Environmental Science & Technology

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# Enhanced Bioavailability and Microbial Biodegradation of Polystyrene in an Enrichment Derived from the Gut Microbiome of *Tenebrio molitor* (Mealworm Larvae)

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Cite This: *Environ. Sci. Technol.* 2021, 55, 2027−2036

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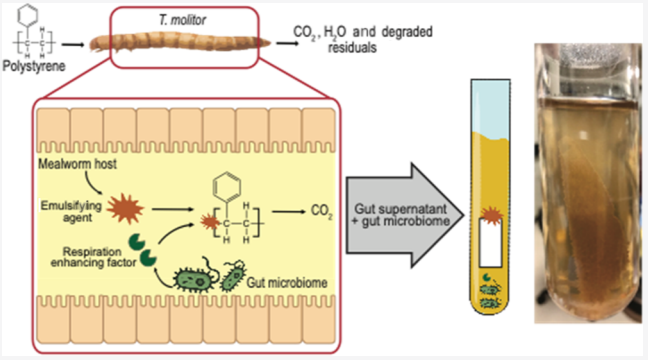
**ABSTRACT:** As the global threat of plastic pollution has grown in scale and urgency, so have efforts to find sustainable and efficient solutions. Research conducted over the past few years has identified gut environments within insect larvae, including *Tenebrio molitor* (yellow mealworms), as microenvironments uniquely suited to rapid plastic biodegradation. However, there is currently limited understanding of how the insect host and its gut microbiome collaborate to create an environment conducive to plastic biodegradation. In this work, we provide evidence that *T. molitor* secretes one or more emulsifying factor(s) (30−100 kDa) that mediate plastic bioavailability. We also demonstrate that the insect gut microbiome secretes factor(s) (<30 kDa) that enhance respiration on polystyrene (PS). We apply these insights to culture PS-fed gut microbiome enrichments, with elevated rates of respiration and degradation compared to the unenriched gut microbiome. Within the enrichment, we identified eight unique gut microorganisms associated with PS biodegradation including *Citrobacter freundii*, *Serratia marcescens*, and *Klebsiella aerogenes*. Our results demonstrate that both the mealworm itself and its gut microbiome contribute to accelerated plastic biodegradation. This work provides new insights into insect-mediated mechanisms of plastic degradation and potential strategies for cultivation of plastic-degrading microorganisms in future investigations and scale-up.




## INTRODUCTION

Plastic pollution is a well-known threat to human and environmental health. Strategies are needed for effective management of recalcitrant plastic wastes because current cradle-to-grave lifecycles result in plastic accumulation in landfills and in the environment.[1] Thus, there is an urgent need to permanently and completely eliminate plastic waste from this linear lifecycle. Recent work to achieve this goal has focused on natural plastic biodegradation, mainly identifying and isolating bacterial strains capable of biodegrading plastic polymers.[2−6] Among the microbes tested against non-hydrolyzable[7] or vinyl plastics (*e.g.*, polystyrene (PS), polyethylene, and polypropylene), the rates of plastic degradation vary but are typically low, ranging from ∼5% plastic degradation (by mass) within 6 months (∼0.03% per day) to ∼8% within 2 weeks (∼0.6% per day).[8,9] Hydrolyzable plastics (*e.g.*, polyesters such as polyethylene terephthalate) are susceptible to significantly higher rates of microbial degradation.[10,11]

A fundamental limitation to microbial biodegradation of plastics is the limited bioavailability of plastics to microbial attack.[4,12,13] Petroleum-derived plastics have high molecular weights and are highly hydrophobic, both of which limit the ability of microorganisms to interact with these materials, especially in aqueous environments.[4,12,13]

Efforts to improve plastic biodegradation rates have revealed that the gut environment of insect larvae can support comparatively rapid plastic degradation. To date, plastic degradation has been demonstrated in several holometabolic insect species: *Tenebrio molitor* (yellow mealworms),[14−17] *Tenebrio obscurus* (dark mealworms),[18] *Zophobas atratus* (superworms),[19,20] *Plodia interpunctella* (Indianmeal moth),[21] *Achroia grisella* (lesser wax moths),[22] and *Galleria mellonella* (greater wax moths).[23−27] Of these, the best characterized is the yellow mealworm.[17] Previous research has established that ingested plastic is retained within the gut of yellow mealworms for 15−20 h, during which time nearly 50% of the plastic is mineralized to $CO_2$ (∼60% per day).[14,16,17] Researchers have also demonstrated that the gut microbiome of yellow mealworms can degrade several chemically dissimilar plastics (including PS and polyethylene), suggesting an initial, non-specific attack.[28]

**Received:** July 24, 2020
**Revised:** November 1, 2020
**Accepted:** December 31, 2020
**Published:** January 12, 2021







https://dx.doi.org/10.1021/acs.est.0c04952
*Environ. Sci. Technol.* 2021, 55, 2027−2036

Plastic-degrading microorganisms demonstrate expedited degradation within the insect gut environment, implying that the insect host may play a role in the plastic biodegradation process. When the gut microbiome activity is suppressed, *G. mellonella* retains the capacity to metabolize polyethylene.[23] In addition, significant changes are observed in the proteins secreted by the insect's salivary glands, underscoring its role in plastic degradation.[23] When the gut microbiome is left intact, the microbial community composition shifts during degradation and one plastic-degrading bacterium was isolated: *Enterobacter* sp. D1.[24,26] This suggests a complex system in which the insect host and its gut microbiome collaborate to degrade plastic.

Mealworms are a useful model organism for plastic biodegradation studies because they are readily cultivated and are the subject of a rapidly growing body of research.[17,28−30] As with *G. mellonella*, previous research has demonstrated that the mealworm gut microbiome undergoes notable changes during plastic degradation.[17,28] Suppression of gut microbiome by supplementing their diet with antibiotics indicates that the microbiome is necessary for PS biodegradation.[15] PS-degrading *Exiguobacterium* sp. YT2 has been isolated from the mealworm gut microbiome,[15] and researchers have investigated enzyme activity in both the mealworm and the gut microbiome, though the specific mechanisms and the role of the mealworm itself have yet to be identified.[30]

In this work, we describe the efforts made to increase plastic biodegradation rates by enhancing gut microbiome-derived enrichments outside the gut microenvironment. To do this, we first investigated the roles of the mealworm and its gut microbiome in plastic degradation. We then applied these insights to enhance plastic degradation in microbial enrichments outside the mealworm.

