Environmental Science & Technology Letters

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Letter

Biofilm Formation Influences the Wettability and Settling of Microplastics

Amber J. Pete, Philip J. Brahana, Mustapha Bello, Michael G. Benton, and Bhuvnesh Bharti*

Cite This: *Environ. Sci. Technol. Lett.* 2023, 10, 159−164

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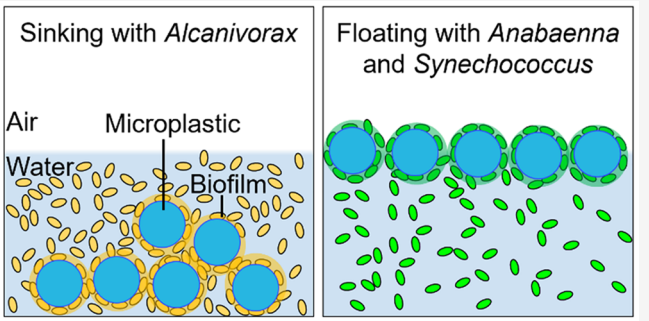
ABSTRACT: The fate of 99% of the plastics present in oceans is unknown. It is presumed that biofilm formation on plastics leads to their sinking to the ocean floor, thus making them undetectable at the surface. While it is established that biofilms lead to sinking of plastics, it is the mechanism by which biofilms enhance the vertical transport of plastics that remains unknown. It is commonly assumed that biofilms increase the effective mass density of the plastics, which drives their sinking. Here, we show that such an assumption is not always true, and formation of biofilms alone is an insufficient criterion to predict the sinking or floating of plastics. We study the biofilm formation and vertical transport of polyethylene microplastics in the presence of *Alcanivorax borkumensis*, *Anabaena* sp., and *Synechococcus elongatus*. We find that while all three microorganisms formed biofilms on microplastics, only *Alcanivorax* led to their sinking. The sinking of microplastics is attributed to the production of highly active biosurfactants by *Alcanivorax* and its adsorption onto microplastics, which is not the case for *Anabaena* and *Synechococcus*. Our study highlights that it is not only the formation but the properties of the formed biofilms that govern the sinking or floating of plastics.



KEYWORDS: *Biofilm formation, microplastics, biosurfactants, plastic waste, microplastic transport*

INTRODUCTION

Plastics have been produced since the early 1900s,[1] and their widespread use continues to increase globally. Most commodity plastics are used for short times and thereafter discarded as waste.[2] It is estimated that 6300 t of plastic waste was generated between 1950 and 2015, with nearly 80% of that waste accumulating in landfills or aquatic environments.[3,4] Out of all the plastic waste released into the oceans, nearly 99% remains unaccounted for, presumably sunk to the bottom of the ocean.[5] The plastics could sink to the ocean floor either in their macroscopic form or as debris less than 5 mm in size, commonly known as microplastics.[6] Despite clear evidence for sinking of macroplastics and microplastics, various environmental factors which could contribute to vertical transport of plastics are yet to be identified.

Microplastics have become ubiquitous, but their persistence in water bodies is especially concerning as these plastics are in an environment conducive to integration into the aquatic food web. Microplastics have been recognized as vectors of common pollutants,[7−9] thus their integration within the food chain poses a severe hazard to both aquatic life and human health. Therefore, it is critical to understand the mechanisms driving the transport of microplastics and correspondingly predict their fate in the environment.[10]

Microplastics entering the aquatic environment in their microscopic state are called *primary* microplastics, while microplastics forming in the environment by gradual weathering and abrasion of macroscopic plastics are called *secondary* microplastics.[10] In both forms, microplastics come into immediate and direct contact with microorganisms when released/formed in fresh or marine waters. Microorganisms can attach to the surfaces of microplastics and secrete extracellular polymeric substances (EPS), driving the formation of biofilms.[8] The biofilm formation is linked to the increased mass densities of the plastics leading to their sinking in aquatic environments.[11−14] However, two questions remain unanswered: (1) Do all microbial biofilms lead to the sinking of microplastics? (2) How do biofilms enable the vertical transport and sinking of microplastics? The lack of answers to these questions stems from the inherent complexity of not only plastic−microbe interactions but also our ability to characterize the compositions and properties of the biofilms formed on microplastics. To predict the fate of microplastics in real environments, it is critical to understand the mechanism of

