



Interaction of Soil Microbes with Organoclays and their Impact on the Immobilization of Hg under Aerobic Conditions

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Abstract Immobilization of mercury (Hg) leaching from bank soils of East Fork Poplar Creek (EFPC) is considered part of remediation strategies to mitigate the amount of Hg entering the creek. Different approaches are currently being evaluated, such as utilizing engineered sorbents to immobilize Hg species in EFPC bank soils. However, the influence of environmental microbes on the immobilization of Hg by sorbents is unknown. Organocation-modified phyllosilicate clay minerals (organoclays) are widely used as sorbents for the immobilization of contaminants. This study evaluates the interactions of *Serratia marcescens* and *Burkholderia thailandensis* with the sorbent Organoclay PM-199 and their impact on the immobilization of Hg under aerobic conditions. We evaluated the competitive binding of Hg between sorbents and selected microorganisms in a series of pure culture studies using bacterial strains identified in EFPC bank soil samples. Our results suggest that Hg sorption by Organoclay PM-199 is not significantly impacted by common soil bacteria present

in EFPC, specifically *Serratia marcescens* and *Burkholderia thailandensis*, which are known to form biofilms. These findings suggest that sorbent amendments are an effective strategy for the remediation of Hg contamination in natural ecosystems.

Keywords Mercury · Sorbent · Competitive interactions · Microorganisms · Organoclay

1 Introduction

Mercury (Hg) emissions are a global problem that entails major environmental challenges with severe implications on human health (Horowitz et al., 2014; Selin, 2009). Historic use of elemental Hg at the Y-12 National Security Complex within the Oak Ridge Reservation (ORR) in East Tennessee has led to widespread contamination of East Fork Poplar Creek (EFPC), which originates within the plant boundaries. Hg is transformed by microorganisms to the highly toxic form methylmercury (MeHg), which biomagnifies up trophic levels and accumulates in fish. Due to the high environmental impact involved with Hg transformations in aquatic environments, the ORR has been placed on the US National Priorities List as part of the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) since 1989 (Brooks & Southworth, 2011). Some applicable measures that were put in place to reduce Hg inputs into the headwaters of EFPC include collection and treatment of contaminated sump waters, dechlorination of all effluents flowing into the creek,

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capping of a contaminated pond, and stabilization of eroding stream banks (Southworth et al., 2000; Turner & Southworth, 1999). These remedial actions led to a decrease of waterborne Hg concentrations in EPFC by more than 90%; however, MeHg levels in water and fish remain elevated, particularly in the downstream environment of EPFC (Peterson et al., 2016).

Regardless of the different efforts to mitigate the inputs of Hg to the headwaters of EPFC, the erosion of Hg-contaminated bank soils contributes to the total Hg flux into the creek. During precipitation and storm events, high levels of Hg are mobilized from stream bank soils increasing the overall Hg concentration in the creek by approximately 10% (Watson et al., 2016). A distinct soil layer in the creek banks has been described as the historical release deposit (HRD) and is known to contain high amounts of Hg (Watson et al., 2016). The HRD is a sandy texture soil found approximately 50 cm below the top of the bank and contains varying concentrations of Hg (Dickson et al., 2017). Strategies considered to mitigate Hg release from EPFC bank soils include bank stabilization to limit soil erosion and the deployment of sorbents either as a soil amendment or contained in a permeable reactive barrier to stabilize Hg in situ (Peterson et al., 2016).

