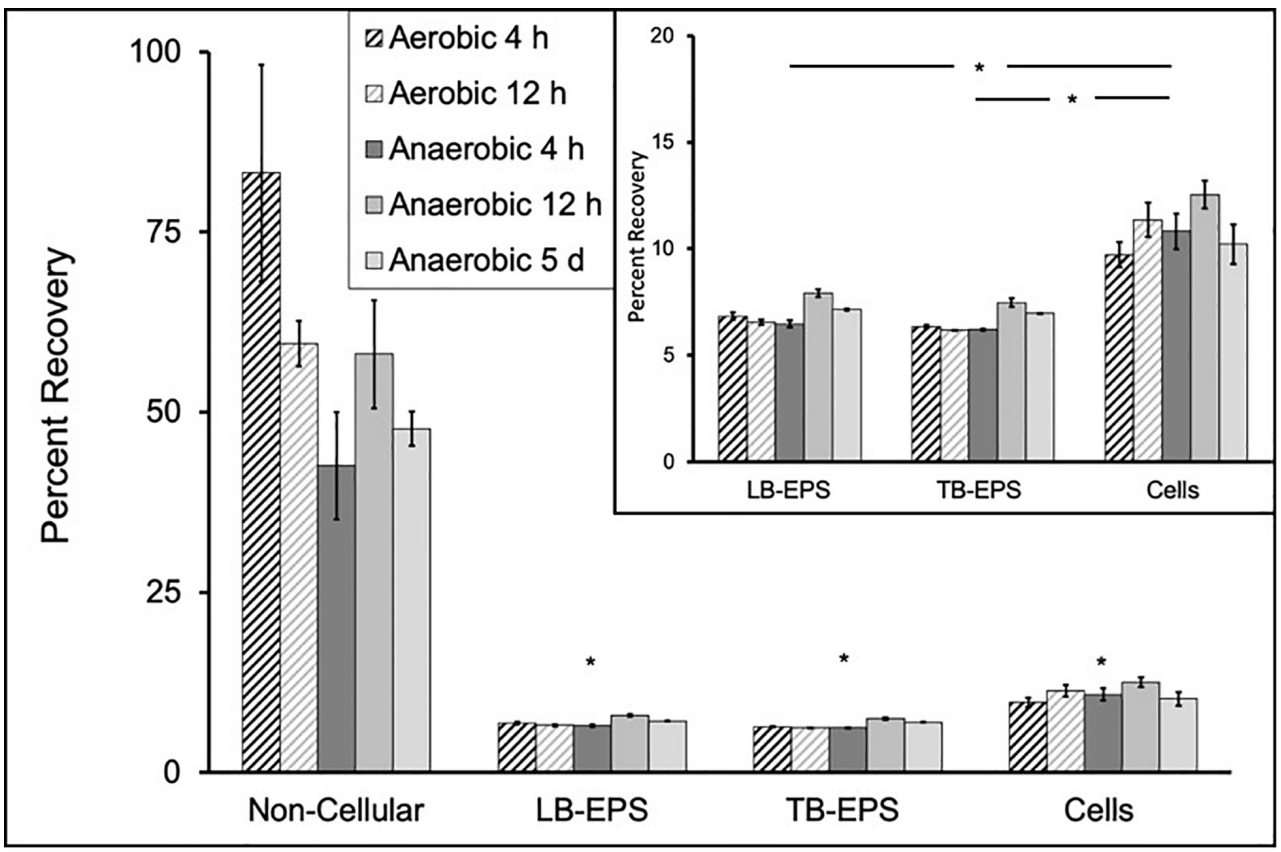

FIGURE 5 | The distribution of nano-PS in the four components of the exposed bacterial culture after exposure to 150 mg/L nano-PS, including the non-cellular growth broth, which is significantly greater ($p < 0.05$) than lightly bound (LB)-EPS, tightly bound (TB)-EPS and association with cells. Inset shows statistical comparison of the nano-PS in LB-EPS and TB-EPS to the cells (*$p < 0.05$). Error bars represent the SEM of triplicate biological replicates.

suggests that the nano-PS material itself, and not simply bacterial interactions with particles, is important in inducing changes in cellular behavior for S. *oneidensis*.

