Assessing the fate of plastic debris in the marine environment

Final Report

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Prepared by:

Jeffrey W. Turner, Principal Investigator
Lee J. Pinnell, Co-Principal Investigator
Texas A&M University-Corpus Christi
6300 Ocean Dr., Unit 5858
Corpus Christi, Texas 78412
Phone: 361-825-2132
Email: jeffrey.turner@tamucc.edu

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1700 Congress Ave.
Austin, TX 78701-1495

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EXECUTIVE SUMMARY

Plastic debris is increasingly abundant in marine systems. Previous studies have shown that microorganisms colonize and form biofilms on plastic debris. Further, recent studies have reported the discovery of rare microorganisms capable of biodegrading plastic. The purpose of this study was to assess the fate of plastic in the Laguna Madre, TX. For this purpose, a microcosm experiment was conducted, exposing plastic (polyethylene terephthalate; PET) and bioplastic (polyhydroxyalkanoate; PHA) to the benthic marine environment for 15 months. The primary Aims were 1) Quantify the biodegradation of plastic and bioplastic debris in the marine environment, 2) Identify the algae fouling plastic and bioplastic debris in the marine environment, 3) Identify the bacteria fouling plastic and bioplastic debris in the marine environment, 4) Determine which bacterial enzymes play a role in plastic and bioplastic biodegradation, 5) Image colonization and biodegradation with scanning electron microscopy (SEM) analysis of plastic and bioplastic debris, and 6) Engage the community with a comprehensive outreach and education campaign relating to plastic pollution.

To fulfill Aim 1, the biodegradation of PET and PHA was quantified by measuring weight loss over time. The total exposure period was 424 days. The PET particles did not lose weight even after 424 days but the PHA particles lost 51% of their original weight. This result suggests that microorganisms capable of biodegrading PET are rare or not present in Laguna Madre sediment. To date, most reports of plastic biodegration have occurred in heavily polluted or highly specialized habitats. PHA however was biodegraded but the rate of biodegradation was slower than anticipated given that a variety of marine bacteria are known to product and biodegrade PHA naturally. The complete biodegradation of a ~3 mm diameter PHA nurdle would require approximately 830 days if the biodegradation rate remained constant.

To fulfill Aim2, algal species fouling PET, PHA, and a ceramic biofilm control were identified using algal pigment biomarkers with High Performance Liquid Chromatography (HPLC). The Chl *a* and fucoxanthin biomarkers was detected in both PET and PHA biofilms. Further, the concentration of both biomarkers increased over time. The Chl *a* and fucoxanthin biomarker are indicators of diatom colonization. Diatoms are known to colonize biotic and abiotic surfaces in marine environments. Here, Chl *a* and fucoxanthin abundance increased over time and both were more abundant in PHA biofilms verses the PET biofilms. This result suggests that algae

successively colonized the surfaces over time. Additional, the introduction of PHA may enhance primary production in marine sediments.

To fulfill Aim 3, the bacteria fouling PET, PHA, and a ceramic biofilm control (i.e. surface-associated bacteria) were identified with 16S rRNA gene sequencing. For comparative purposes, the bacteria in the water column (i.e. free-living bacteria) were identified by the same method. The free-living microbial community was distinct in comparison to all substrates. This result confirms that surface-associated and free-living communities are fundamentally different. The microbial communities colonizing the PET and ceramic biofilm control were not significantly different and thus, the inclusion of the ceramic biofilm control revealed that PET was not colonized by a novel microbial community. The PHA microbial community however was novel. This result suggests that previous literature claiming that plastic biofilms are novel is in fact suspect seeing that those studies did not include an appropriate biofilm control.

To fulfill Aim 4, the bacterial species and enzymes enriched by PET and PHA were identified by shotgun metagenomic sequencing. The PET biofilm was not enriched for bacterial species or enzymes. In contrast, the PHA biofilm was dominated by sulfate-reducing bacteria (SRB). The PHA biofilms were also enriched with adenylyl sulfate reductases (*aprBA*) and dissimilatory sulfite reductases (*dsrAB*). SRB play an important role in carbon and sulfur cycling in marine sediments and their stimulation has been shown to limit methanogenesis in coastal systems. Thus, trading plastic pollution for bioplastic pollution may affect biochemical cycling by altering the flow of carbon from methane to carbon. The PHA biofilms were also enriched with esterases and depolymerases which are enzymes that play a primary role in polymer biodegradation. Together, these results show that PHA stimulated the growth of SRB including the enrichment of sulfate-reducing enzymes as well as polymer degrading enzymes.

To fulfill Aim 5, the colonization and biodegradation of PET and PHA was monitored by SEM. The SEM images provided an exceptional time-course visual. PET displayed no size reduction or pitting. The PHA particles experienced progressive and significant size reduction and pitting. The extensive pitting suggests that anaerobic bacteria, such as SRB, could be active in the deeper recesses of the biofilm. Although the images show a dramatic contrast, it is important to remember that 49% of the PHA weight remained after 15 months, showing that biodegradation of PHA is a slow process.

To fulfill Aim 6, we partnered with the Aquatic Education Program (AEP) at the Center for Coastal Studies (TAMU-CC) to conduct a comprehensive outreach and education campaign. The campaign connected with students by organizing wetland field trips during which students received a lecture on plastic pollution before participating in a beach cleanup. The AEP also connected with students and the general public through a plastic pollution display that was part of a mobile Wetland on Wheels diorama. The diorama visited schools and community events for the purpose of educating the community per the increasing problem of plastic pollution. In total, the AEP connected with over 1,300 participants at 11 different events. We also disseminated the results of this study and 8 scientific meetings and 5 stakeholder meetings with more than 11,960 attendees.

In summary, this study showed that PET was not colonized by a novel microbial community nor was it biodegraded during the 15-month study period. This finding runs counter to previous scientific literature claiming that plastic debris are colonized and biodegraded by novel microbial communities. Here, the inclusion of a biofilm control highlighted the importance of conducting controlled experimentation that includes appropriate controls. Meanwhile, PHA did stimulate the growth of a novel microbial community dominated by SRB and enriched for sulfate reductases and hydrolases. Thus, PHA biofilms were sites of enhanced enzyme activity that contributed to its biodegradation. Seeing that SRB play an integral role of carbon and sulfur cycling, trading petroleum-based plastics for bioplastics could have unintended effects on biogeochemical cycling. Such consequences should be thoroughly investigated before best-management practices related to plastic pollution are instated. It would therefore be prudent to focus efforts on education and outreach that discourages single-use items regardless of whether they are made from plastic or bioplastic.

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INTRODUCTION

The mass production of plastics in the 1940's was celebrated as the dawn of a new era – the Age of Plastic (Thompson et al. 2009). Today, our landscapes and oceans are awash with plastic waste. A recent study calculated that 275 million tons of plastic waste was generated in 2010 and 4.8 to 12.7 million tons entered the oceans (Jambeck et al. 2015). Over 5.25 trillion pieces of floating plastic waste litters the ocean's surface (Eriksen et al. 2014). The ingestion of plastic debris by marine animals, along with its toxic additives (e.g. plasticizers, flame retardants, antimicrobials and other chemicals used in plastics manufacturing), poses a threat to healthy ocean ecosystems (Seltenrich et al. 2015). Moreover, the concentration of plastic debris and toxic additives in marine food webs and their eventual ingestion by humans poses an emerging threat to human health.

The accumulation of plastic waste is greatest in bays, lagoons and coastal seas surrounded by densely populated coastlines (Eriksen et al. 2014). Closed bays or lagoons with limited flushing (i.e. limited exchange with larger water bodies) are sinks for the accumulation of plastic debris (Barnes et al. 2009). In the United States, the world's longest barrier island (Padre Island) isolates the Laguna Madre from the Gulf of Mexico. Consequently, the lagoon is a semi-closed, hypersaline system with limited flushing (Kennish et al. 2010). The lagoon is also a highly productive system supporting many finfish, sea turtles and migratory birds. Sections of the lagoon have been impacted by recent changes in coastal population growth and watershed development. An abundance of plastic waste near the Upper Laguna Madre inspired a local artist to raise awareness of plastic waste through an art exhibit entitled "Oceans of Plastic" at the Art Museum of South Texas. (see http://www.sheliarogersfineart.com/OceansofPlasticExhibitAMST/).

Conventional wisdom suggests that plastic does not biodegrade and is therefore a permanent part of the ecosystem. Yet marine microorganisms are known to quickly form biofilms on floating plastic debris (Oberbeckmann et al. 2014) and recent research has shown that rare microorganisms can biodegrade plastic (Sudhakar et al. 2008). Marine microorganisms may hold the key to effective bioremediation of this plastic waste. Invisible to the naked eye, an estimated 3.6×10^{28} trillion microorganisms play an invaluable role in the maintenance of marine ecosystems (Whitman et al. 1998). The sum of the metabolic and evolutionary potential stored in the microbial community defines microorganisms as nature's first responders. It follows that an abundance of plastic debris will select for the enrichment and evolution of microorganisms capable of plastic biodegradation. The potential of marine microorganisms for bioremediation was manifest following the Deepwater Horizon oil spill when researchers concluded that hydrocarbon-degrading microbes were largely responsible for the disappearance of oil (Kimes et al. 2014). Similarly, microbes indigenous to the Gulf of Mexico and Laguna Madre may be effective at biodegrading plastic waste.