Received: October 10, 2022
Revised: October 20, 2022
Accepted: October 21, 2022
Published: October 25, 2022

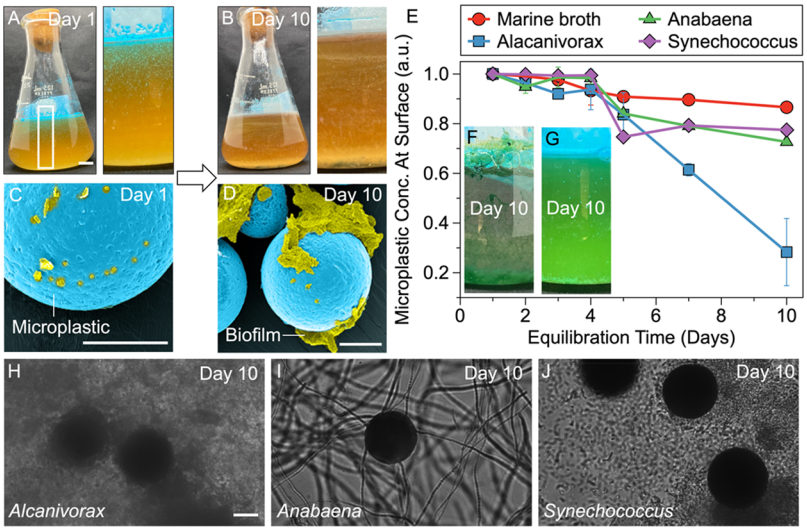


Figure 1. Biofilm formation and vertical transport of microplastics. (A, B) Images of flasks containing microplastics (blue) and *Alcanivorax* on Days 1 and 10. Scale bar = 1 cm. (C, D) False colored SEM image showing the formation of biofilm (yellow) by *Alcanivorax* on microplastics (blue) after Day 1 and Day 10 of equilibration. Scale bar = 20 μm. (E) Change in microplastic concentration at the air−water interface (blue value) as a function of equilibration time for each culture. The obtained concentrations were normalized to the value on Day 1. (F, G) Zoomed-in image of the air−water interface showing the accumulation of microplastics (blue) and biofilm formation by *Anabaena* and *Synechococcus* after 10 days of equilibration. (H−I) Optical microscopy images showing the existence of biofilms after Day 10 of equilibration with *Alcanivorax*, *Anabaena*, and *Synechococcus*. Scale bar = 30 μm. Microplastics sink in culture of *Alcanivorax* but remain floating in cultures of *Anabaena* and *Synechococcus*.

biofilm-driven sinking or floating of microplastics which is the focus of this Letter.

The current study provides a heuristic relationship between the sinking of microplastics and the nature of microbial biofilm formation on their surfaces. This Letter explores the contribution of biofilm formation to the sinking of microplastics in aquatic environments. We use polyethylene microspheres as model microplastics because of their widespread presence in aquatic environments.[4] We investigate the formation of biofilms onto polyethylene microplastics using three microorganisms: alkane-degrading *Alcanivorax borkumensis* − marine water, cyanobacterium *Anabaena* sp. − freshwater, and cyanobacterium *Synechococcus elongatus* − freshwater. These microorganisms are environmentally relevant,[15−19] making them good models to understand the mechanism of vertical transport of polyethylene microplastics. The mixture of microorganisms and microplastics is studied for 10 days and monitored for the formation of biofilms and corresponding changes in their vertical transport through the water column.

MATERIALS AND METHODS

Microplastics. Polyethylene model microplastics (spheres) with diameters of 70 μm (±10%) were purchased from Cospheric, Inc. (Figure S1). The microplastics were dyed blue in color for ease of visualization in our experiments. The microplastics used in the study were a blend of polyethylene (>70%) with proprietary additives (<30%) and engineered to be neutrally buoyant with mass densities of 1 g cm^{-3}. Using these model microplastics with precise and consistent sizes, densities, and surface properties was necessary to understand the microbial−microplastic interactions and corresponding impacts of biofilm formation on microplastic sinking. Note that in the absence of microorganisms, the particles float to the air−water interface due to their inherent hydrophobicities. These particles remain hydrophobic even after 10 days in water, indicating that the additives in the model microplastics do not leach into the water and result in less buoyant microplastics over time.[20]

Microbial Incubation. *Alcanivorax* was cultured in a modified marine broth,[21] and *Synechococcus* and *Anabaena* were cultured in a modified BG-11 medium[22] (see SI for details). The pH was 7.5 ± 0.2 for each medium. The mixtures of cells and microplastic were incubated at 27 °C in a Forma Scientific Incubator for the entire duration of the experiment.