## MATERIALS AND METHODS

**Plastic Materials.** Commercially available expanded PS foam [thickness: 5.2 cm, weight-averaged molecular weight ($M_w$): 170,700 ± 9800 Da, and number-averaged molecular weight ($M_n$): 88,500 ± 5900 Da] was used as a feed for the mealworms (Carlisle Construction Materials, Puyallup, WA). PS foam blocks were cut into 2−3 cm cubes and cleaned with a stream of air to remove any residues. PS microplastics (size: ∼150 μm, $M_w$: 76,400 ± 4700 Da, and $M_n$: 41,500 ± 3300 Da) were used in microbial cultures (Jinshuowang Plastic Materials Co., Ltd, Dongguan, Guangdong, China). PS microplastics were either used directly in microbial cultures or heat-pressed at 260 °C using PHI Manual Compression Press (PW-22 Series, Industry, CA) into uniform thin films (thickness: 17.6 ± 1.1 μm) for use in microbial cultures.

**Mealworm Maintenance.** Mealworms, larvae of *T. molitor* Linnaeus (average weight: 75−85 mg/worm), were purchased online from Rainbow Mealworms (Compton, CA) and shipped overnight to the laboratories at Stanford University (mealworms from this source have previously been shown to degrade PS).[16,17,28,31] Prior to arrival, the mealworms were fed bran; after arrival, they were subject to a 48 h starvation period before initiating tests with the experimental diet of either natural wheat bran (Exotic Nutrition, Newport News, VA) or PS foam. Mealworms (∼1200 per experimental condition) were bred in food-grade polypropylene containers (volume: 780 mL) and kept in incubators maintained at 25 °C and 70% humidity.[14,16] Each diet condition was duplicated.

To elucidate the respective roles of the mealworm and its gut microbiome as well as the effects of a natural versus plastic diet, we bred mealworms under four conditions: (1) bran (control diet), (2) PS, (3) bran + antibiotic, and (4) PS + antibiotic in the experimental setup described above. Gentamicin sulfate (Acros Organics, Fisher Scientific, Pittsburg, PA) was the antibiotic used to suppress the gut microbiome as described in previous studies.[15−17] Antibiotic experiments were used to assess the role of the mealworm in the absence of significant gut microbiome activity. Gentamicin was added at 30 mg/g bran to the bran + antibiotic group and an equivalent mass was added to the PS + antibiotic group. The PS-fed mealworms were initially given 2.6 g of PS foam. The bran-fed mealworms were initially given 12 g of bran (the mass of each diet supplied was based on previous studies[14,16,17,28]). Bran and gentamicin were supplemented every 3 days, and dead mealworms were removed. The mealworms were maintained on the experimental diets for ∼3 weeks to allow for adaptation to the experimental diets[16,28] before use in any experiments. Prior to testing, the mealworms were subjected to a 24 h starvation period to ensure removal of antibiotic residuals.

**Collection of Gut Microbiome and Supernatant.** To collect the mealworm gut microbiome and produce a supernatant containing secreted factors from the mealworm and its gut bacteria (henceforth, "supernatant"), the following procedure was used. Fifty mealworms from each diet were scarified following established methods.[15] Briefly, the mealworms were immersed in 75% ethanol for 1 min and then rinsed with deionized (DI) water. Mealworm guts were harvested via dissection into 0.6 mL of sterile saline (0.75% NaCl) in a sterile microcentrifuge tube. The samples were vigorously vortexed (Vortex-Genie 2, MO BIO, Carlsbad, CA), and the liquid phase was collected and replaced with sterile saline water. This was repeated ∼3−4 times until the liquid phase was clear. The collected liquid was then passed through a 40 μm nylon cell strainer (Falcon, Fisher Scientific, Pittsburg, PA) and then centrifuged at 4000 rpm for 10 min to collect gut bacteria.

Following centrifugation, the supernatant was filtered through a 0.2 μm syringe filter (25 mm, nylon, Fisher Scientific, Pittsburg, PA) to remove the remaining particulates. The filtered liquid was then either saved for future use in experiments ("supernatant") or subject to fractionation by molecular weight to better understand the role(s) of secreted factors. Molecular weight spin columns (Amicon Ultra-15 Centrifugal Filters, MilliporeSigma, St. Louis, MO) were used to split the supernatant into three fractions: <30 kDa, 30−100 kDa, and >100 kDa (SI M1).

All supernatant samples were stored at 4 °C until used in experiments. An additional supernatant was collected as needed to ensure that the samples were not stored for longer than 3 weeks.

**Enrichment and Isolation of PS-Degrading Bacteria.** A microbial enrichment was used to study the potential for cultivation of key gut microbiome members outside the mealworm gut. The gut microbiome was collected from 50 PS-fed mealworms as described above. The collected gut bacteria were then inoculated into 30 mL of carbon-free Bushnell Haas (BH) media containing the following chemicals (g/L): 0.2 $MgSO_4$, 0.02 $CaCl_2$, 1.0 $KH_2PO_4$, 1.0 $K_2HPO_4$, 1.0 $NH_4NO_3$, and 0.05 $FeCl_3$.[32] The PS film (0.5 g) was added to the culture along with the supernatant (0.18 mL, 0.6% v/v) from the PS-


https://dx.doi.org/10.1021/acs.est.0c04952
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fed mealworms. The enrichment was grown in a round-bottomed glass culture tube (25 mm outer diameter, Fisher Scientific, Pittsburg, PA), sealed with a Kim-Kap (25 mm outer diameter, Fisher Scientific, Pittsburg, PA), and incubated at 30 °C on an orbital shaker table (150 rpm). The enrichment was cultured for 3 months with weekly 10 mL media changes and supplemented with the PS supernatant (0.18 mL, 0.6% v/v) until a stable biofilm is developed on the PS film (Figure S3).

Serum bottle assays were conducted to assess the volume of $CO_2$ produced by PS-degrading enrichments. The cultures were incubated in 25 mL serum bottles (Wheaton, Millville, NJ) capped with butyl-rubber stoppers (MilliporeSigma, St. Louis, MO) and crimp-sealed (MilliporeSigma, St. Louis, MO) under an air headspace of 1 atm. The liquid volume was 15 mL and the headspace volume was 10 mL. The cultures were incubated at 30 °C on an orbital shaker table (150 rpm). All serum bottle assays were conducted in triplicate. The serum bottles were inoculated with either bacteria from the mealworm gut or bacteria from the enrichment. To obtain sufficient inoculum, bacteria from either sample were first grown in 15 mL tryptic soy media for 24 h at 30 °C. The bacteria were then collected by centrifugation, washed with BH media, and re-spun prior to use.