The fate of plastic debris in the Laguna Madre, TX and neighboring systems is unknown. Knowledge gaps can be addressed by controlled *in situ* experimentation using techniques that allow full analysis of all plastic- and bioplastic-associated microbial biofilms. For this purpose, the culture-independent DNA sequencing of the biofilms (i.e. metagenomics) will identify the microbial species and enzymes that play a role in plastic colonization and biodegradation. Further, the growth requirements of specific bacteria can be conferred from the in-depth analysis of genomes assembled from metagenomes.

One proposed solution for dealing with the problem of plastic pollution is to replace petroleum-based plastics with biodegradable plastics. As a result, the global production of

bioplastics has increased recently and is forecasted to increase substantially in the near future. For example, in the next few years the production of the biodegradable plastics polyhydroxyalkanoate (PHA) and polylactic acid (PLA) are expected to increase tenfold and fourfold, respectively (Florence et al. 2015). The subsequent increase in bioplastic waste entering coastal seas will provide yet another new particle for microbial colonization. Recent studies have investigated the response of marine microorganisms to bioplastics (Eich et al. 2015) but *in situ* studies comparing plastic and bioplastic biofilms are lacking.

The purpose of this study was to assess the fate of plastic (polyethylene terephthalate; PET) and bioplastic (polyhydroxyalkanoate; PHA) in the Laguna Madre, TX. This study was designed to complete the following Aims: 1) Quantify the biodegradation of plastic and bioplastic debris in the marine environment, 2) Identify the algae fouling plastic and bioplastic debris in the marine environment, 3) Identify the bacteria fouling plastic and bioplastic debris in the marine environment, 4) Determine which bacterial enzymes play a role in plastic and bioplastic biodegradation, 5) Image colonization and biodegradation with scanning electron microscopy (SEM) analysis of plastic and bioplastic debris, and 6) Engage the community with a comprehensive outreach and education campaign relating to plastic pollution.

To complete this study, the principle investigator (PI) conducted a 15-month *in situ* microcosm study of plastic and bioplastic degradation. Briefly, plastic (polyethylene terephthalate; PET) and bioplastic (polyhydroxyalkanoate; PHA) were submerged in the Upper Laguna Madre for a period of 15 months and sampled monthly. The PET and PHA nurdles were contained within "microbial time capsules" that permited the flow of water, nutrients and microbes but prohibit mixing with surrounding sediments. A ceramic biofilm control was employed for comparative purposes. We hypothesized that the PET biofilms would be indistinguishable from the ceramic

biofilms while the introduction of PHA would select for the growth of a distinct microbial assemblage involved in biogeochemical cycling and capable of producing polymer degrading enzymes. Predicted benefits included important baseline data for monitoring the bioremediation of plastic and bioplastic waste. The study was also predicted to provide data that will inform the future development of best-management practices related to plastic pollution.

METHODS

Microcosms. A controlled microcosm experiment was conducted to circumvent the limitations of sampling found plastic debris. Unlike previous studies that targeted found plastic debris, this controlled experiment included ceramic nurdles as a biofilm control. The experiment was comprised of multiple microbial “time capsules” or MicroCaps (Figure 1). Each MicroCap contained randomized assortments of three substrates: either 6.0 g of ceramic nurdles (Lyman Products, Middletown, CT, USA), 3.0 g of PET nurdles (M&G Chemicals, Ettelbruck, Luxembourg), or 3.0 g of PHA nurdles (Doctors Foster and Smith, Rhinelander, WI, USA). MicroCaps were submerged in the Upper Laguna Madre (ULM) at the TAMU-CC Laguna Madre Field Station: 27°32'39.0"N and 97°17'07.7"W (Figure 2). In total, 45 sampling devices containing 180 MicroCaps were deployed.

Sample collection. The MicroCaps were collected in quadruplicate on a monthly schedule for 15 months. Additionally, quadruplicate 1 L water samples were collected. All samples were stored on ice during transport to the laboratory and sample processing proceeded immediately after transport. Three of the four replicates collected at each sampling event were used for both the determination of biodegradation rates and community composition analysis. The fourth replicate was stored in the dark at -80 C immediately upon return to the laboratory and was only used for visualizing substrate colonization with scanning electron microscopy (SEM). Triplicate substrates were rinsed 3X with 25 mL of 0.22 µm filter-sterilized lagoon water to remove loose debris. Water samples (100 mL) were filtered on 0.22 µm polycarbonate filters (Millipore Sigma, Burlington, MA, USA).

Weight loss. The loss of weight over time was used to quantify rates of biodegradation. Prior to exposure, the weight of the aggregated nurdles was measured to the nearest 0.001 mg.

Subsamples of each substrate type were collected on November 15, 2016 (28 days exposure), December 13, 2016 (56 days exposure), January 12, 2017 (86 days exposure), February 6, 2017 (113 days exposure), March 7, 2017 (142 days exposure), April 6, 2016 (172 days exposure), May 2, 2017 (202 days exposure), and May 30, 2017 (230 days exposure), June 29, 2017 (260 days exposure), July 25, 2017 (286 days exposure), August 21, 2017 (313 days exposure), September 20, 2017 (343 days exposure), October 18, 2017 (371 days exposure), November 27 (404 days exposure), and December 16, 2017 (424 days exposure). Following sample collection, the organic matter fouling the nurdles was sloughed off via the first two steps of the DNA isolation procedure (see below). The nurdles were then washed twice with 50 mL of ultrapure water, and then placed in 50 mL of fresh ultrapure water for 12-15 hours at room temperature. The ultrapure water was then flushed away, and the nurdles were again rinsed twice with 50 mL of ultrapure water. Each sample was then transferred to an individual glass dish, and the nurdles were dried for 24 hours at 60 °C. To control for any loss of mass as a result of the digestion, unexposed nurdles (0 days exposure) were also treated with the same procedure. The weight of the aggregated nurdles was measured again and compared to the pre-exposure weight.

Scanning electron microscopy. Microbial colonization of each substrate and changes in the physical appearance of the ceramic, PET, and PHA particles were visualized with SEM. For each time point (above), an additional sample of each substrate type that did not undergo the organic matter digestion outlined in Task 1, was immediately frozen in the dark at -80 °C upon return to the lab. Following the digestion of the other samples from the same time point, both sample types (PET and PHA) were removed from -80°C and freeze dried. Both the digested and freeze-dried samples were then mounted to stubs using double-stick tape and imaged using a Jeol JCM-6000 Neoscope at 4 magnifications (~20X, 70X, 220X, 700X).

Algal pigment analysis. The presence of algal species was quantified via analysis of two algal pigments: Chl *a* and fucoxanthin. For each time point (above), an additional sample of each substrate type that did not undergo the organic matter digestion outlined in Task 1, was immediately frozen in the dark at -80 °C upon return to the lab. 1 g was subsampled and then freeze-dried. 1 mL of 80% acetone was added, and the sample was then incubated in the dark for 24 hours at room temperature. Following incubation, 300 µL sample was filtered through a 0.7 µm disk filter to remove algal particles. Filtered extracts (200 µL) were injected directly into a Hewlett–Packard model 1100 HPLC equipped with one Hewlett-Packard ODS-Hypersil C18 column and two Vydac 201TP C18 columns in series and a diode array detector set to 436nm. Column temperature was set to 38 °C.

DNA isolation. Genomic DNA was isolated as described previously (Zhou et al. 1996). Isolated DNA was quantified with a BioPhotometer D30 (Eppendorf, Hamburg, Germany) and a Qubit Fluorometer (ThermoFisher, Waltham, MA, USA). The DNA was stored at -20 ºC.

Metagenome sequencing. Metagenomic library preparation and sequencing was completed by Molecular Research LP (Shallowater, TX, USA). Triplicate samples of water, ceramic, PET and PHA collected in the first month were subjected to shotgun metagenome sequencing. Triplicate samples collected every month (N = 15 months) were subjected to 16S rRNA sequencing. Raw sequence reads were process with the European Bioinformatics Institute's (EBI) Metagenomics Pipeline (Mitchell et al. 2016) which contains a workflow for merging overlapping reads, trimming adapter sequences and low-quality bases, identification of 16S rRNA reads, and identification of predicted coding sequences (pCDS).

Microbial community composition. Operational taxonomic units (OTUs) were assigned with QIIME (Caporaso et al. 2010) using the SILVA 128 SSU 97% database (Quast et al. 2013).

Beta-diversity was calculated using weighted UniFrac values in QIIME. A Permutational Multivariate Analysis of Variance (PERMANOVA) was run to test for significant differences between communities. Monte Carlo simulations were used to compute p-values for all pair-wise comparisons.

Enzyme enrichment. To identify the enrichment of hydrolases potentially involved in biodegradation, predicted proteins were aligned against the ESTHER database of alpha/beta-hydrolases (Hotelier et al. 2004). Similarly, to identify the enrichment of enzymes integral to sulfate reduction, predicted proteins were aligned against custom sulfate adenyltransferase (Sat/Met3) and APS reductase (AprBA) databases and a published dissimilatory sulfite reductase (DsrAB) database (Müller et al. 2014). Alignments were made with blastp with an expect value of 10^{-5}, a minimum alignment length of 30 amino acids, and a 50% minimum percent identity. The number of positive alignments was normalized and compared between all sample types (i.e. water, ceramic, PET, and PHA)

Recovery of genomes from metagenomes. To identify specific bacterial species enriched by either substrate, draft genomes were recovered from metagenomes as described previously (Parks et al. 2017). Genomes were retained for further analysis when meeting the following criteria: an estimated quality of ≥ 50 (completeness – 5x contamination), scaffolds resulting in an N50 ≥ 10 kb, containing < 100 kb ambiguous bases, and consisting of < 1 000 contigs and < 500 scaffolds.