RESULTS AND DISCUSSION

Vertical Transport of Microplastics. *Alcanivorax* formed biofilms on the surfaces of polyethylene microplastics, enhancing their vertical transport and causing them to sink to the bottom of the water column. Microplastics show a dynamic response to the presence of microorganisms. Immediately after introducing microorganisms, i.e., Day 1, microplastics floated at the air−water surface (Figure 1A). In the presence of *Alcanivorax*, the microplastics began to sink in the growth medium by Day 3 (Figure S2). The air−water surface was completely depleted of microplastics by Day 10 (Figure 1B). The dynamic changes in the locations of microplastics can be readily observed in the zoomed-in view of the incubation flask as shown in Figure 1A and B and Figure S2. We used ImageJ to integrate the blue value, representing microplastics, over the air−water interface of the growth medium. We normalized the integrated blue values to the value at Day 1; hence, the values of 1 and 0, respectively, represent all and none of the microplastics present at the air−water interface. The integrated values provided a semiquantitative measure of the change in concentrations of microplastics at the air−water interface and thus the sinking of the microplastics over time (Figure 1E). We found that microplastic concentration at the interface rapidly decreases after Day 4, highlighting the sinking of microplastics in the presence of *Alcanivorax*. The biofilm formation was confirmed by bright-field optical microscopy and false colored scanning electron microscopy (SEM). The samples for SEM were extensively

https://doi.org/10.1021/acs.estlett.2c00728
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washed, and the majority of the biofilm was removed to allow for simultaneous visualization of microplastics (blue) and biofilm (yellow) as shown in Figure 1C and D. A more realistic view is provided by the optical microscope image, which shows the formation of a thick biofilm on the microplastics by Day 10 (Figure 1H). The formation of biofilms and corresponding sinking of microplastics are consistent with the literature that suggests that biofilm formation (biofouling) leads to the vertical transport of plastics.[14,23,24] However, as we demonstrate below, the origin of the sinking of microplastics by *Alcanivorax* is not merely biofilm formation but likely due to biosurfactant production by the microorganism.

To investigate the universality of the relation between biofilm formation with microplastic sinking, we studied the vertical transport of microplastics in cultures of common cyanobacteria, *Anabaena* sp. and *Synechococcus elongatus*. We found that despite the formation of biofilms on the surfaces of microplastics (Figure 1F, G), neither cyanobacterium elicited considerable sinking (Figure 1E; Figures S3 and S4). Instead, the biofilm appeared to hold the microplastics together at the air−water surface (Figure 1F, G, I, J). This observation is in contrast with previous findings of Bose and co-workers,[13] where the cyanobacteria induced aggregation and settling of polystyrene particles. The observed difference can be attributed to the dissimilarity of the surface chemistry and compositions of the commercial polystyrene particles used in the previous study, which are expected to be highly hydrophilic unlike our polyethylene microplastics which are hydrophobic. In our study, we found that despite biofilm formation in all three cultures (Figure 1H−J) microplastics sank only in the presence of *Alcanivorax*.

Biofilm Formation on Microplastics. The formation of biofilms at the microplastic surface is not the only factor that governs the vertical transport of microplastics in the environment. After Day 10 of equilibration, microplastics in all three cultures showed distinct biofilms adhered to their surface (Figure 1H−J; Figures S5 and S6). No sinking of microplastics was observed with *Anabaena* and *Synechococcus* even for extended equilibration times of 120 days. The observed biofilm formation in all three cultures, yet sinking only in *Alcanivorax*, suggests that the microbial attachment and biofilm formation alone are insufficient to influence the vertical transport of microplastics.