To isolate the bacterial strains involved in plastic degradation from the PS-degrading enrichment, 30 $\mu$L of liquid culture from the enrichment was plated on non-selective agar plates (tryptic soy agar, Difco, Fisher Scientific, Pittsburg, PA). Colonies were picked with sterile inoculum loops and streak-plated to isolate individual pure colonies, which were then grown in 3 mL tryptic soy media (MilliporeSigma, St. Louis, MO) for 24 h at 30 °C. A bacterial pellet was then collected by centrifugation, rinsed with BH media, and re-spun prior to use. The rinsed bacteria were then inoculated in 5 mL BH media with the PS film, supplemented with the PS supernatant (30 $\mu$L, 0.6% v/v), and cultivated at 30 °C on an orbital shaker (150 rpm).

To identify bacteria associated with plastic degradation, colonies were isolated from the enrichment and sequenced. Liquid media from the enrichment and isolates described above was first plated on tryptic soy agar plates. For each isolate capable of growth on PS (16 total), an individual colony was randomly selected for sequencing. For the enrichment, individual representative colonies were selected for sequencing (16 total). DNA was extracted from the selected colonies using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) following manufacturer's protocols. The 16s rRNA V4 region was then PCR amplified using 8F (5′AGAGTTTGATCCTGGCTCAG3′) and1492R (5′GGTTACCTTGTTACGACTT3′). Amplification was confirmed by denaturing gel electrophoresis and clean PCR products (QIAquick PCR Purification Kit, Qiagen, Hilden, Germany) were sent to MCLAB (San Francisco, CA) for sequencing on ABI 3730xl automated sequencers. Pair-end reads were aligned in Geneious Prime (2020.1.1, de novo assembled using the Geneious assembler with standard settings) and strains were identified by a BLAST sequence similarity search of the NCBI 16s rRNA database with a sequence similarity threshold of >97%. Additional information on the sequenced strains is available in Table S1.

**Microbial Activity Analysis.** Biolog MT2 Microplates (Biolog Cat. # 1013, Hayward, CA) were used to screen the ability of cultivated bacteria and the mealworm gut microbiome to metabolize PS as their sole carbon source. Each MT2 microplate is pre-loaded with buffered nutrient medium (without any carbon sources) and a redox dye (tetrazolium violet), a well-established colorimetric indicator of carbon oxidation (respiration) by bacteria.[33,34] Bacteria from the gut microbiome were collected via centrifugation as described above and resuspended in sterile saline (0.75% NaCl) such that the final optical density was ∼0.9 ($OD_{595}$).[35] The bacterial suspension was inoculated into the MT2 microplate at 130 $\mu$L per well, followed by the addition of the supernatant and/or PS microplastics (∼3 mg) to the appropriate wells. All experimental conditions were tested in triplicate with a negative control (loaded with inoculum and supernatant without PS) and positive controls (loaded with inoculum and glucose and supernatant as described below) in duplicate. The microplate was then incubated at 30 °C with gentle shaking in the Synergy HTX Multi-Mode Plate Reader (BioTek Instruments, Winooski, VT) with hourly colorimetric measurements (absorbance: 595 nm).[36,37]

To assay the $CO_2$ produced in the headspace of the serum bottles, 0.25 mL of gas from each serum bottle was injected onto a GOW-MAC gas chromatograph with an Altech CTR 1 column and a thermal conductivity detector. The following method parameters were used: injector, 120 °C; column, 60 °C; detector, 120 °C; and current, 150 mV. The peak area of $CO_2$ was compared to standards and quantified using the software ChromPerfect (Justice Laboratory Software, Denville, NJ, USA) to determine the concentration (measured as the percent of the headspace volume) of $CO_2$ in the headspace of each vial.

**Characterization Methods.** Contact angle analyses were performed to quantitatively explore differences in the supernatant hydrophobicity for each experimental condition. A Contact Angle Goniometer (Rame-Hart model-290, Succasunna, NJ) was used to measure supernatant hydrophobicity (*i.e.*, the ability of the tested supernatant to wet a hydrophobic surface, PS film), with DI water tested as a control. Increased surface wetting indicates increased hydrophobicity of the liquid. For each sample, the PS film was placed on a level platform and 5 $\mu$L of liquid supernatant was dropped onto the film in quadruplicate. Contact angle analysis was also utilized to characterize changes in PS films after incubation with microbial cultures. Control and treated films were placed on a level platform and 5 $\mu$L of DI water was dropped onto the film in quadruplicate. Contact angles were quantified using the DROPimage Pro Software (Rame-Hart, Succasunna, NJ).

To characterize the chemical changes in the PS film after incubation with microbial cultures, films were visualized using Fourier transform infrared (FTIR) spectroscopy on a Nicolet iS50 FTIR spectrometer (Thermo Fisher Scientific, Inc., Pittsburg, PA), as previously described.[28] Absorbance spectra were recorded and transformed into transmittance for graphing. The spectra were recorded between 4000 and 500 $cm^{-1}$ with at least 16 scans at a spectral resolution of 0.482 $cm^{-1}$. Peaks were identified using OMNIC software (Thermo Fisher Scientific Inc., Pittsburg, PA).