Community engagement. We partnered with the Aquatic Education Program (AEP) at the Center for Coastal Studies (TAMU-CC) to raise public awareness of plastic pollution and help to change consumer habits of single use plastic products. The AEP designed a multifaceted plastics-based education and outreach program to provide relevant, scientific, field-based

education to a wide spectrum of the community including school children, college students, and the coastal community at large. The program accomplished this through several venues including wetland field trips, community talks, and programs at schools and community events utilizing the AEP's mobile Wetland On Wheels display. Additionally, press releases describing the study and the presentation of results at scientific conferences and stakeholder meetings furthered outreach efforts.

Statistical analyses. Data analysis was completed in R. Shapiro-Wilks tests and quantile-quantile plots were used to test normality. One-way analysis of variance (ANOVA) was conducted with the R multcomp package using a Tukey post-hoc test and Westfall values.

Data availability. Raw sequence reads were deposited in the European Nucleotide Archive under accession number ERP017130 (https://www.ebi.ac.uk/ena/). The six high-quality metagenome-assembled genomes (MAGs) were deposited in GenBank (https://www.ncbi.nlm.nih.gov/) under the following accession numbers: Madre1 QZKZ00000000, Madre2 QZLA00000000, Madre3 QZLB00000000, Madre4 QZLC0000000, Madre5 QZLD00000000, and Madre6 QZLE00000000. SEM images were made publicly available at https://www.flickr.com/photos/153260055@N04/collections. A short video depicting an education/outreach activity, designed to raise plastic pollution awareness, can be found here https://www.flickr.com/photos/153260055@N04/albums/72157698412361434.

RESULTS

Weight loss. After the final collection date following 424 days of exposure, the weight of the PHA samples decreased by an average of 1,512 mg (Figure 3), representing a loss of approximately 51% from their original mass. The rate (3.57 mg day^{-1}) was linear and was not significantly affected by seasonal changes in environmental parameters (e.g. temperature, salinity, DO, etc.). In contrast, the mass of PET samples remained unchanged.

Scanning electron microscopy. SEM imaging revealed that PHA samples experienced size reduction and pitting (Figure 4 and 5). Figure 4 illustrates the biofilm formation over time while Figure 5 illustrates the size and structure of the samples post biofilm digestion. The PET samples however experience no reduction in size and no pitting. All SEM images of exposed and unexposed samples can be found at https://www.flickr.com/photos/153260055@N04/collections.

Algal pigment analysis. Absorption spectra show peaks at 9-10 minutes and 17-19 minutes representing fucoxanthin and Chl *a*, respectively (Figure 6), providing an indication of the concentration of algae in each sample. Throughout the course of the experiment there were higher amounts of algae associated with PHA biofilms than PET biofilms based on Chl *a* concentration per gram of substrate (Figure 7).

Microbial community composition. The relative proportion of sequences within each sample that mapped to the designated taxonomic classification was used to visualize microbial community structure at the community level for the 28 (Figure 8) and 113 day (Figure 9) sampling events. To make community composition shifts more interpretable, Figure 10 shows PHA-associated communities only at 28, 113, and 202 days exposure. In addition, a principal coordinate analysis was performed on weighted UniFrac values to visualize the differences between sample types (Figure 10). Figure 11 shows how samples clustered based on the phylogenetic makeup of

each sample. In addition, we determined the bacterial community structure based on metagenomic sequencing from a parallel 4-week exposure. The generation of a tree based upon Bray-Curtis dissimilarity values of the community structure demonstrated there are unique communities between the biofilms and seawater, and that PHA harbors the most unique community amongst substrate types (Figure 12).

Enzyme enrichment. The predicted proteins from each of the 12 metagenomes were aligned against the ESTHER database of the alpha/beta-hydrolase fold superfamily of proteins. Profiles followed a similar pattern to that of community composition with PET and ceramic being the most similar sample groups (Figure 13), while the PHA had the most distinct profile. Over half of the hydrolases within PHA-associated communities were aligned to the same poly(3-hydroxybutyrate) depolymerase gene sequence. The abundances of depolymerase, esterase, cutinase, and lipase enzymes were compared between sample types (Figure 14) and demonstrated a significant increase in depolymerase and esterase enzymes within PHA-associated microbial communities versus all other community types. A maximum-likelihood tree of 46 PHB depolymerase enzyme gene sequences illustrated the diversity of the enzymes (Figure 15).

Recovery of genomes from metagenomes. Six high-quality genomes were recovered from the PHA biofilm metagenomes. Three of these genomes belonged to novel species of sulfate-reducing bacteria: *Desulfovibrio*, *Desculfobacula*, and *Desulfofustis*. Close inspection of these genomes revealed the presence of genes that likely play a role in PHA biodegradation and sulfate reduction: depolymerases, *sat/met3*, *aprBA*, and *dsrAB*.

Community engagement. To make the issue of plastic pollution visible, accessible, and accountable to the people of Texas, the AEP designed, developed and implemented a unique small plastics collection device (Figure 16). This device was a short length of PVC capped on one end

with modified “stopper” in the other, with has a short rope handle to allow easy transport. After a short lecture about plastics in the environment, participants walked along various wetland areas (and school grounds) collecting small plastics. The idea was for participants to focus on the amount and type of plastic debris found within the various environments. Many were shocked with the amount and how little time it took to fill their containers. After a short hike the students emptied their contents into a larger container and trained AEP staff were able to guide them into a discussion of the different items and their origin. Special attention was given to single use plastic items (bottle caps, candy wrappers, straws, etc.). The topic of surface area and size of plastics in the environment was also discussed with participants learning that large plastics ultimately become small plastics, which can potentially enter the food web. The AEP was able to reach over 1,300 participants at 11 different events including wetland field trips, school visits, and community events (Table 1). This short video shows students collected plastics in during a wetland field trip: (https://www.flickr.com/photos/153260055@N04/albums/72157698412361434). In addition to the AEP outreach program, the results of this study were disseminated at scientific conferences and stakeholder meetings in the form of poster and oral presentations (Table 2). In total, the results of this study were presented at 8 scientific meetings and 5 stakeholder meetings attended by more than 11,960 attendees. This study and its results were also shared with the general public through the following TAMU-CC news articles. A third news article reporting the study was published by KRISTV titled *Solving Plastics Pollution* (2016) but the web-link is no longer active.

- *Experiment seeks to ID plastic-eating microorganisms* https://tamucc.edu/news/2016/05/032416 Plastic Capsule.html - .XA6ThxNKgUE
- *Island university PhD student studies plastic-eating microbes* http://www.tamucc.edu/news/2018/02/021918_Plastic_Research_Laguna_Madre.html - .WoxZr1XwYy4

DISCUSSION

This study assessed the fate of plastic and bioplastic in the Laguna Madre, Texas and addressed critical knowledge gaps with regards to microbe-plastic interactions. First, while earlier studies focused on floating plastic debris in pelagic systems (Zettler et al. 2013; Oberbeckmann et al. 2014), this study focused on plastic debris in a benthic habitat. The focus on benthic habitats was critically important seeing that floating plastic debris commonly sinks under the weight of biofouling (Barnes et al. 2009) and the majority of debris sinks immediately as the specific gravity of many plastics is greater than that of seawater (Andrady, 2011). Further, coastal benthic habitats are especially vulnerable to plastic debris loading due to their proximity to coastal population centers and urban environments (Barnes et al. 2009), with an estimated 4.8 to 12.7 million metric tons entering coastal seas annually (Jambeck et al. 2015). Second, while earlier studies utilized 16S rRNA gene sequencing to assess the taxonomic diversity of plastic-associated biofilms (Bryant et al. 2016), this study utilized shotgun metagenomics to characterize enzymatic gene pools and recover individual genomes. Third, while earlier studies based their conclusions on comparisons between surface-associated and free-living communities, this study recognized the need for a biofilm control to make accurate conclusions.

The paradox of plastic debris is that estimates of plastic pollution far exceed the observed concentration of debris in the marine environment and it was proposed that this disparity is evidence of biodegradation (Thompson et al. 2004; Sole et al. 2017). Concurrently, the discovery of rare microorganisms capable of biodegrading plastic suggest bioremediation is possible (Yoshida et al. 2016). Here, we used weight loss measurements to show that PET did not biodegrade during the course of a 15-month exposure study. Moreover, scanning electron microscope images (see Figures 4 and 5) confirmed the absolute absence of pitting which is a sign

of biodegradation. By contrast, PHA lost 51% of its weight and was extensively pitted. This result was not surprising seeing that PHA is an energy and carbon storage polymer that's produced naturally by a diversity of bacteria (Dawes and Senior 1973). The rate of loss however was concerning seeing that complete biodegradation of a ~3 mm diameter PHA nurdle would require approximately 830 days if the biodegradation rate remained constant.

Microorganisms colonizing plastic and bioplastic debris could play a role in its biodegradation. Alternately, colonizing microorganisms could affect biogeochemical cycling or alter primary and secondary production in marine environments. Previous studies of plastic debris in the Pacific gyre reported a correlation between plastic, Chl *a*, and diatom concentrations to suggest that plastic debris accumulation enhances primary production (Carson et al. 2013; Bryant et al. 2016). In this study, both plastic and bioplastic were colonized by algae as evidenced by the detection of Chl *a* and fucoxanthin pigments. Additionally, the increasing concentration of these pigments over time was indicative of microbial succession. That bioplastic biofilms contained higher pigment concentrations confirms that bioplastic particles were sites of enhanced biological activity.