Sorbents are insoluble materials or mixtures of materials that are designed to absorb contaminants through physical or chemical sorption with high affinity (USEPA, 2016). The material characteristics of a sorbent, such as pore size, surface chemistry, surface area, and hydrologic conductivity, determine the effectiveness of the sorbent for contaminant removal (Chen et al., 1999). Various types of sorbent materials have been considered for Hg remediation including materials derived from biomass precursors (biochar, activated carbon, bone apatite), materials specifically engineered to capture Hg (thiol-functionalized sorbents, polysulfides) (Chen et al., 1999; Worthington et al., 2017). Activated carbons and biochars have been investigated in microcosm and field studies and were found to exhibit low toxicity while lowering bioavailability of Hg and MeHg (Gilmour et al., 2013; Gilmour et al., 2018; Gomez-Eyles et al., 2013). Organoclays are organocation-modified phyllosilicate clay minerals (bentonite clay) that are frequently used for the remediation of organic contaminants (Lee et al., 2004). More recently, organoclays also have been considered for the remediation of heavy metal contamination due to favorable properties, such as high internal surface area and

excellent hydraulic conductivity (Guerra et al., 2011; He et al., 2015; Sarkar et al., 2010; Stathi et al., 2007). In a recent study, organoclays were shown to be highly effective in removing environmentally relevant Hg species better than activated carbon or sulfur-impregnated activated carbon (Gai et al., 2019). Natural organic matter (NOM) can interact with Hg species and result in the formation of strong Hg-NOM complexes, which has significant impacts on sorbent performance and microbial transformations (Chiasson-Gould et al., 2014; Johs et al., 2019; Miller et al., 2012). In addition to the competitive interactions between Hg, sorbents, and NOM, understanding the role of microbial interactions with sorbent materials, such as microbial biofilm formation and attachment at the sorbent-porewater interface, is essential for evaluating the overall impact of sorbent amendments in a contaminated environment (Muller & Brooks, 2019). Recent studies demonstrated that at least a fraction of sorbed mercury remains bioavailable for methylation by sulfate-reducing bacteria (Hamelin et al., 2015; Lin et al., 2013; Olsen et al., 2016). The formation of biofilms on sorbents may inhibit effective immobilization of Hg species. Furthermore, biofilms and periphyton have been identified as potential sources of MeHg species as they may harbor anaerobic microorganisms known to methylate Hg (Hamelin et al., 2011; Lin & Jay, 2007; Olsen et al., 2016). Thus, the potential impact of microbial activity and biofilms on the removal effectiveness of sorbents requires further investigation.

Burkholderia thailandensis (Tseng et al., 2016) and *Serratia marcescens* (Rice et al., 2005) are facultative anaerobic Gram-negative bacteria that occur naturally in soils. These model organisms also tend to form biofilms through a chemical signaling pathway (quorum sensing) that regulates microbial community composition and biofilm structure (Fazli et al., 2014; Tseng et al., 2016). Biofilms consist of cells embedded in an extracellular matrix of exopolysaccharides, which might block the pores of the sorbent, thereby rendering the sorbent ineffective. Therefore, it is important to understand the complex interactions between abiotic and biotic processes on the long-term impact of sorbent. Here, we evaluate the impact of microbial interactions on the effectiveness of the sorbent Organoclay PM-199 (PM-199). We investigate in series of laboratory batch experiments how biotic processes, such as biofilm formation, may affect Hg sorption by PM-199 over extended periods of time. The goal of this study was to gain insights

into the role of microbial biofilms on the immobilization and transformation of Hg species by sorbent amendments.

2 Results

2.1 Variability of Total Hg Concentrations in EFPC Soils

Overall, HgT was the highest in HRD layer samples collected from location EFK 18.2 (Fig. 1a). HgT was 45% higher in soil samples from EFK 18.2 collected in the fall (695.9 $\mu\text{g Hg/g}$ soil dry wt) compared to samples collected during other seasons at this site, which is likely the result of variations between individual samples. At site EFK 11.2, overall concentrations were approximately one order of magnitude lower with the highest concentrations recorded in the winter samples, which were 33% (54.4 $\mu\text{g Hg/g}$ dry wt.) higher compared to other seasons. HgT in the uncontaminated control sample (Hinds Creek) was three orders of magnitude lower relative to samples from site EFK 11.2 (Fig. 1b).