**Statistical Analysis.** Statistical analyses were performed in Prism (version 7.0a). To compare respiration activity and supernatant contact angles between experimental conditions, ANOVA tests were performed, followed by pairwise comparisons using Student's *t*-test with Tukey's correction. Normality of data was assessed with Shapiro−Wilk test prior to further analysis ($p > 0.05$ for all data). All *p*-values are adjusted


https://dx.doi.org/10.1021/acs.est.0c04952
Environ. Sci. Technol. 2021, 55, 2027−2036

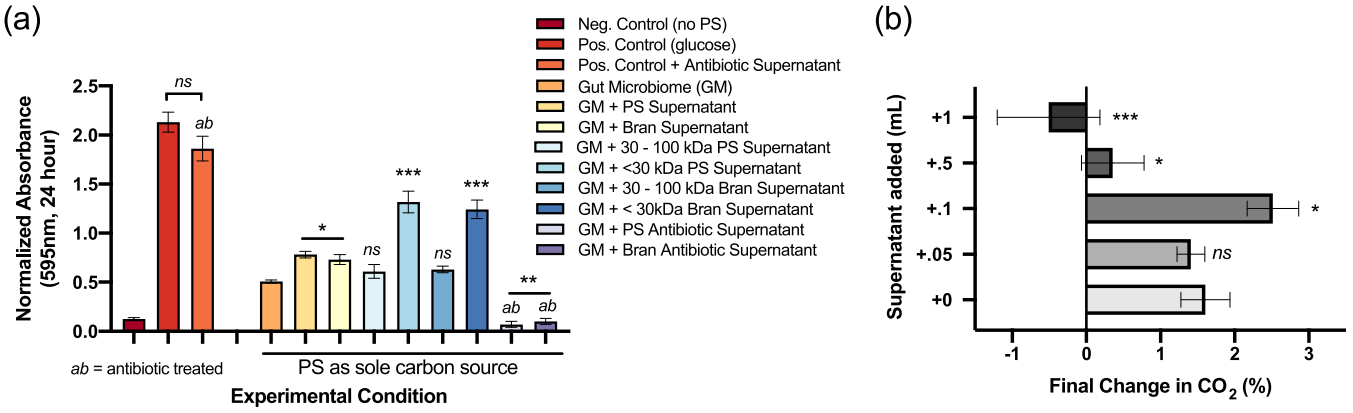


**Figure 1.** Effects of mealworm gut supernatant on *ex situ* gut microbiome growth on PS. (a) The respiration activity of the gut microbiome in Biolog MT2 plates was measured using tetrazolium violet redox dye as a colorimetric indicator at absorbance: 595 nm after 24 h. The bacteria were cultured in carbon-free media supplemented with PS microplastics (carbon source) and supernatant fractions (<30 kDa, 30 to 100 kDa) from each experimental condition. The supernatant condition is indicated by color with antibiotic-treated supernatant indicated by ab. (b) Final concentration of $CO_2$ respired (final−initial percent $CO_2$ in the headspace of serum bottles after 24 h) by bacterial cultured supplemented with different doses of PS supernatant. Statistical significance calculated relative to the gut microbiome not supplemented with supernatant is indicated by * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$; no statistical significance is indicated by ns.

*p*-values, and all error values are reported as average ± standard deviation of experimental replicates.

## RESULTS AND DISCUSSION

**Effects of Supernatant on *Ex Situ* Microbial Growth.** A Biolog MT2 plate assay was used to assess respiration by the gut microbiome immediately after extraction from mealworms. This assay demonstrated that the gut microbiome was able to respire on PS as the sole carbon source and respiration increased significantly in cultures supplemented with gut supernatant from mealworms fed either PS or bran ($p \leq 0.05$ for both, Figure 1a). No increase in respiration activity occurred after the addition of gut supernatant from mealworm-fed antibiotic-treated diets; rather, inhibition was observed (Figure 1a). Inhibition could be due to carryover of antibiotics in the gut supernatant or secretion of other factors (*e.g.*, immune responses proteins[38]) that are inhibitory or toxic to the gut microbiome. To test whether inhibition was due to residual antibiotics, positive controls (glucose-fed wells) were supplemented with supernatant from antibiotic-treated mealworms. No loss in activity was observed ($p = 0.16$). This suggests that the gut microbiome produces and secretes factors that enhance PS degradation.

To better understand the beneficial effect of the supernatant on respiration, the supernatant was fractionated by molecular weight into three fractions: <30 kDa, 30−100 kDa, and >100 kDa. The two smallest of these fractions (<30, 30−100 kDa) from each condition were then subject to the same respiration assay described above. This assay revealed a two-fold increase in respiration activity in the lowest molecular weight fraction (<30 kDa), from both PS and bran diets (Figure 1a). Taken together, our results suggest that the smallest supernatant fraction contains a respiration-enhancing factor secreted by the gut microbiome of mealworms fed PS or bran. This factor increases the ability of the gut microbiome to respire on and thus degrade PS outside the mealworm gut. SDS-PAGE gel analysis (SI M2) revealed differences between the low molecular weight PS and bran supernatant (Figure S4). Further research should focus on differences between these supernatants as they may give insights into pathways of microbially mediated PS biodegradation. Additional research is also needed to identify any emulsifying agents shared by these supernatants that may promote plastic degradation (Figure 1a).

After identifying the fraction of the supernatant that contributed to increased PS degradation, the amount of supernatant needed to achieve this enhanced effect was assayed. To do this, five dosages of supernatant were tested: 0.0, 0.05, 0.1, 0.5, and 1 mL (0.0, 0.3, 0.6, 3.0, and 5.6% v/v). Supernatant dosages were used to supplement the gut microbiome from PS-fed mealworms cultured in carbon-free media with PS microplastics in serum bottles. $CO_2$ production was measured over 2 weeks. The ideal dose was determined as the concentration of the supernatant that led to the largest net volume of $CO_2$ produced over 14 days, measured as the final minus initial concentration of $CO_2$ in the headspace. The initial concentration of $CO_2$ was measured 24 h after the initiation of experiments. To assess whether the increase in respiration was due to addition of an external carbon source (rather than the presence of degradation-enhancing secreted factors), the $CO_2$ production of cultures supplemented with an equivalent amount of glucose (based on chemical oxygen demand) was tested for each supernatant dose (Figure S1). The plots of each dose along with the negative controls (gut microbiome supplemented with the supernatant but no PS) and glucose equivalent are provided in the Supporting Information (Figure S1).