Importantly, measures of microbial community composition showed that PET and ceramic biofilms were indistinguishable. The similarity between the PET and ceramic biofilm communities suggests that inert substrates such as petroleum-based plastic and hard substrates such as ceramics are not colonized by novel microbial communities. Rather, inert and hard substrates most likely serve as neutral surfaces that catalyze biofilm colonization. By contrast, the PHA biofilms were novel. The novel response to bioplastic was most likely the result of the lability of PHA, as earlier laboratory, culture-based studies have shown that marine microorganisms are capable of colonizing and biodegrading PHA (Imam et al. 1999; Volova et al. 2010). This study extended the

findings of those culture-based investigations by identifying genera and species that are part of the bioplastic-specific response *in situ,* and in particular, identified sulfur-reducing bacteria (SRB) as dominant members of the naturally-occurring PHA-associated benthic community.

Sulfate-reducing bacteria are integral to carbon and sulfur cycling, and sulfate-reduction is the dominant remineralization pathway in coastal sediments (Jørgensen, 1982). The SRB *Desulfobacterales, Desulfobulbacaea,* and *Desulfovibrionacaea* commonly comprise less than 20% of the total bacterial community in marine sediments (Purdy et al. 2002). In this study, these families collectively comprised 9 and 12% of the PET and ceramic community, but they comprised over 39% of the bioplastic community. This overwhelming dominance of SRB in the PHA biofilm is a strong indication that bioplastic accumulation stimulates sulfate reduction in marine sediments. The stimulation of SRB could have unintended consequences for biogeochemical cycling. A previous study showed that stimulation of SRB, through the application of gypsum ($CaSO_4$) in coastal wetlands, can divert the flow of carbon from methane (produced by methanogenic archaea) to carbon dioxide (produced by sulfate reduction) (Denier van del Gon et al. 2001). Thus, bioplastic SRB stimulation could affect the balance between sulfate reduction and methanogenesis in marine sediments, fundamentally altering marine sediment greenhouse gas emissions.

The novel microbial communities colonizing the PHA were microhabitats of enhanced enzyme activity. Specifically, the bioplastic biofilms were enriched for two classes of hydrolases known to be the primary enzymes used by microbes to interact polymers: depolymerases and esterases (Volova et al. 2010; Yoshida et al. 2016). The enrichment primarily reflected a significant increase in polyhydroxybutyrate (PHB) depolymerases. PHB depolymerases are known to contribute to bioplastic biodegradation (Mukai et al. 1993) but less is known about their distribution or diversity in natural microbial assemblages. In this study, the phylogenetic analysis

of PHB depolymerases (see Figure 15) revealed that a large diversity of PHB depolymerases were present in the bioplastic biofilms and therefore, the distribution of these enzymes could extend to a mixed consortium. The substantial increase of depolymerases suggests the consortium is capable of biodegrading PHA, as was confirmed by weight loss and pitting (see Figures 4 and 5).

In addition to hydrolases, enzymatic gene pools for sulfate reduction were analyzed due to the previously discussed dominance of SRB in bioplastic-associated communities. Results showed that bioplastic biofilms were significantly enriched with two enzymes critical to dissimilatory sulfate reduction: dissimilatory adenylyl sulfate reductase (*aprBA*) and dissimilatory sulphite reductase (*dsrAB*). Previous studies have utilized the detection of these enzymes to show that sulfate reduction is widely distributed and taxonomically diverse (Meyer and Kuever, 2007; Müller et al. 2014). Through the recovery of genomes from metagenomes, the results of this study expand on that known diversity with the discovery of three novel *Desulfovibrionaceae*, *Desulfobacteraceae*, and *Desulfobulbaceae* species. Moveover, the presence of esterases and depolymerases lends support to the hypothesis that *Desulfovibrionaceae* sp. Madre1, *Desulfobacteraceae* sp. Madre2, and *Desulfobulbaceae* sp. Madre3 play a role in PHA biodegradation.

In conclusion, the results of this study demonstrated that PET did not promote a significant shift in microbial community composition of enzyme pools. By contrast, PHA introduction stimulated the growth of a novel microbial community that was enriched for SRB, hydrolases, and sulfate reductases. Further, recovered genomes, representing the three most common families of SRB, contained genes integral to both sulfate reduction and bioplastic biodegradation, providing evidence that the two processes are linked in marine sediments. The significant shift in microbial diversity and enzyme pools, in response to the introduction of PHA, could prove insightful when

considering bioplastics as a replacement for traditional petroleum-based plastics i.e., trading plastic pollution for bioplastic pollution could have unintended effects on carbon and sulfur cycling in marine sediments. We strongly recommend that biochemical effects be considered in the identification of and development of best-management practices concerning plastic and bioplastic pollution. It would be prudent to focus efforts on education and outreach that discourages single-use items regardless of whether they are made from plastic or bioplastic.

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Table 1. Summary of education and outreach activities conducted by the AEP's plastics-based program.

Date	Event	No. of Participants
July 21, 2017	Oyster Camp – Goose Island SP	20
September 21, 2017	King High School field trip	18
October 31, 2017	Halloween at the Beach	200+
November 4, 2017	Storyfest – George West, TX	700+
November 8, 2017	Notoce Nature – Duval County	142
November 15, 2017	Windsor Park Elementary school visit	187
December 2, 2017	TAMU-CC Biology class trip	25
February 15, 2018	TAMU-CC Estuarine Organisms class	22
February 16, 2018	Carrol High School field trip	15
	TOTAL	**1,329+**

Table 2. Summary of the scientific presentations where the results of this study were shared with the scientific community.

Date	Event/Presentation Title	No. of Attendees
Spring 2016	Marine Biology Graduate Student Symposium / *Bacterial response to plastic in a Texas lagoon*	100+
Spring 2016	Surfrider Foundation Coastal Bend Chapter Meeting / *Characterizing the microbial response to plastic debris in the marine environment*	25+
Fall 2016	Texas Branch Meeting American Society for Microbiology / *Bacterial response to plastic in a Texas lagoon*	150+
Spring 2017	American Cetacean Society TAMU-CC Chapter Meeting / *Plastic soup and the microbes that live in it*	30+
Spring 2017	Texas Branch Meeting American Society of Microbiology / *A tale of two nurdles: plastic-microbe interactions in Texas' coastal waters*	150+
Fall 2017	Surfrider Foundation Coastal Bend Chapter Meeting / *Nurdles for dinner? The microbial response to plastic and bioplastic in Texas' waters*	25+
Fall 2017	National Meeting Canadian Society of Microbiology / *A tale of two nurdles: how microbes respond to plastic and bioplastic in coastal waters*	1,000+
Fall 2017	International Society of Microbial Ecology / *A tale of two nurdles: how microbes respond to plastic and bioplastic in coastal waters*	3,000+
Spring 2018	Texas Branch Meeting American Society of Microbiology / *Life on a nurdle: the microbial response to plastic and bioplastic in the Upper Laguna Madre, TX*	150+

Spring 2018	American Association for the Advancement of Science / *Nurdles for dinner? The microbial response to plastic and bioplastic in coastal waters*	7,000+
Fall 2018	International Society of Microbial Ecology / *A tale of two nurdles: how microbes respond to plastic and bioplastic in coastal waters*	3,000+
Fall 2018	Texas Plastic Pollution Symposium / *Life on a nurdle: how microbes respond to plastic in coastal waters*	150+
Fall 2018	TAMU-CC Islander Green Team Meeting / *Life on a nurdle: the microbial response to plastic vs. bioplastic*	30+
Fall 2018	Texas Branch Meeting American Society of Microbiology / *Nurdles for dinner? The microbial response to plastic and bioplastic in coastal waters*	150+
	TOTAL	11,960+

FIGURES

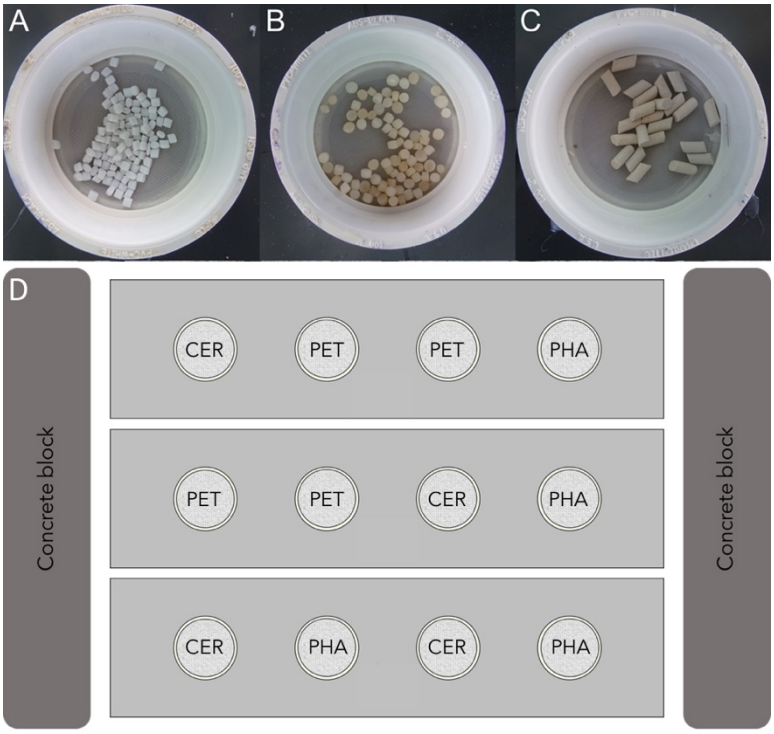

Figure 1. Each sampling device (D) consists of a PVC frame containing 4 MicroCaps that hold either PET nurdles (A), PHA nurdles (B), or ceramic nurdles (C). Devices were submerged at the Laguna Madre Field Station. Sampling devices were anchored to the seafloor with concrete blocks (D).