2.2 Microbial Impact on Hg Sorption

S. marcescens and *B. thailandensis* were selected for these studies because they commonly appear in EFPC microbial communities, they are facultative anaerobes, and are known to form biofilms. To investigate seasonal variations, soil samples collected during the summer, fall, and winter seasons were used for the biosorption experiments. The results indicate that the presence of *S. marcescens* did not significantly influence immobilization of Hg(II) by PM-199 (Fig. 2). The fall sample from site EFK 11.2 showed about 29% reduction of HgT when PM-199 was added to the solution compared to a sample from EFK 18.2 from the same season that showed about 99% decrease when PM-199 was added to the solution. Conversely, soil samples obtained during the summer season showed 61% reduction of HgT in the presence of bacteria or sorbent in EFK 11.2 samples, which is 32% reduction difference between the fall and summer samples from the same site (EFK 11.2) when compared to the control. Because *S. marcescens* mostly thrive in the presence of reduced Hg concentration (François et al., 2012), the results from EFK 11.2 best describes the interaction of *S. marcescens* with PM-199 compared to the EFK 18.2 that contains 45% more of the HgT recorded in the original soil samples.

To further assess the competitive interactions of microorganisms and PM-199 for the sorption of Hg in the absence of soil, we also compared removal efficiencies in the presence of *Burkholderia thailandensis*, which is another genus found to be relatively abundant in EFPC bank soils (Egbo et al., 2017). Sorbents were inoculated with *Burkholderia thailandensis* at the same time and incubated at 25 °C. The influence of *B. thailandensis* on PM-199 was consistent with the results from the experiments with *S. marcescens* in the presence of soil samples. No significant inhibition of Hg sorption was observed when PM-199 was incubated with *B. thailandensis* (Fig. 3). The average residual concentrations of HgT in solution in samples containing both PM-199 and *B. thailandensis* compared to samples containing either PM-199 or *B. thailandensis* only were not statistically different across all Hg(II) concentrations. To put the difference in perspective, the HgT concentrations decreased by 68%, 27%, and 56% in samples initially containing 68 μM , 13.6 μM , and 6.8 μM Hg(II), respectively, when PM-199 alone was compared with PM-199 and *B. thailandensis*. When PM-199 was compared to PM-199 and *B. thailandensis* combined, the Hg(II) concentration in the sample with PM-199 only, although not significantly different, 36%, 23%, and 4% HgT lower than initial 68 μM , 13.6 μM , and 6.8 μM Hg(II), was observed, respectively. Thus, PM-199 was effective in immobilizing Hg(II) in the presence of *B. thailandensis*, demonstrating that no microbial inhibition of Hg(II) sorption by PM-199 occurred at the biomass concentrations and sorbent to liquid ratios used in these experiments.

2.3 Structural Evaluation of PM-199

The SEM micrographs showed that PM-199 incubated with either *B. thailandensis* or Hg indicated a possible change in the morphology of PM-199 in the presence of Hg and bacteria (Fig. 4). As shown by the SEM, PM-199 is uniform with visible layers and edges. However, the bacteria-treated sorbent appeared smoother with nonuniform distinct surface roughness.

3 Discussion

The goal of sorbent amendments is to reduce Hg flux into the environment. However, there is a need to understand microbial interactions and long-term impacts