To understand the difference in microplastic transport behavior in the three cultures, we correlated biofilm formation to the kinetics of microplastic sinking. We quantified the change in locations of microplastics immobilized on the air−water interface within a quartz capillary of diameter 3.6 mm (Figure S7). In a typical experiment, 300 μL of dilute cells ($\sim 10^6$ cells mL^{-1}) and 5 mg of microplastics were added to the capillary. We used a high-speed camera to image the air−water interface where the microplastics were initially present. We quantified the equilibration time-dependent changes in the mean gray value of the region immediately below the interface shown by the yellow rectangle in Figure 2B. The gray value is the measure of the concentration of microplastics and/or microorganisms in the water phase directly below the air−water interface. Note that this experimental setup is unique and enabled the time-dependent quantification of vertical transport of microplastics, which is not the case with traditional methods.[14,23]

Initially all microplastics remain adsorbed at the air−water interface despite being neutrally buoyant, due to their

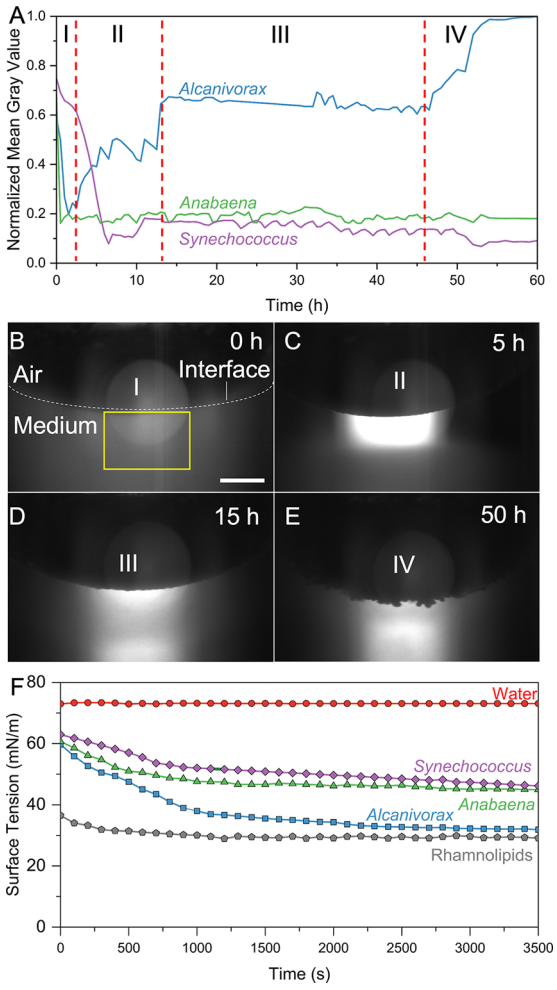

Figure 2. Transport of microplastics in the presence of microbes and interfacial activity of the media. (A) Time-dependent variations in the gray value in the air−water interfacial region. The gray values are obtained by analyzing the 1.8 mm × 1.2 mm region immediately below the interface as represented by the yellow box in B. The intensity is the mean gray value within the box. The 8-bit grayscale images have 256 intensity levels; in our case, we assign black as 255 and white as 0. In our case, higher gray values indicate the presence of microorganisms and/or microplastics in the yellow observation box. (B−E) Sequence of images showing the sinking of microplastics with *Alcanivorax*. The mechanism of biofilm formation and sinking of microplastics with *Alcanivorax* is divided into four stages. Stage I: Initially dispersed cells settle to the bottom of the capillary (B → C). Stage II: Cells migrate back toward the interface (C → D). Stage III: *Alcanivorax* cells accumulate and grow at the microplastics surface (D). Stage IV: Microplastics covered with biofilms protrude down from the air−water interface and sink through the water column (E). (F) Dynamic surface tensions of water, rhamnolipids, and supernatant from each of the cultures, indicating that *Alcanivorax* produces a highly active biosurfactant.

hydrophobicity. The mean gray value in the vicinity of the interface rapidly decreases and then remains constant for *Anabaena* and *Synechococcus*, highlighting that the majority of these cyanobacteria sink to the bottom, but microplastics remain at the interface (Figure 2A; Figure S8). No notable change in the vicinity of the air−water interface containing microplastics was observed throughout the 120-h experiment (not shown). The biofilms formed by these two cyanobacteria

https://doi.org/10.1021/acs.estlett.2c00728
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on microplastics (Figure 1I, J) is likely the result of a small fraction of the cells trapped at the air−water interface.