Figure 2. The Laguna Madre Field Station (LMFS) is located in the Upper Laguna Madre (near Intercoastal Waterway Marker 83) and operated by Texas A&M University – Corpus Christi.

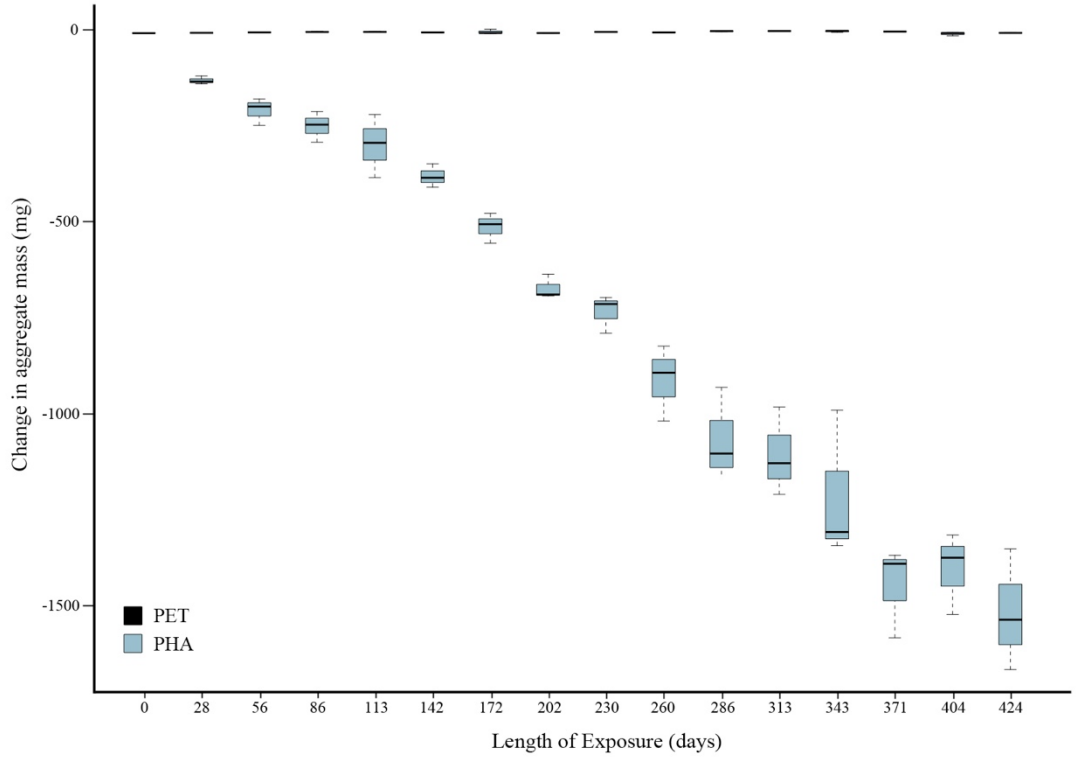

Figure 3. Changes in the aggregated mass of PET and PHA nurdles exposed to microorganisms at the water-sediment interface of the Upper Laguna Madre, TX for exposure periods of 28, 56, 86, 113, 142, 172, 202, 230, 260, 286, 313, 343, 371, 404, and 424 days.

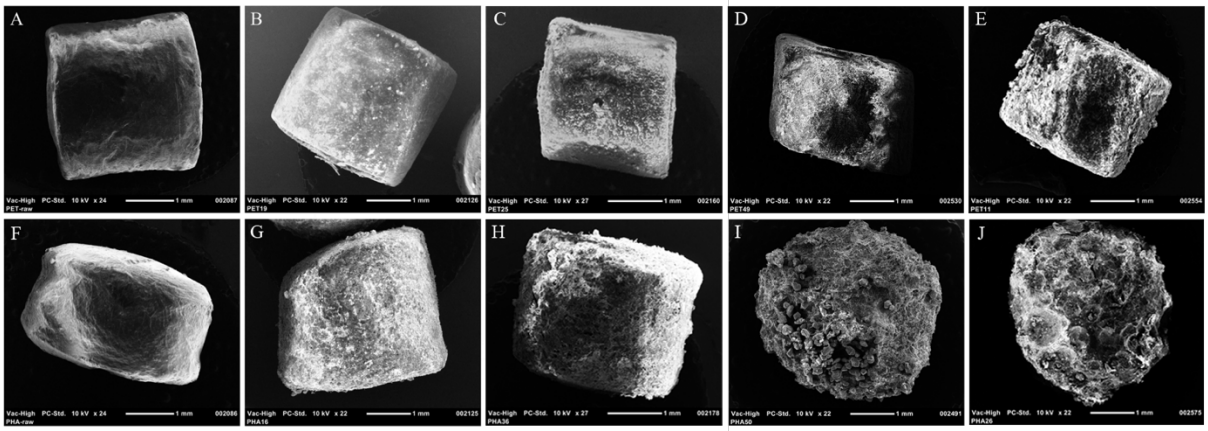

Figure 4. SEM images of PET and PHA biofilms (top and bottom panels, respectively) at approximately 20X magnification. Images show a time series: 0 (A and F), 142 (B and G), 230 (C and H), 343 (D and I), and 424 (E and J) days exposure.

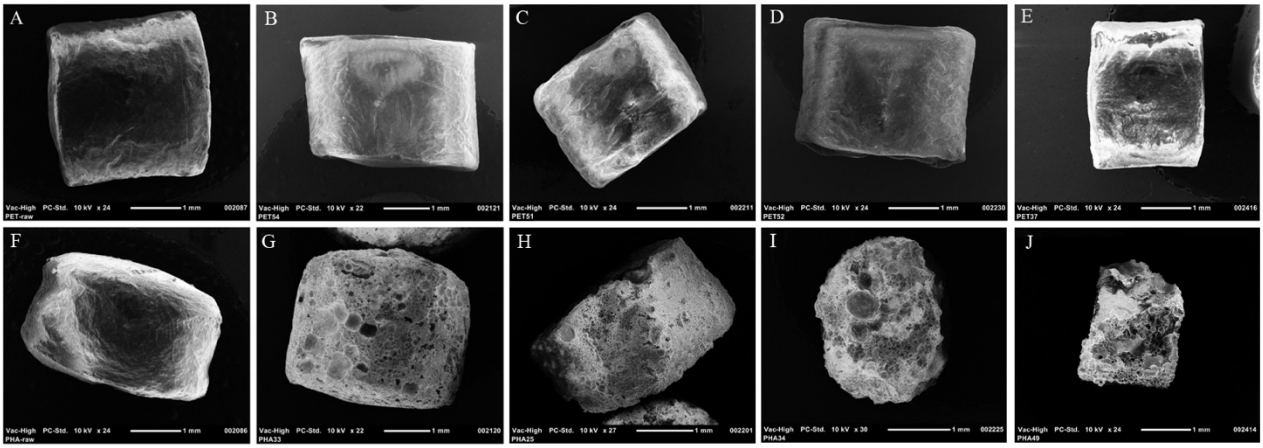

Figure 5. SEM images of PET and PHA samples post biofilm digestion (top and bottom panels, respectively) at approximately 20X magnification. Images show a time series: 0 (A and F), 142 (B and G), 230 (C and H), 343 (D and I), and 424 (E and J) days exposure.