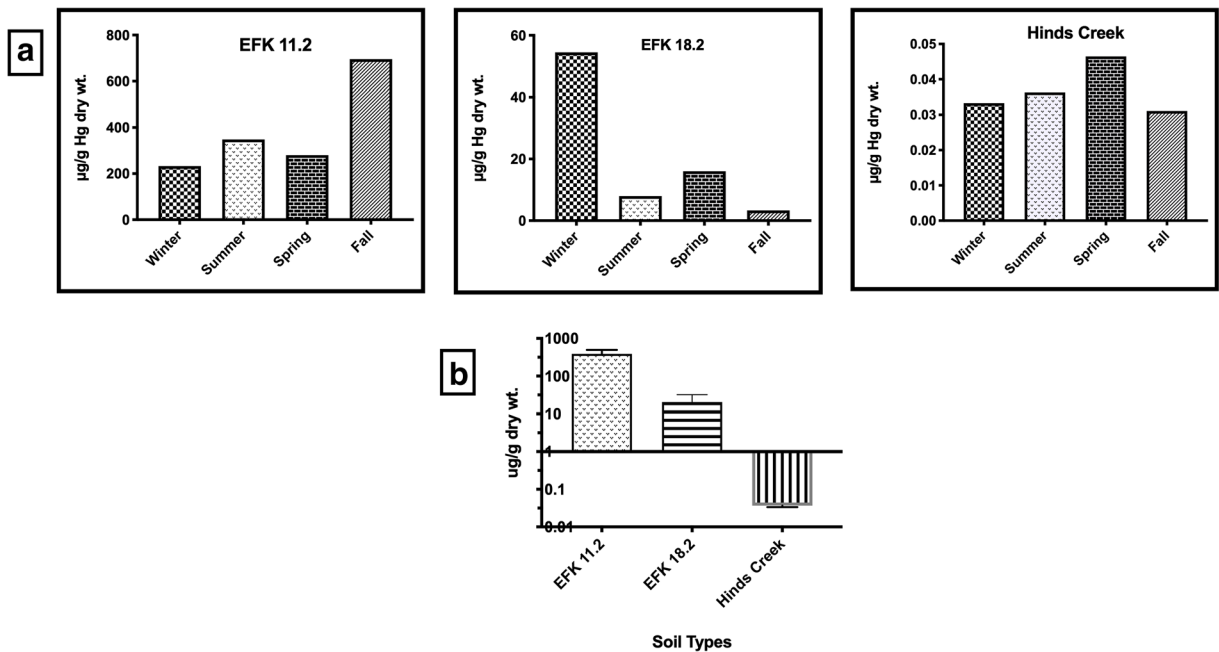


Fig. 1 Hg concentration in EFPC bank soil collected at different seasons (a), and average of all samples collected during the three seasons (b). Variable total Hg concentrations were recovered with increased concentrations in the fall (EFK 11.2) and winter season

Soil Types

(EFK 18.2), while concentrations at Hinds Creek remained unchanged. The average Hg concentrations at EFK 11.2 and EFK 18.2 were 2-3 orders of magnitude higher compared to the reference site (Hinds Creek)

of the in situ application of sorbents. Sorbents like activated carbon are known to influence and support rapid attachment of bacteria and favoring biofilm

formation, while at the same time reducing contaminant levels as previously demonstrated for PCB (Mercier et al., 2013). PM-199 is a montmorillonite-based