At the lowest doses (0.0, 0.05, and 0.1 mL), addition of the supernatant increases the amount of $CO_2$ produced compared to the negative control, again highlighting the benefit of supernatant addition for PS biodegradation (Figure S1a−c). Supplementation with the two highest doses (0.5 and 1.0 mL) did not increase the respiration of PS overtime; instead, high doses led to a rapid increase in $CO_2$ production in the first 24 h, after which the $CO_2$ concentration stabilized (Figure S1d,e). This suggests that supplementation at high doses with supernatant or glucose enables the growth of bacteria that can grow quickly on readily available soluble carbon rather than bacteria that can degrade PS. Competitive pressure from the fast growing bacteria could actively select against biodegradation of PS, which may explain why the concentration of $CO_2$ plateaued after the initial respiration. The final


https://dx.doi.org/10.1021/acs.est.0c04952
Environ. Sci. Technol. 2021, 55, 2027−2036

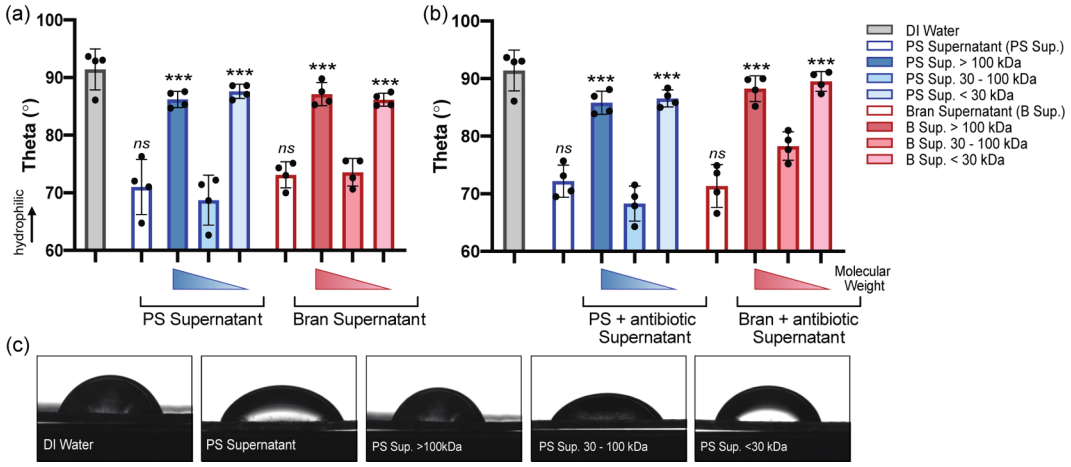


**Figure 2.** Hydrophobicity of mealworm gut supernatant. Contact angle analysis on the supernatant fractions from each of the mealworm diets, colored by mealworm diet for each supernatant and ordered by molecular weight fraction (<30 kDa, 30−100 kDa, >100 kDa, and size gradient indicate below), legend on the right. DI water was used as a negative control. (a) Contact angle (°) of the supernatant fractions from the PS and bran-fed diets. (b) Contact angle (°) of the supernatant fractions from PS and bran + antibiotic fed diets. (c) Representative photos of DI water, whole PS supernatant, and PS supernatant fractions on the PS film. Statistical significance relative to the intermediate molecular weight fraction (30−100 kDa) fraction is indicated by * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$; no statistical significance is indicated by ns.

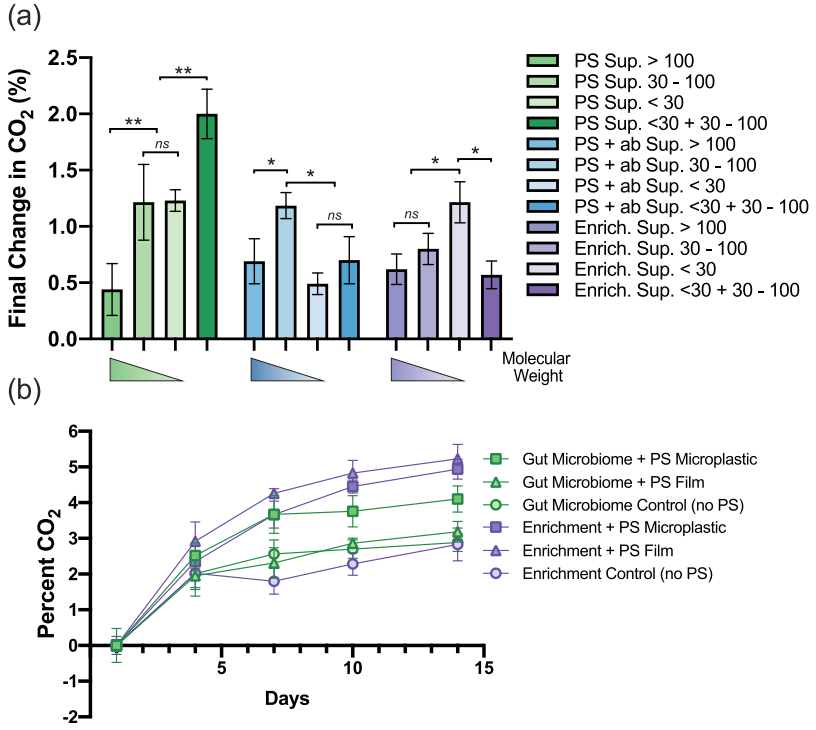


**Figure 3.** Microbial respiration on PS supplemented with PS supernatant. (a) Final percent change in $CO_2$ produced (measured in the headspace of serum bottles) by the gut microbiome supplemented with different supernatant fractions from mealworms fed PS. The supernatant fractions were tested separately and in combination to assess the impact on respiration (final−initial percent $CO_2$ in the headspace of serum bottles), colored by supernatant condition (PS in green, PS + ab (antibiotic) in blue, enrichment in purple) and ordered by molecular weight fraction (size gradient indicated below) legend on the right. (b) PS degradation (represented by percent $CO_2$ produced) of extracted gut microbiome (green) and cultured enrichment (purple) fed with PS microplastics (squares) or film (triangles), relative to control (no PS, circles). Statistical significance is indicated by * $p \leq 0.05$ and ** $p \leq 0.01$; no statistical significance is indicated by ns.

change in $CO_2$ produced over the course of the experiment was plotted by dose to determine the ideal dose of supernatant to support PS degradation (Figure 1b). The lower doses of supernatant led to greater increases than the highest two doses, with 0.1 mL (0.6% v/v) being the clear ideal dose. These results reveal that supplementing *ex situ* gut microbiome cultures with supernatant improves PS respiration beyond the levels achieved by addition of bioavailable carbon, supporting the hypothesis that the supernatant contains factor(s) that enhance the capacity of the gut microbiome to respire and biodegrade PS.