The *Alcanivorax* cultures with microplastics showed a nonlinear variation of gray values with time. We identified four distinct stages of *Alcanivorax* growth and microplastic transport. In Stage I, we observed that the *Alcanivorax* cells were highly concentrated near the interface (Figure 2B), but after 4 h, the cells started to settle to the bottom of the capillary as reflected by the decrease in the gray value. In Stage II (after 15 h), the gray value increased indicating the migration of cells back toward the air−water interface and accumulation in the vicinity of the microplastics (Figure 2C). *Alcanivorax* prefers to use hydrocarbons as an energy source, thus its observed migration back to the interface and subsequent biofilm formation can be attributed to its affinity for polyethylene microplastics.[25,26] Note that the migration of *Alcanivorax* cells back to the interface after initial settling was not observed in the absence of microplastics (Figure S9). In Stage III, the gray value remains constant as the cells adhere to the microplastic surface, replicate, and form biofilms (Figure 2D). The biofilm-covered microplastics enter the observation box as they began to sink in Stage IV (Figure 2E). The aggregation and sinking of microplastics upon contact with biological matter (e.g., biofilms) are believed to be responsible for "missing" microplastics, i.e., microplastics predicted to exist in the ocean but unaccounted for on the ocean surface.[27] The observed formation of biofilms in all three cultures but sinking only in the case of *Alcanivorax* highlights that the correlation of biofilm formation with the sinking of microplastics is not as strong as previous literature suggests. Ionic strength of growth media may also lead to increased adhesion of *Alcanivorax* onto hydrophobic surfaces, as shown in previous studies.[28,29] However, ionic strength does not influence the ability of extracellular DNA to assist with the attachment of bacteria to surfaces.[30] Hence, although cells may attach faster in high ionic strength media, the number of cells attached to a surface at equilibrium is not significantly impacted by ionic strength. We hypothesize that biofilms alter the surface properties of microplastics which impact their wettabilities, possibly through the release of biosurfactants. To verify this hypothesis, we studied the surface tension of the cell medium after the cultures reached the stationary phase of growth.

Surface Activity of Medium. A highly surface-active biosurfactant produced by *Alcanivorax* could alter the wettability of microplastics and thus drive the observed sinking of microplastics. The reduction in surface tension by the microbes is a strong indicator of the presence of biosurfactants. We quantified the interfacial activities of the three microbial cultures using a pendant drop optical tensiometer (Biolin Scientific). For the measurements, we centrifuged out the cells at 5000*g* (*Alcanivorax* and *Synechococcus*) and 7000*g* (*Anabaena*) for 30 min; here, *g* is the gravitational acceleration. No further filtering of the supernatant was performed. The dynamic surface tensions of the biosurfactant extracts (supernatants) from all three cultures and 0.4 mM rhamnolipids, a model glycolipid, are shown in Figure 2F. Two critical observations from the tensiometry data can be made: (1) The surface tension reaches its equilibrium value after a delay of ∼1200 s for all three microorganisms. The observed delay is attributed to the presence of nanometer- and micrometer-sized interfacially active aggregates in the supernatants, which require a finite time to diffuse and adsorb onto the interface. (2) Each of the microbes showed a substantial reduction in surface tension in comparison to water, but *Alcanivorax* resulted in the lowest surface tension value, comparable to rhamnolipids. The surface tension reduction is likely from the release of biosurfactants comprised of fatty acids, lipids, and lipopeptides from the cells and within the biofilms.