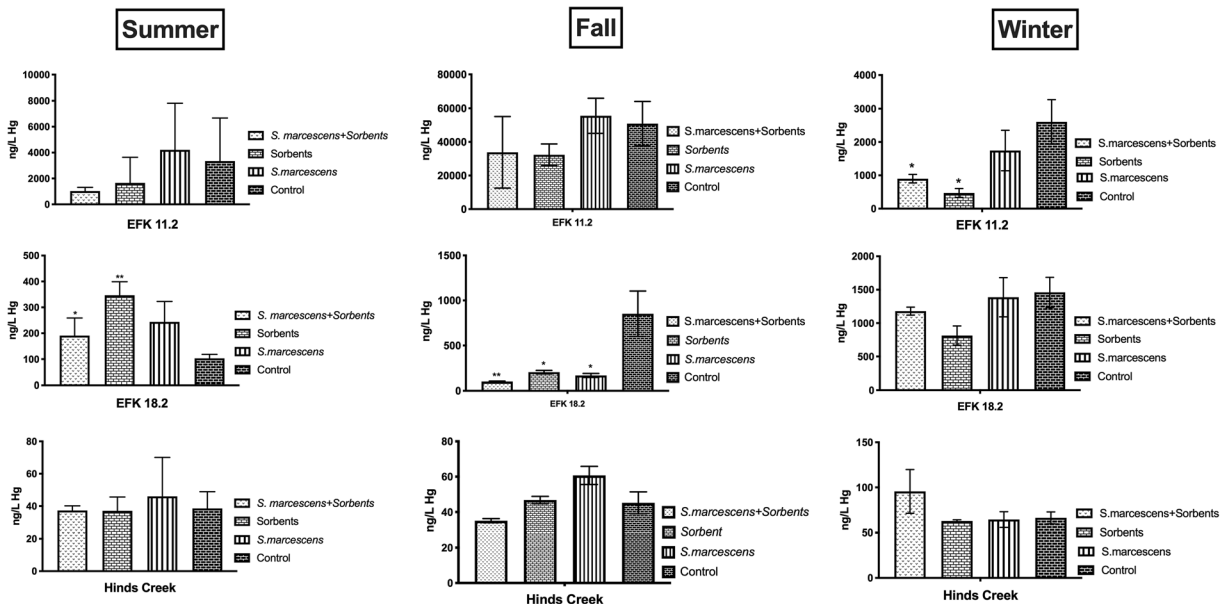


Fig. 2 Microbial actions on the sorbents' ability to immobilize Hg. Concentration of HgT in solution after 48 h of biosorption using *S. marcescens* and PM-199 in bank soils of EFPC. Rows

represent seasons and columns represent the different sampling sites. Error bars represent standard deviation of triplicates

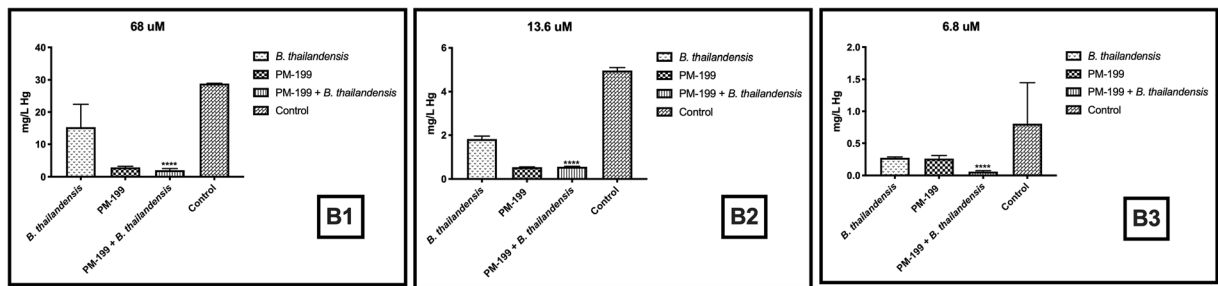


Fig. 3 Microbial actions on sorbent’s ability to immobilize Hg. Concentration of HgT in solution after 48 h of biosorption using *B. thailandensis* and PM-199 at different concentrations of mercuric nitrate. B1 to B3 represent different Hg dilutions. Statistical

relationship between the control and PM-199 or PM-199 and *B. thailandensis* was significant for B1, B2, and B3, but not when PM-199 and PM-199 and *B. thailandensis* are compared

modified clay sorbent with high hydrologic conductivity exhibiting a very small pore sizes (Chen et al., 1999) The interaction of bacteria or natural organic matter with the sorbent may block fine pores and thus inhibit adsorption (Fahey et al., 2010; McDonough et al., 2008). PM-199 efficiently sorbs Hg as a result of its porosity and accessible surface area (Gai et al., 2019; Johs et al., 2019), making it very suitable candidate for Hg remediation applications.