### Identification of Endogenous Emulsification Activity.

In conducting the experiments described above, addition of gut supernatant resulted in rapid emulsification of hydrophobic PS



https://dx.doi.org/10.1021/acs.est.0c04952
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microplastics into the aqueous media (Figure S2a). Further, this emulsification was stable over time, enabling the microplastics to settle in the aqueous phase as they do when treated with Tween-80, a known surfactant (Figure S2b). Emulsification was observed in all diets tested (PS, bran, ± antibiotic treatment), suggesting that the production and secretion of an emulsifying agent is independent of both diet and the gut microbiome, implying that it must be endogenous to the mealworm itself. Emulsifying surfactants are known to increase degradation rates of hydrophobic pollutants, including plastics, where bioavailability of hydrophobic substrates is rate limiting.[13,39−41] It is possible that the supernatant acts similarly in the mealworm gut environment, coating hydrophobic plastic particles, with an emulsifying agent that increases bioavailability and accelerates biodegradation.

To further quantify this emulsification activity, contact angle analysis was used to measure the ability of the supernatant to wet a hydrophobic surface (PS film) relative to DI water as a control (Figure 2). Increased surface wetting is analogous to increased hydrophobicity of the supernatant, implicating the supernatant as an emulsifying agent for plastics. Whole supernatant from both PS- and bran-fed mealworms was more hydrophobic than DI water (increased surface wetting on a hydrophobic surface, $p < 0.001$ for PS and bran supernatant, Figure 2a). When fractionated, the intermediate supernatant fraction (30−100 kDa) was significantly more hydrophobic than the other two fractions (<30 kDa, >100 kDa) for all experimental diets: PS, PS + antibiotic, bran, and bran + antibiotic (Figure 2, PS: $p < 0.0001$ relative to both >100 kDa and <30 kDa; bran: $p = 0.002$, $p < 0.001$ relative to >100 kDa, <30 kDa, respectively). In each case, the intermediate fraction was significantly better at wetting the hydrophobic surface, as indicated by a distinctly smaller contact angle (theta, deg). This further confirms that the emulsification activity is endogenous to the mealworm itself and indicates that the emulsifying agent lies within the molecular weight range of 30−100 kDa, which will facilitate the identification of the factor.

This analysis reveals a significant role of the mealworm itself in PS biodegradation. The absence of emulsifying agents in *ex situ* microbial cultures could help explain the low rate of PS biodegradation relative to conditions *in situ*. Emulsifying agents produced by microorganisms are known to enhance remediation of liquid hydrocarbons;[42,43] the mealworm microbiome may similarly enhance biodegradation of solid hydrocarbons, such as plastic. Further research into identifying and characterizing this emulsifying agent is needed to maximize and exploit its potential utility.

**Enhanced *Ex Situ* Microbial Growth.** The insights from the previous sections were then applied to enhance the *ex situ* PS degradation using the extracted mealworm gut microbiome. Serum bottles were inoculated with PS-fed mealworm gut microbiome in carbon-free media with PS microplastics added as the sole carbon source. Cultures were supplemented with different fractions, tested separately and in combination as follows: <30 kDa, 30−100 kDa, <30 + 30−100 kDa, and >100 kDa. The supernatant was supplemented at the previously determined ideal dose (0.6% v/v) and $CO_2$ production was measured over 10 days. When the supernatant fractions were added separately, both the <30 kDa and 30−100 kDa fractions stimulated similar increases in $CO_2$ production ($p \geq 0.99$), both of which caused a significantly higher increase than the >100 kDa fraction ($p < 0.01$ relative to both <30 kDa and 30−100 kDa, Figure 3a). These results are further evidence of microbial secreted factor(s) in the <30 kDa fraction (Figure 1). Further, these results suggest that supplementation with either microbial secreted factors in the <30 kDa fraction or mealworm-produced emulsifying agent in the 30−100 kDa fraction (Figure 2) increases *ex situ* microbial respiration on plastic. When combined, the <30 kDa + 30−100 kDa fractions promoted the highest increase in $CO_2$ production ($p < 0.01$ relative to both <30, 30−100 kDa, Figure 3a). The observed respiration of the <30 kDa + the 30 to 100 kDa fractions is significantly higher than the respiration observed when cultures were supplemented with unfractionated supernatant (Figure 1a). This could suggest an inhibitory effect due to an agent in the supernatant >100 kDa. Further research is needed to confirm and explore this difference.

The only fraction from antibiotic-treated supernatant that increased activity was 30−100 kDa, further confirming that the low molecular weight fraction provides no added benefit in the absence of the gut microbiome. These results demonstrate that the contributions of both the mealworm and its gut microbiome are necessary to achieve enhanced plastic biodegradation.

To explore the long-term effect of these respiration-enhancing factors on plastic biodegradation, a microbial enrichment was inoculated with PS-fed mealworm gut microbiome in carbon-free media supplemented with PS film (as the carbon source) and PS gut supernatant. This mixed community enrichment was cultured for several months until a stable biofilm is formed (Figure S3). The PS respiration activity of the enrichment was then compared to freshly extracted PS-fed mealworm gut microbiome to determine whether the enrichment conditions selected for more efficient plastic biodegrading microbes. This was done using serum bottles seeded with inoculum (either the microbial community from the enrichment or the freshly extracted gut microbiome) in carbon-free media, supplemented with 0.6% v/v PS-fed mealworm gut supernatant and either PS film or PS microplastics as a carbon source. Cultures were grown for 14 days. The microbial enrichment exhibited significantly higher PS respiration activity ($CO_2$ production) on both PS microplastics and PS film, suggesting that the enrichment was selecting for more efficient plastic degrading bacteria than those found in the gut microbiome (Figure 3b). This enhanced plastic biodegradation activity was especially prominent when comparing the $CO_2$ produced from respiration on PS films; while the microbial enrichment produced slightly more $CO_2$ on PS film than PS microplastics, the gut microbiome produced significantly less $CO_2$ on PS film (Figure 3b). This suggests that in addition to selecting for better PS-biodegrading bacteria, the conditions of the microbial enrichment also selected for bacteria that thrive in biofilms, an unsurprising result given that the microbial enrichment was cultivated with PS film and formed a stable biofilm on its surface (Figure S3). These promising results highlight the ability to extrapolate insights drawn from the mealworm gut environment (*e.g.*, the addition of a gut supernatant) to select for bacteria capable of enhanced plastic biodegradation outside the mealworm.