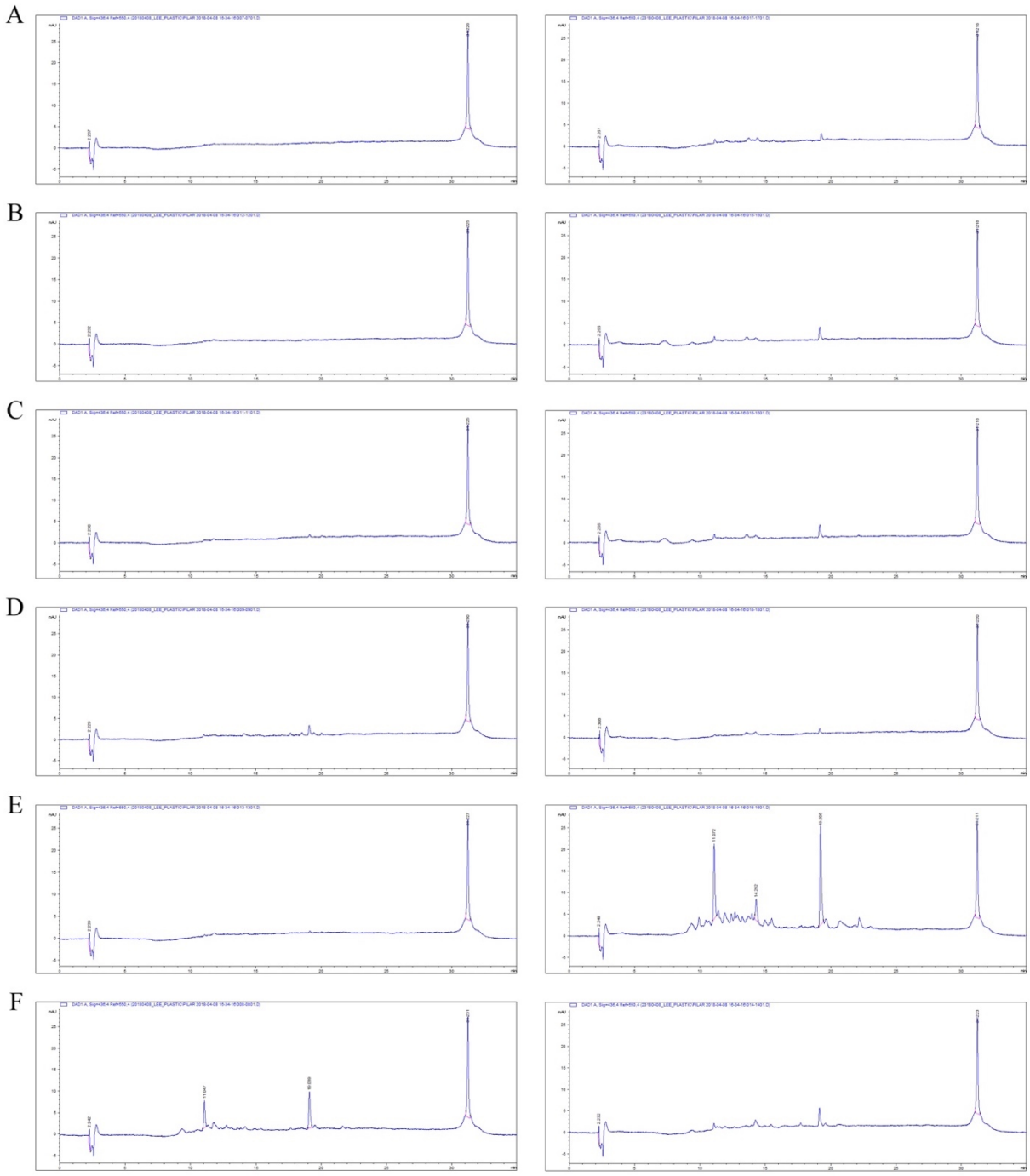

Figure 6. Absorbance spectrum of algal pigments from PET (left side) and PHA (right side) samples exposed for 86 days (A), 142 days (B), 313 days (C), 371 days (D), 404 days (E), and 424 days (F), as determined by HPLC. The peak at 9-10 minutes represents fucoxanthin. The peak at 17-18 minutes represents Chl *a*.

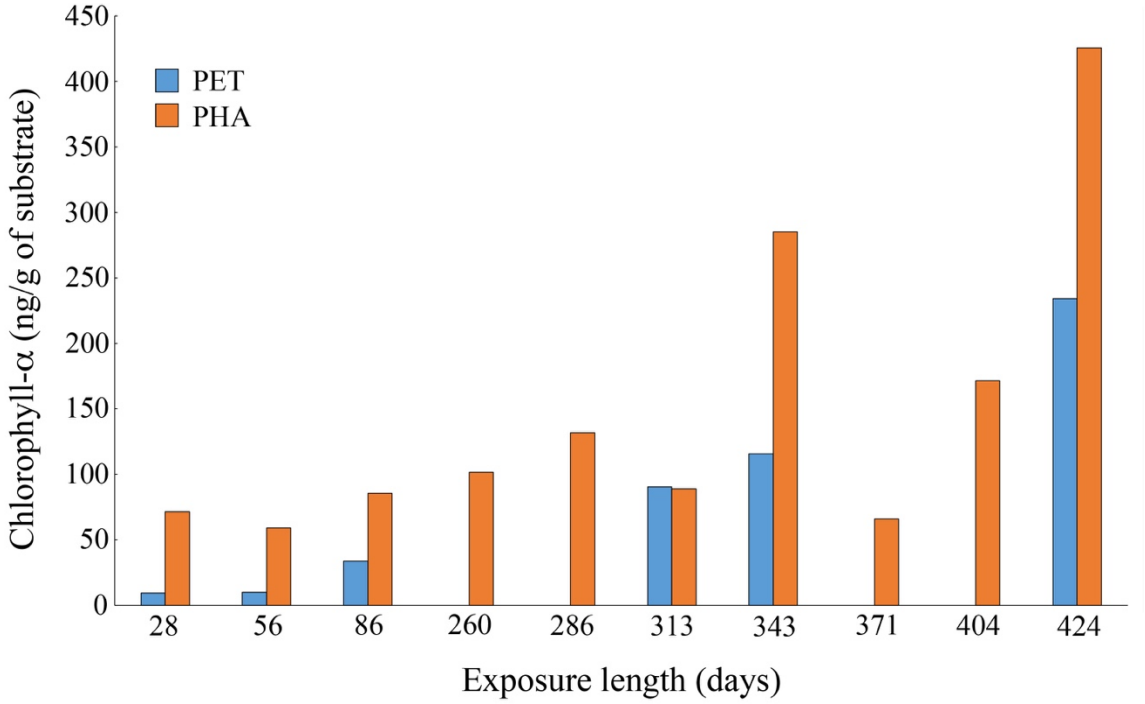

Figure 7. Chl *a* concentrations in the PET and PHA biofilms over the course of the 15-month exposure. Concentrations were calculated using the area under each peak at the 17-19 minute mark as seen in the absorption spectra (see Figure 6) and normalized to the amount of substrate in each sample.

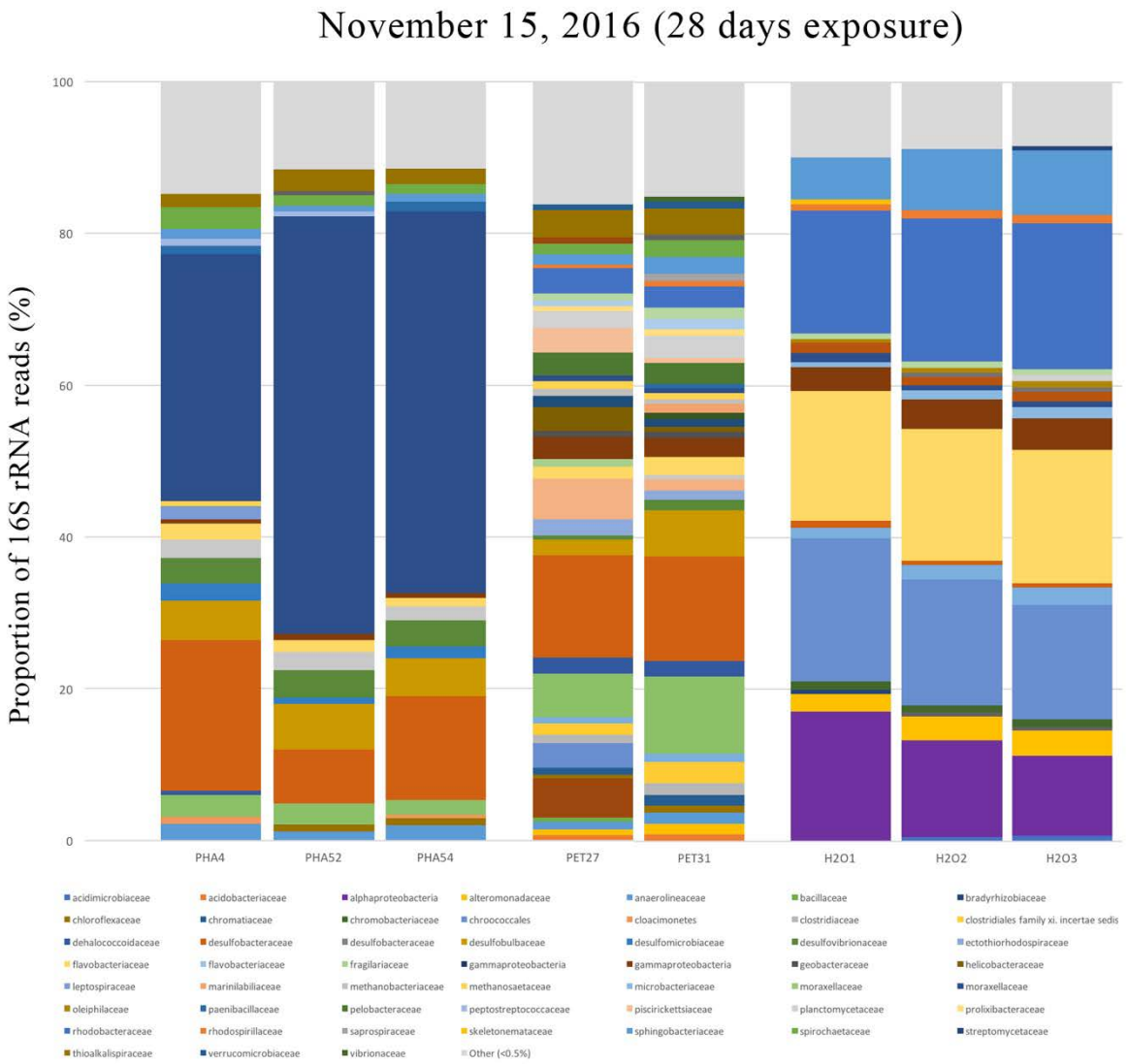

Figure 8. PHA and PET biofilm microbial community composition after 28 days exposure based on 16S rRNA gene sequences at the family level. Additionally, a seawater sample taken at the time of sample collection was included. Taxonomic families making up less than 0.5% are shown in the category of "other (<5%).