We evaluated the effectiveness of PM-199 for the removal of Hg from solution in the presence of soils leaching Hg and investigated the potential impact of bacterial biofilms in a series for laboratory incubation experiments. *S. marcescens* and *B. thailandensis* are known for their ability to use quorum sensing to form biofilms (Fazli et al., 2014; Mangwani et al., 2016; Queck et al., 2006; Rice et al., 2005; Tseng et al., 2016). The formation of multispecies biofilms advances at a consistent rate during initial growth, while biofilm biomass may decrease with age (Macedo et al., 2005; Mercier et al., 2013). Biofilm formation was observed in

batch experiments where the sorbent was incubated in the presence of bacteria, while batch experiments containing only sorbent did not exhibit biofilm formation (data not shown). It was expected that biofilm attachment to the sorbent would occur during the early stage of formation. Indeed, changes in the morphology of sorbent particles visualized using SEM are consistent with biofilm formation under the experimental conditions (Fig. 4). However, the present results show that the presence of microbial biomass did not decrease Hg(II) absorption or increase release of Hg from soils.

Results from the biosorption experiments showed no significant inhibition in the sorption of Hg released from contaminated soils in the presence of *S. marcescens* by PM-199. There was no significant difference in the final HgT concentrations in solution when the soil samples were incubated with *S. marcescens* and PM-199 compared to when treated with PM-199 only. However, we observed a high variability in the Hg solution concentrations between individual samples (Fig. 3), which is likely a result of seasonal variations in soil Hg

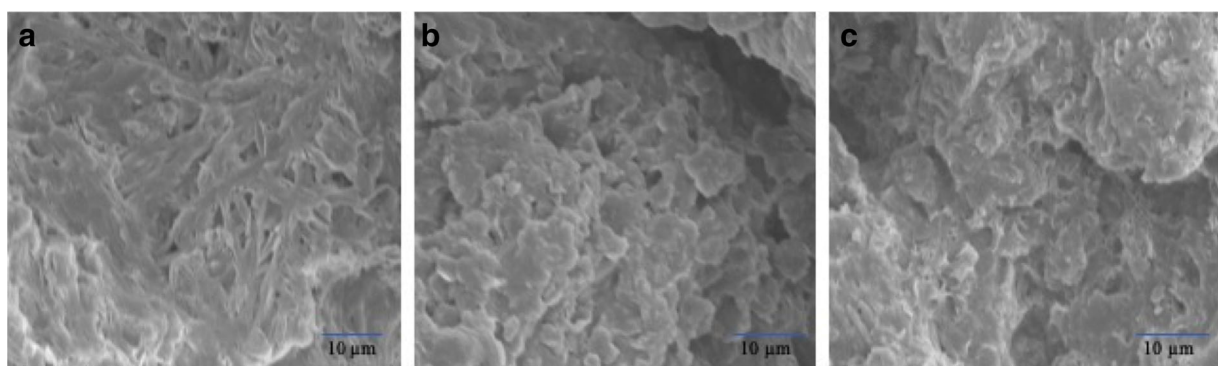


Fig. 4 SEM micrographs showing the morphology of **a** PM-199 prepared in diH₂O, which served as the control; **b** PM-199 and *B. thailandensis*; **c** PM-199 and Hg. All three samples were

incubated at 30 °C for 72 h. The control retained its neatly crafted layers compared to the sorbents treated with either bacteria or Hg

concentrations. Larger quantities of Hg are expected to be released from soils, particularly during the wet season and storm events due to erosion of bank soils with high Hg content and high permeability. The physical characteristics of EFPC soils favor Hg release, especially the HRD soils, which were previously described as a channery soil with a very low water holding capacity (Dickson et al., 2017).