To further understand the mealworm's role(s) in PS biodegradation, the supernatant from the mixed community enrichment was subject to the same supernatant fractionation process used for the mealworm gut supernatant. The enrichment supernatant fractions were then assayed using the


https://dx.doi.org/10.1021/acs.est.0c04952
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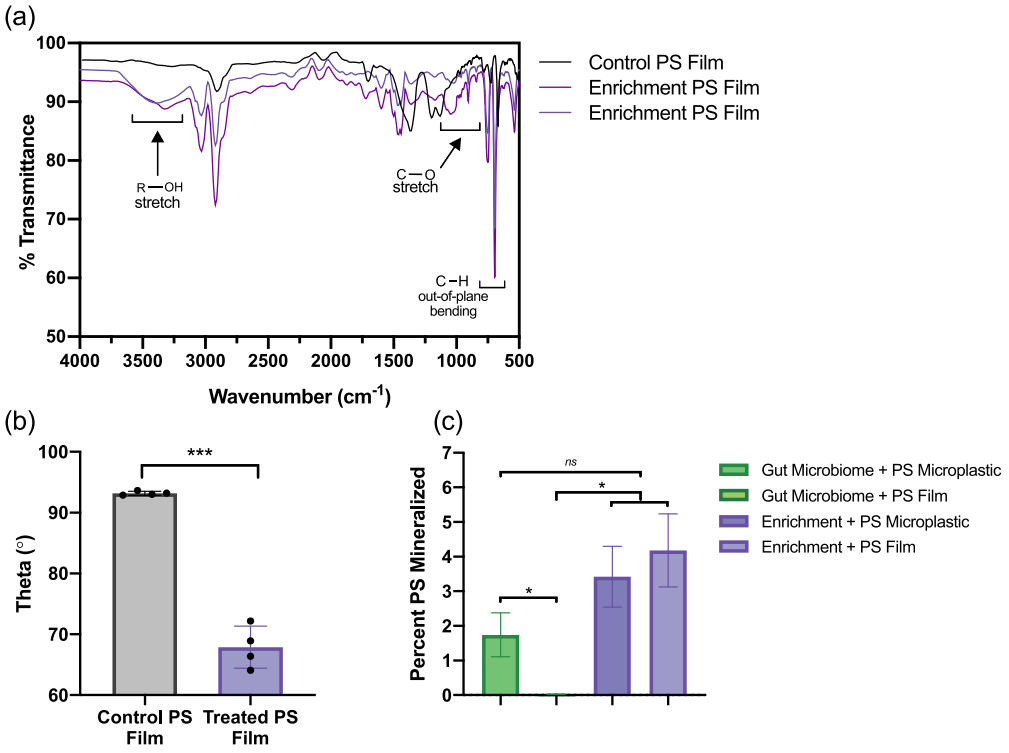


**Figure 4.** Characterization of PS degradation by gut microbiome and cultured microbial enrichment through changes in polymer surface chemistry and mass loss. (a) FTIR spectra (in percent transmittance) of PS films after incubation with microbial enrichment (purple) and a microbial isolate (green) relative to the control PS film; annotations show functional groups associated with key peaks based on wavenumber. (b) Contact angle analysis of DI water on the control PS film and the PS film after incubation with the microbial enrichment. (c) Percent of initial PS mineralized to $CO_2$ over 14 days in serum bottle experiments, colored by condition: extracted gut microbiome (green) and cultured enrichment (purple). Statistical significance calculated relative to the control PS film is indicated by * $p \leq 0.05$ and *** $p \leq 0.001$; no statistical significance is indicated by ns.

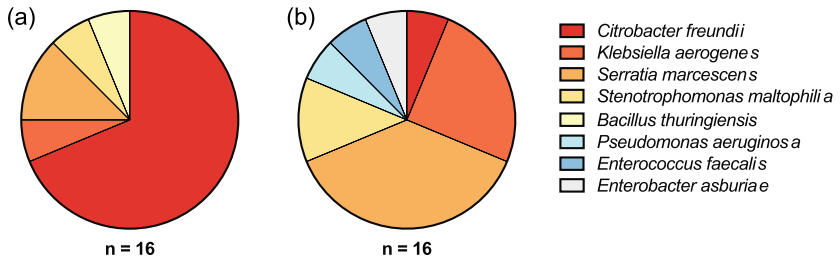


**Figure 5.** Relative abundance of PS-degrading bacterial species identified by 16S rRNA sequencing of individually isolated colonies from (a) the mixed microbial enrichment (16 representative colonies) and (b) pure cultured isolates capable of PS degradation (16 total). Additional data on species and sequence information is available in Table S1.

same serum bottle experiment described above (Figure 3a). Unlike the supernatant fractions from the PS-fed mealworm, only the smallest fraction (<30 kDa) from the enrichment increased $CO_2$ production. This provides additional confirmation that the PS degradation-enhancing effect of the 30–100 kDa supernatant fraction is due to a factor(s) produced and secreted by the mealworm itself.

The effects of PS degradation by the gut microbiome and by the microbial enrichment were further assayed by monitoring changes in functional groups indicative of oxidative attack and hydrophobicity of PS films (via FTIR visualization and contact angle analysis) and mass loss. The FTIR spectra of the PS films incubated with the microbial enrichment revealed oxygen incorporation as indicated by the appearance of peaks associated with the R–OH stretch (3000–3500 $cm^{-1}$) and C–O stretching (1000–1300 $cm^{-1}$), as well as C–H bending (700–750 $cm^{-1}$) often associated with monosubstituted benzene derivatives (Figure 4a). Changes in the surface of incubated PS films were further explored by measuring changes in contact angle of DI water on control versus incubated PS films (Figure 4b). These analyses revealed a significant difference between the surface hydrophobicity of the films ($p < 0.001$), with a significant increase in surface wetting on the treated PS film. This is further evidence of changes in surface chemistry of the PS film, including oxygen incorporation, which would facilitate increased surface wetting by water. The gut microbiome has a significantly lower rate of plastic degradation on the PS film (measured as the percent of the initial PS mineralized to $CO_2$) than the enrichment ($p < 0.05$, Figure 4c). However, the rate of plastic degradation on PS microplastics between the gut microbiome and the enrichment were not significantly different ($p = 0.56$, Figure 4c). While the


https://dx.doi.org/10.1021/acs.est.0c04952
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rates of plastic degradation observed by both *ex situ* cultures were still slower than within the mealworm gut (Figure 4c), these results are an important first step toward higher rates of plastic degradation outside the mealworm and suggest that further enhancements are possible.