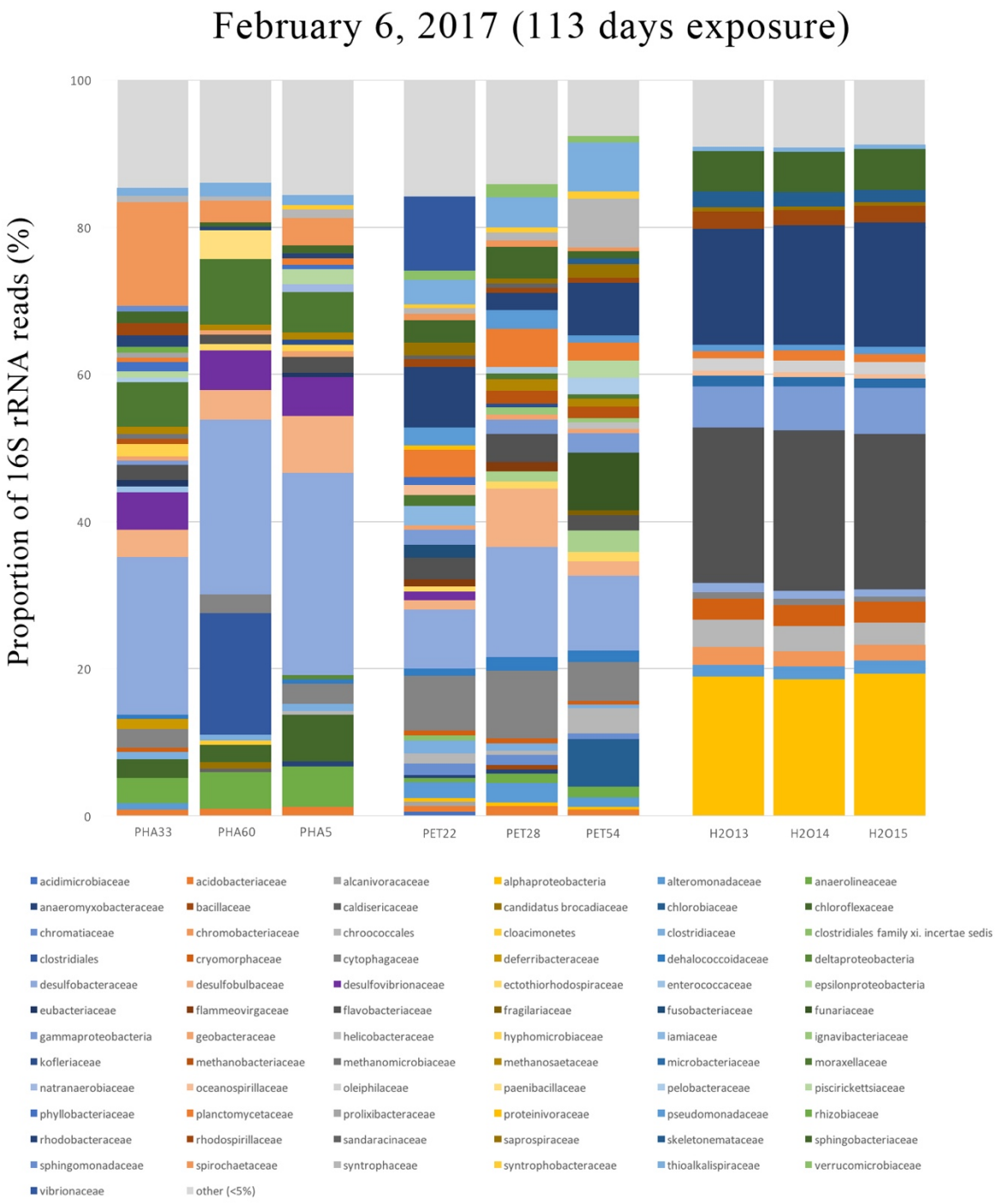

Figure 9. PHA and PET biofilm microbial community composition after 113 days exposure based on 16S rRNA gene sequences at the family level. Additionally, a seawater sample taken at the time of sample collection was included. Taxonomic families making up less than 0.5% are shown in the category of "other (<5%).

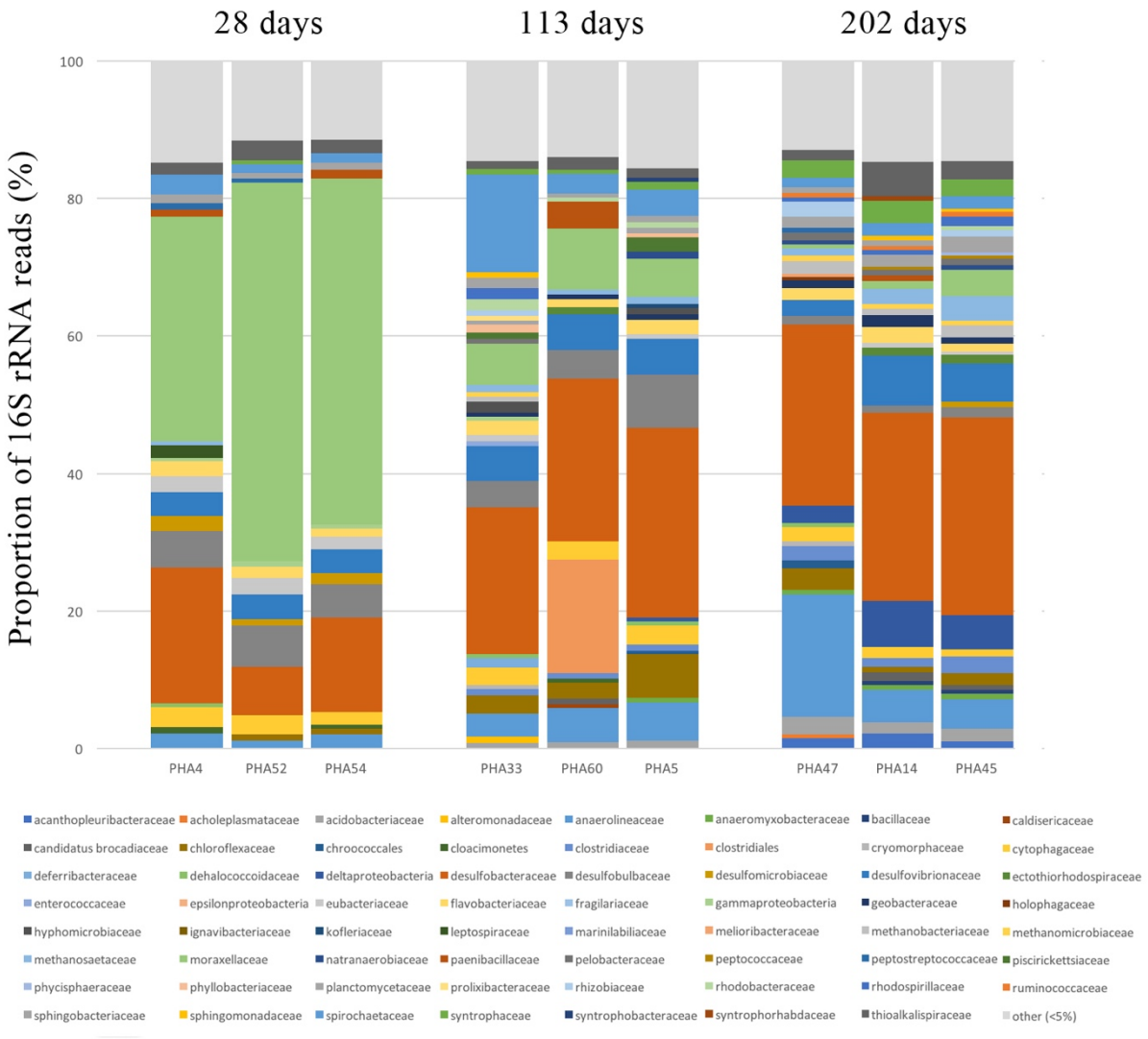

Figure 10. The PHA biofilm microbial community composition after 28, 113, and 202 days exposure based on 16S rRNA gene sequences at the family level. Taxonomic families making up less than 0.5% are shown in the category of "other (<5%).

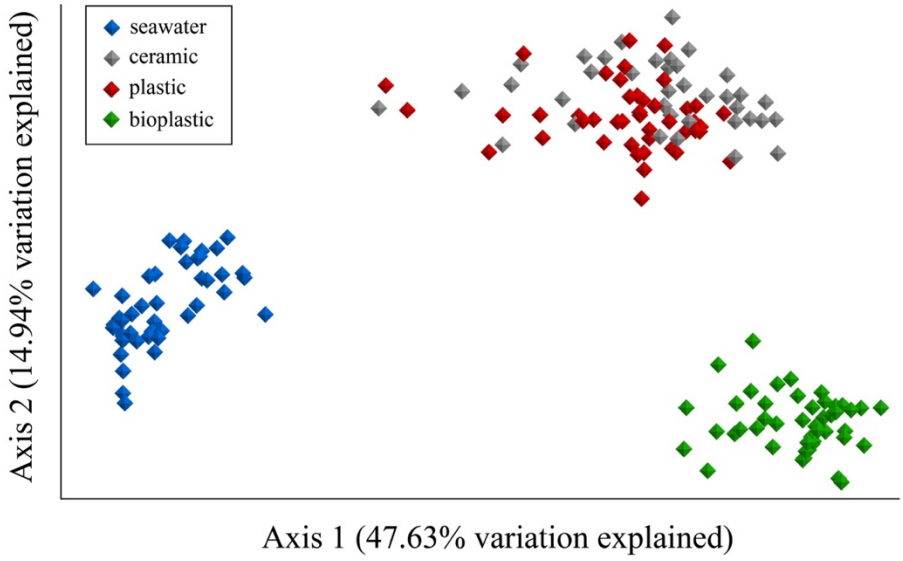

Figure 11. Principal coordinate (PCO) analysis showing relationships between microbial community composition in 152 samples. PCOs representing the similarity between seawater, ceramic, plastic (PET), and bioplastic (PHA) biofilm communities are shown based on weighted UniFrac values. The first PCO, represented on the x-axis, explains 47.63% of the variation seen between samples, while the second explains 14.94%.