Competitive binding experiments to evaluate the sorption of Hg(II) to PM-199 in the presence of *B. thailandensis* showed that microbial interactions with the sorbent do not interfere with its ability to immobilize Hg. No significant differences were observed between sorption of Hg(II) to PM-199 in the presence and absence of *B. thailandensis*. *B. thailandensis* in the absence of PM-199 sorbed some of the added Hg(II) compared to an unamended control, but the added biomass did not outcompete the sorption of Hg(II) by PM-199 (Fig. 4). In a different study, it was shown that regardless of increase in biofilm formation over time, sorbents such as activated carbon, were still able to effectively absorb organic contaminants (Mercier et al., 2013). The layered pore structure of PM-199 is an important characteristic of this material increasing its ability to absorb contaminants such as Hg effectively. However, microbial biofilms may impact the pore structure over long timescales and can as such reduce the overall effectiveness of the sorbent. Therefore, microbial growth and biofilm formation can become problematic during the long-term application of any sorbent. Our results showed that *S. marcescens* and *B. thailandensis* have a low impact on PM-199's ability to immobilize Hg, which is important for limiting the amount of Hg leaching off contaminated soil layers into aquatic systems, such as EFPC. Microbial community composition can vary significantly with soil geochemistry, location, and depth. Therefore, remediation strategies involving the deployment of sorbents for the stabilization of Hg-contaminated soils requires site-specific studies to evaluate the long-term effectiveness of sorbent amendments.

4 Material and Methods

4.1 Soil Sampling

EFPC bank soil samples were collected from two sites with soil profiles as previously described (Egbo et al., 2017). Briefly, the samples were collected from site

EFK 11.2 (latitude 35.982243, longitude - 84.32755), which is 11.2 km from the mouth of the creek, and site EFK 18.2 (latitude 36.00438, longitude - 84.28246), which is 18.2 km downstream from the mouth of the creek. Samples from EFK 11.2 were collected from a soil layer also referred to as the historical release deposit (HRD), which is typically observed at > 24" above the water level in the creek as described previously (Dickson et al., 2017). A reference soil sample was collected from an uncontaminated reference site (Hinds Creek) which is located approximately 45 km northeast of EFPC in a watershed with similar geochemistry, hydrology, and underlying geology (Egbo et al., 2017). Samples were collected in summer (July 2015), fall (October 2016), spring (March 2017), and winter (December 2017). The soils were collected from the stream banks of the creek and stored in sterile sampling bags (Whirl-Pak, Nasco, Madison, WI, USA) and then transported on dry ice to Alabama State University within 24 h of sampling and kept at - 20 °C until analysis.

4.2 Isolation of Microorganisms

All glassware was soaked in 25% HNO₃ overnight and rinsed three times with deionized water (diH₂O), and then soaked with 10% HCl overnight then rinsed three times with diH₂O before autoclaving. Prevalent bacteria (*Serratia marcescens* and *Burkholderia* sp.) known to form biofilms previously identified in EFPC soils were cultured using nutrient-specific media as described previously (Egbo et al., 2017). *Burkholderia thailandensis* (ATCC 700388) was cultured in Nutrient Broth No. 3 containing 5 g/L peptone, 1 g/L meat extract, 2 g/L yeast extract, and 5 g/L sodium chloride (Sigma-Aldrich, St. Louis, MO, USA) for 24 h at 30 °C while *S. marcescens* was cultured in nutrient broth containing beef extract and pancreatic digest of gelatin (Carolina Biological Supply, Burlington, NC, USA) for 24 h at 25 °C. All bacteria were grown to OD₆₀₀ of 0.5 before inoculating the biosorption assay samples.

4.3 Impact of Soil Bacteria on Hg Release from Soils Amended with PM-199

The impact of soil bacteria on Hg stability in contaminated soils amended with Organoclay PM-199 (CETCO, Hoffman Estates, IL, USA) was investigated using soils collected from EFPC and Hinds

Creek over different seasons. Approximately 1 g of soil was added to 25 mL of diH₂O and allowed to stand at room temperature for 14 days. The purpose of this step was to release any unbound Hg from the soil. After day 14, the soil samples were spun down at 4000 rpm for 15 min at 25 °C before discarding the supernatant. The soils were resuspended in 23 mL of diH₂O and 2 mL of the *Serratia marcescens* culture grown to an OD₆₀₀ of 0.5 and 0.2 g of PM-199 was added. The culture was incubated at 25 °C for 48 h on an orbital shaker (160 rpm). After 48 h of incubation, the mixture was spun down at 4500 rpm for 15 min, and the supernatant collected and preserved in 0.1% trace metal-grade HCl before analyzing the total Hg concentration (HgT) in the solution. The control was just the supernatant from soil without bacterial culture and/or sorbent after 14 days and incubated under the same conditions as the treated samples.