Beyond particulate matter, no significant differences in the secretion of riboflavin was measured when bacteria were exposed to polystyrene or polyethylene thin films, indicating that the

material and/or size in which the bacteria are exposed to the material impacts its response (**Figure 4B**). For context of the size comparison, the surface area of the thin films is 2.0×10^{14} nm^2 is very similar to the nano-PS exposure concentration of 75 mg/L that has a surface area of 2.2×10^{14} nm^2. This highlights the significance of the elevated riboflavin secretion in the presence of nano-PS. While the thin film polystyrene and polyethylene induced a similar result, it remains unclear if nano-polyethylene would elicit the same size-dependent response that was observed with nano-PS. Polystyrene and polyethylene are both considered to have fairly recalcitrant backbones and would be harder for microorganisms to degrade compared to polymers such as polylactic acid (Amaral-Zettler et al., 2020). Based on this similarity, we would predict that nano-polyethylene may similarly increase riboflavin secretion, although studies of natural microbial communities show that microbes respond differently to these polymers (Zettler et al., 2013; Debroas et al., 2017).

Characterizing Association of Nano-PS With *S. oneidensis*

To understand the association of nano-PS with respect to the bacteria in an effort to interpret the observed changes in functionality, we analyzed various components of the bacterial culture and organisms by ICP-MS. After exposure, the majority of the nano-PS remained in the non-cellular culture media under all experimental conditions (averaging 59.2 ± 15.7% of the recovered nano-PS), which was significantly greater ($p < 0.05$) than all the other culture components (**Figure 5**). The remaining fractions of nano-PS were found in the LB-EPS (7.0 ± 0.6%), TB-EPS (6.6 ± 0.6%), and directly associated with the cellular biomass (10.9 ± 1.1%) (**Figure 5**, inset). The mass balance for nano-PS was ranged between 66 and 106% of the total nano-PS in the exposure, which was the result of efforts to separate the aggregated nano-PS from the pelleted bacteria cell (see **Supplementary Figure S6**). Collectively, our results suggest that nano-PS interact both indirectly with the bacteria through entrapment in the EPS and directly with the bacterial membranes. Particles which were associated with the membrane were presumed to be strongly bound because the associated nano-PS remained attached after a series of cell washing steps. Bacteria exude EPS as a mechanism for improved chemical reactions, nutrient capture, and protection (Costa et al., 2018). This work supports previous nanoparticle toxicity studies, where nanoparticles are shown associated with the EPS, likely a protective response by the bacteria (Kang et al., 2014; Nomura et al., 2016). Interestingly, though, there is a significant increase ($p < 0.05$) of nano-PS associated with the bacterial biomass as was as compared to the LB-EPS or TB-EPS samples. While it would be unusual for a particle with a diameter of 160 nm to cross the bacterial membranes (Neal, 2008; Ivask et al., 2014), an association with the membrane is supported by previous work with a variety of materials and bacterial organisms (Ivask et al., 2012, 2014; Zhao et al., 2013). Specifically, for *S. oneidensis*, Jacobson et al. (2015) has shown that Au nanoparticles (12.8 nm) associate with the bacteria as a function of the lipopolysaccharide (LPS) within the outer cell membrane, we would hypothesize *S. oneidensis* would have a similar mechanism for our nano-PS results. The exposure times did not influence the percent of associated nano-PS, suggesting that the interaction with the EPS and membranes occurs within the first 4 h of exposure. Other nanoparticle association studies have suggested that this association could be even faster, within the first 60 min of exposure (Butler et al., 2014; Moore et al., 2017).

The presence of nano-PS associated to the cell biomass indicates that a direct interaction occurs between the nano-PS and the bacteria. To determine if there is a correlation between the amount of nano-PS particles associated with the cell and the changes in riboflavin secretion, we quantified the amount of nano-PS in the cell biomass upon increased exposure doses (**Figure 6**). An increase in the number of nano-PS associated with the cell was observed with increased exposure concentrations. There was a significant increase in the nanoparticles per cell at 300 mg/L in comparison to the 75 and 150 mg/L exposure results, although we did not observe a doubling in membrane nano-PS concentration with a doubled exposure concentration. The 300 mg/L exposure concentration correlates with the nano-PS exposure concentration in which the riboflavin secretion had significant decreases in comparison to the control. Based on the determined low number of nano-PS/cell (12–15 nano-PS particles/cell), we do not anticipate nano-PS aggregates on the cell surface but that the particles would be individually bound onto the cell membrane. This association behavior has been observed by Jahnke et al. (2016) in which *S. oneidensis* was exposed to 50 and 100 nm gold nanoparticle at similar nanoparticles/cell dosages. The corresponding surface area coverage between the cells exposed to 150 mg/L and 300 mg/L nano-PS was 13.4 and