**Identification of Plastic Degradation Microbial Community.** Once the microbial enrichment formed a stable biofilm on the surface of the PS film, a sample of the biofilm was collected and streak-plated in order to isolate and identify strains involved in plastic biodegradation. Sixteen individual representative colonies were randomly selected for sequencing ("enrichment strains"). The same process was then repeated, and 24 individual representative colonies were randomly selected and grown overnight in carbon-rich media. The cultures were then centrifuged, and the cultured bacteria transplanted to carbon-free media with PS films, supplemented with PS gut supernatant at 0.6% v/v. The cultures were monitored for biofilm development and changes in optical density as an indication of growth and respiration on the PS film. After 14 days, the media from each isolate the culture that showed signs of respiration on PS (16 total). To obtain isolated strains, these cultures were plated, and representative colonies were picked for sequencing ("isolate strains").

Overall, eight unique bacterial species were isolated and identified (Figure 5 and Table S1). The identified species are predominantly aerobes or facultative anaerobes, supporting previous findings that oxygen incorporation is critical for rapid plastic biodegradation.[28] While the mixed enrichment and identified isolates included many of the same species, they were not the same, suggesting that a different subset of species thrive in the mixed enrichment than can survive alone on PS (Figure 5). However, to fully understand this shift, further information, such as that from next-generation sequencing of the microbial enrichment, is required.

Of the identified plastic-biodegrading strains, many are associated with the degradation and bioremediation of hydrocarbons and polymeric substrates (Table S2). In the mixed enrichment, the most prevalent species was *Citrobacter freundii*, a bacterium known to degrade long-chain polymers (*e.g.*, tannic acid[44]), linear hydrocarbons (*e.g.*, oil[45]), and cyclic hydrocarbons (*e.g.*, phenylenediamine,[46] 4-nitroaniline,[47] and pentachlorophenol[48]). Further, *Citrobacter* sp. are significantly associated with gut microbiome in PS- and PE-fed mealworms,[28] and several *Citrobacter* enzymes have been used for degradation of phenylenediamine.[46] These previously identified enzymes may also be involved in PS biodegradation, suggesting possible directions for future research on the enzymatic degradation of PS.

Of the identified isolates, the most prevalent strains are *Serratia marcescens*[49−51] and *Klebsiella aerogenes*,[47,52,53] both of which are associated with hydrocarbon degradation and bioremediation. *S. marcescens*, *Klebsiella* sp. (closely related to *K. aerogenes*), and *Pseudomonas aeruginosa* (another identified isolate) were found in the gut microbiome of PS-degrading mealworms but were incapable of PS degradation when cultured in isolation.[29] This could suggest that these strains are dependent on mealworm-secreted factors to enable PS biodegradation. *P. aeruginosa* is also known to degrade crude oil[54,55] and produce a rhamnolipid,[56] a glycolipid that acts as a biosurfactant that accelerates hydrocarbon degradation. More recently, *P. aeruginosa* was isolated from the gut of PS-degrading superworms (*Zophobas atratus*) and found capable of growth on PS alone.[57] Further investigations should explore when and how *P. aeruginosa* is capable of PS degradation in isolation as it may reveal conditions necessary for plastic degradation and offer further insights into the mechanisms of degradation. Another identified isolate, *Enterobacter asburiae*, was able to degrade polyethylene after its isolation from the gut microbiome of plastic-eating waxworms (*P. interpunctella*).[21] *Stenotrophomonas maltophilia*, another identified species, is capable of cyclic hydrocarbon degradation,[58,59] and the rate of biodegradation can be enhanced in the presence of surfactants, similar to the results of this study.[60]

These results suggest that bacterial strains already known for their ability to degrade hydrocarbon xenobiotics are likely responsible for PS biodegradation. Several of the identified microorganisms are capable of enhanced hydrocarbon degradation in the presence of a biosurfactant,[42,43] which highlights the importance of the emulsifying factor in the mealworm gut for PS biodegradation. Further investigation is required to optimize the conditions for biodegradation of PS and other xenobiotics and to elucidate the metabolic pathway(s) involved.

**Implications.** This work is the first to achieve *ex situ* cultivation of a mixed enrichment derived from the mealworm gut microbiome for enhanced PS biodegradation. This was accomplished by elucidating the respective roles of *T. molitor* and its gut microbiome. We demonstrate a significant role for the insect in secreting an emulsifying agent, increasing the bioavailability of PS, and enabling more rapid microbial attack. The gut microbiome was also found to secrete factor(s) that enhance microbial respiration when PS is the sole carbon source. Additionally, we identify eight unique bacterial species involved in PS degradation, many of which have previously been associated with the biodegradation of hydrocarbons and other xenobiotics. Our results imply that naturally occurring components of the mealworm gut—including those secreted by both the mealworm host and its gut microbiome—must be present and work in concert with the microbiota themselves in order to achieve enhanced plastic respiration and biodegradation. This understanding enables a new approach in which insect gut-derived enrichments can facilitate and inform the design and scale-up of microbial systems for *ex situ* plastic biodegradation. It also sets the stage for biochemical elucidation of emulsifiers and plastic-degrading agents that are produced and secreted by host insects and their associated microbiomes. Understanding the mechanisms of rapid biodegradation within the mealworm may also enable a future in which biodegradation is an alternative end-of-life for plastic waste.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c04952.

Supernatant dose response curves, images from emulsification assays, images of a stable biofilm formed in microbial enrichment, image of SDS-PAGE, sequenced isolates, and known biodegradation abilities of the sequenced isolates (PDF)

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https://dx.doi.org/10.1021/acs.est.0c04952
*Environ. Sci. Technol.* 2021, 55, 2027−2036

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

This work was supported by the Stanford Woods Institute for Environment (award 1197667-10-WTAZB). A.M.B. was supported by the National Science Foundation Graduate Research Fellowship Program (DGE-1656518). A.M.G. was supported by the Stanford Graduate Fellowship (SGF) and the Enhancing Diversity in Graduate Education (EDGE) Fellowship. Part of this work was performed at the Stanford Nano Shared Facilities (SNSF), supported by the National Science Foundation (ECCS-1542152). We thank Dr. Sungeeun Woo (postdoctoral researcher, Stanford University) for his help and support. We also thank Jack Chiueh, Stanford University, for administrative help.

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https://dx.doi.org/10.1021/acs.est.0c04952
*Environ. Sci. Technol.* 2021, 55, 2027−2036