4.4 Competitive Binding of Hg(II) to PM-199 in the Presence of the Soil Bacterium *Burkholderia thailandensis*

To evaluate the impact of soil bacteria on the stability of Hg(II) sorbed to PM-199 at different Hg concentrations. For these experiments, the type strain *B. thailandensis* (ATCC 700388) was used due to its abundance in EFPC soils based on data shown in previous studies (T. E. Egbo and B. K. Robertson unpublished data). A stock solution containing 5.6 g/L of mercuric nitrate was diluted to 68 µM, 13.6 µM, and 6.8 µM in PBS, and the pH was adjusted to 6.8. A total of 6 mL of a suspension containing 0.1 g of PM-199, 1 mL of *B. thailandensis* grown to an OD₆₀₀ of 0.5, and 5 mL of the buffered mercuric nitrate solutions were incubated at 30 °C on an orbital shaker (160 rpm). After 48 h of incubation, the suspensions were filtered through a syringe-driven hydrophilic polyethersulfone filter with a pore size of 0.22 µm (Millipore, Burlington, USA) to remove biomass and sorbent particles prior to analysis. The filtrate was preserved by addition of 0.1% (v/v) trace metal-grade HCl before analyzing the total mercury (HgT) in the solution. All experiments were performed in triplicate.

4.5 Total Mercury Analysis

The total Hg present in each solution was analyzed using a modified version of EPA method 1631 (USEPA 2016). Briefly, each sample was digested with 0.1% (v/v) bromine monochloride (BrCl) for a minimum of 24 h to oxidize any organic matter introduced by the bacteria or the media. Hydroxylamine was added to the solution to remove excess BrCl from the solution before adding 0.5 mL stannous chloride to reduce Hg²⁺ to Hg⁰. A cold vapor atomic fluorescence spectrophotometer (CVAFS) was used for the analysis (Brooks Rand Instruments, Seattle, WA). The Hg⁰ was purged from the solution and trapped on gold-coated bead traps. The traps were heated in a stream of argon to release the Hg⁰ which was then detected using CVAFS (Telliard & Gomez-Taylor, 2002).

4.6 Structural Evaluation of PM-199

The morphology and size of PM-199 after treatment with either the Hg solution or *B. thailandensis* cultured as described above were observed using a high-resolution scanning electron microscopy (SEM). To evaluate the microbial interaction with PM-199, 2 mL of *B. thailandensis* (OD₆₀₀ of 0.5) was incubated with 0.1 g of PM-199 for 72 h at 30 °C. To distinguish the effect of Hg on the morphology of PM-199 compared to *B. thailandensis*, 0.1 g of PM-199 was mixed with 2 mL of 13.6 µM Hg(II) of mercuric nitrate solution in PBS and allowed to stand at 30 °C for 72 h. All samples including the control, 0.1 g of PM-199 was washed thrice with PBS (pH 7.2), fixed with 2% paraformaldehyde, and air-dried at room temperature prior to imaging. Dried grains of PM-199 from the different treatments were mounted on a metal stub using a double-sided carbon tape, sputter-coated with gold, and imaged using SEM (JEM-6010LA model, JEOL USA, Inc., Peabody, MA, USA).

4.7 Statistical Analysis

All statistical analyses were done using GraphPad Prism Software version 7.0 (GraphPad Software, San Diego, CA, USA). For quality control purposes, experiments were conducted in triplicates, and error bars represents the standard deviation of triplicates. Comparisons and relationships were determined to be significant at $p < 0.05$.

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Declarations

Conflict of Interest The authors declare no competing interest.

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