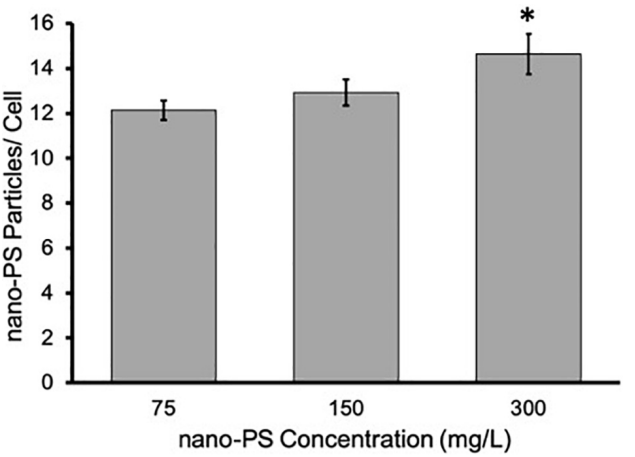

FIGURE 6 | Comparison of the number of nano-PS normalized to the number of cells for samples exposed to 75, 150, and 300 mg/L nano-PS in anaerobic conditions for 12 h. A significant increase (*$p < 0.05$) in the number of nano-PS particles/cell for samples exposed to 300 mg/L nano-PS was observed in comparison to bacteria exposed to 75 and 150 mg/L nano-PS. Error bars represent SEM of biological triplicates.

15.2%, respectively. The significant increase in the nano-PS coverage of membrane surface area paired with the observed significant decrease of riboflavin secretion, suggests that there is a concentration at which the association of nano-PS with the bacterial membrane results in alterations of the bacteria's riboflavin secretion ability. Nanoparticles have been shown to perturb the structural integrity of the cell membranes, resulting in changes within the local lipid environment (Rossi et al., 2014; Contini et al., 2018). Membrane proteins are often sensitive to protein-lipid interactions that may be altered by changes in the morphology of the membrane caused by nanoparticle presence (Lee, 2004; Dowhan and Bogdanov, 2011; Battle et al., 2015; Muller et al., 2019). While the experimental dosages are higher than what would be expected in natural systems, the increase in the number of nano-PS particles associated with cells at higher concentrations may result in the disruption of the membrane shape impacting protein function responsible for riboflavin secretion.

CONCLUSION

Overall, exposing *S. oneidensis* to nano-PS causes a stress response that is not related to viability. Specifically, riboflavin secretion, a critical cell function, is altered in the presence of nano-PS. This work highlights the importance of this riboflavin stress response because it is observed in both anaerobic and aerobic conditions, indicating that the secretion behavior is not a result of its metal reducing function. The observed similar aerobic and anaerobic response is interesting as it indicates that the interaction with nano-PS will be consistent in many different environmental compartments. Furthermore, we reveal that nano-PS associates indirectly with the EPS and directly with the bacteria membranes. This is the first study to characterize *S. oneidensis* nanoplastic or nanoparticle response in both aerobic and anaerobic cultures. By understanding the response of this adaptable single-celled organism under varied conditions, this work has contributed to a better understanding of the impact of plastic waste to the bedrock of our ecosystems.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://conservancy.umn.edu/handle/11299/166578.

AUTHOR CONTRIBUTIONS

VF contributed the data collection and analysis of growth-based viability assays and ICP-MS. LF contributed the data collection and analysis of the riboflavin secretion and iron reduction assays. DM synthesized and characterized nano-PS particles. MM-J and DM contributed the conception of the work. All authors contributed to data interpretation and writing and editing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2020.00097/full#supplementary-material

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