Biosurfactants, such as those produced by *Alcanivorax*, are known to reduce surface tension between 45 and 30 mN/m, with the most surface-active biosurfactants reducing surface tension to ∼30 mN/m. Biosurfactants are surface-active agents produced extracellularly or as part of the cell membrane by microorganisms.[31] *Alcanivorax* produces both glycolipids and phospholipids which could act as biosurfacants.[32] We believe that the hydrophobic tails of the biosurfactants adsorb onto the hydrophobic polyethylene microplastics[33] with the hydrophilic heads toward the solvent. This adsorption process would increase the wettability of microplastics with water and assist with sinking (Figure 1). We isolated the biosurfactant extract from *Alcanivorax* by centrifugation. The extract was then added to *Anabaena* and *Synechococcus* cultures containing microplastics (Figure 1F, G) causing an immediate transfer of microplastics from the surface to bulk water (Figure S10). The vertical migration of the model microplastics into bulk water was also observed upon adding rhamnolipids to the polyethylene microplastics and nanoplastics floating on the surface of water (Figure S11). The observed sinking of microplastics with small amounts of added biosurfactant extract from *Alcanivorax* and rhamnolipid highlights that increase in water wettability of the plastics enhances their vertical transport. The observed changes in the vertical transport of microplastics is further corroborated with contact angle measurements on polyethylene sheets (Figure S12). We find that the water contact angle decreases from 98° to 0° after Day 1 of equilibration of the polyethylene sheets in *Alcanivorax*. Conversely, the water contact angle decreases only to ∼80° for polyethylene sheets equilibrated for 5 days with *Anabaena* and *Synechococcus*. The rapid increase in water wettability of the polyethylene exposed to the *Alcanivorax* culture not only demonstrates the hydrophobic-to-hydrophilic shift that drives the sinking of the microplastics but also alludes to the mechanism of transport being related to the biosurfactant produced by the bacteria. Note that the biosurfactants produced by *Alcanivorax* have yet to be isolated; therefore, our observations of microplastic sinking combined with the low surface tension of the *Alcanivorax* culture highlight the significant role of biosurfactants in governing the vertical transport of microplastics. Further studies on the kinetics of biofilm formation, biosurfactant production, and vertical transport of microplastics are necessary for a comprehensive understanding of the mechanism of plastics sinking to the ocean floor.

In summary, we investigated the vertical transport and sinking behavior of polyethylene microplastics in the presence of *Alcanivorax*, *Anabaena*, and *Synechococcus*. We found that all three microorganisms form biofilms on the surface of polyethylene but sinking of microplastics was observed only for *Alcanivorax*. These findings suggest that the vertical transport of microplastics in aquatic environments is not solely governed by microbial biofilm formation, but the compositions of biofilms also play a significant role. We attribute the observed sinking of microplastics by *Alcanivorax* to the production of interfacially active biosurfactants. Our study outlines a relationship between biofilm formation and the transport of microplastics in aquatic environments, where

https://doi.org/10.1021/acs.estlett.2c00728
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biosurfactants produced by microorganisms could play a governing role. Further investigations are needed to identify the universal relationships (if any) between biofilm formation, biosurfactant production, and corresponding vertical transport of microplastics. Such in-depth knowledge is necessary for understanding the transport of microplastics, which would assist in developing predictive models for determining the fate of microplastics in the environment.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.2c00728.

Experimental details of cultures used in the study, optical micrograph of microplastics, images of microplastic cultures with three bacteria at Days 0, 3, and 10, bright-field microscope images of biofilms formed by bacteria, SEM image of biofilm formed by *Anabaena* on microplastics, schematic of the experimental setup used to study the vertical transport of microplastics, images of the air−water interface showing the sinking/floating of microplastics, sinking of microplastics upon the addition of biosurfactant extracts from *Alcanivorax* and rhamnolipids to cultures of *Anabaena* and *Synechococcus* containing microplastics, contact angle change of the polyethylene sheet upon formation of biofilms by the three tested bacteria (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Bhuvnesh Bharti − *Cain Department of Chemical Engineering, Louisiana State University, Baton Rouge, Louisiana 70803, United States;* orcid.org/0000-0001-9426-9606; Phone: (225)578-3546; Email: bbharti@lsu.edu

Authors

Amber J. Pete − *Cain Department of Chemical Engineering, Louisiana State University, Baton Rouge, Louisiana 70803, United States*

Philip J. Brahana − *Cain Department of Chemical Engineering, Louisiana State University, Baton Rouge, Louisiana 70803, United States*

Mustapha Bello − *Cain Department of Chemical Engineering, Louisiana State University, Baton Rouge, Louisiana 70803, United States*

Michael G. Benton − *Cain Department of Chemical Engineering, Louisiana State University, Baton Rouge, Louisiana 70803, United States;* orcid.org/0000-0001-5681-9398

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.estlett.2c00728

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Ahmed Al Harraq for his assistance with SEM imaging. A.J.P. was supported by the Huel D. Perkins and George A. Daniels fellowships at LSU. B.B. and P.J.B. acknowledge the Division of Chemistry at the National Science Foundation (MPS-2032497) for financial support.

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https://doi.org/10.1021/acs.estlett.2c00728
Environ. Sci. Technol. Lett. 2023, 10, 159−164