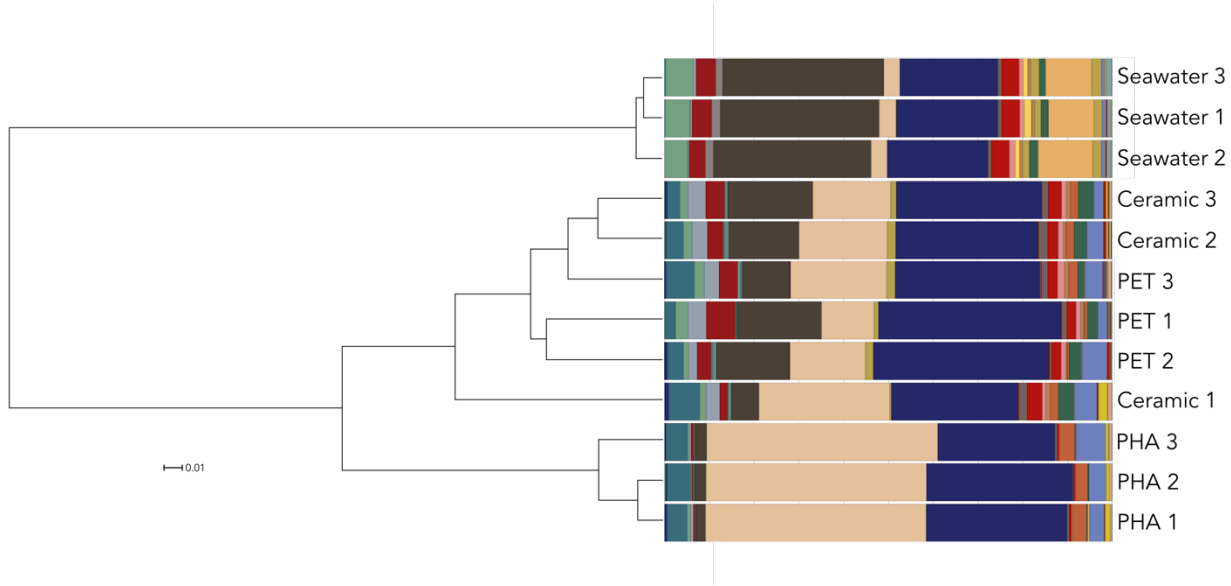

Figure 12. Bacterial community structure at the level of family based on taxonomic analysis of the 16S rRNA gene profiles for each sample using QIIME. The tree on the left demonstrates the relatedness of each sample based upon the Bray-Curtis dissimilarity.

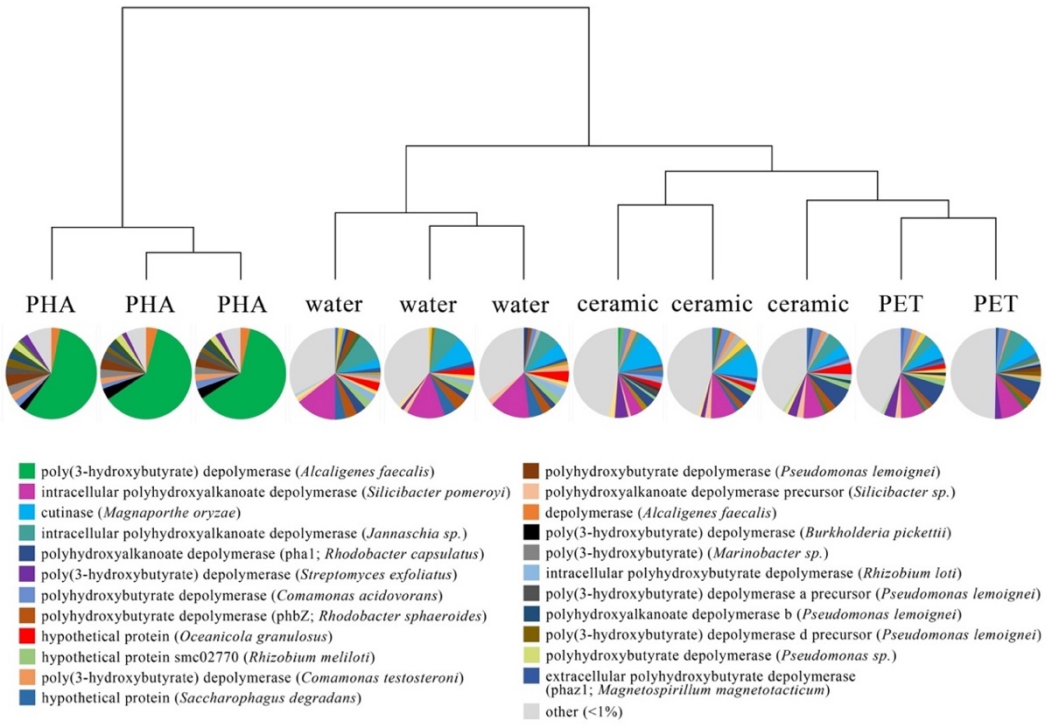

Figure 13. Profiles of hydrolases for each sample based on a blastp alignment of pCDS sequences to a hydrolase database. Sequences representing less than 1% of the total proportion were categorized under other. The hierarchical clustering dendogram is based on Jaccard similarities generated using the R package phyloseq.

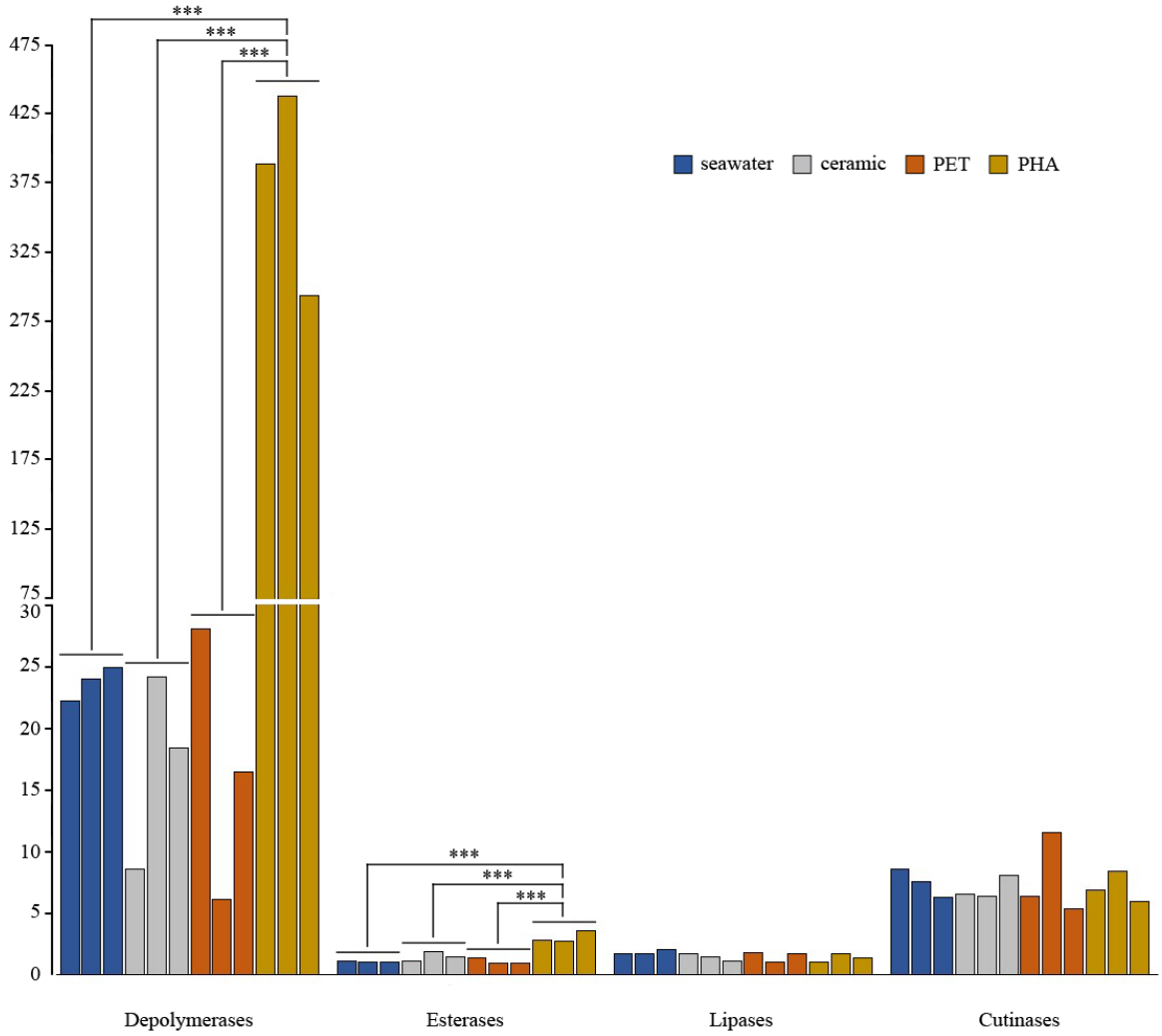

Figure 14. The abundance of depolymerase, esterase, lipase, and cutinase enzyme sequences were normalized to the total number of predicted proteins in each sample, with individual samples plotted and grouped together based on community association (seawater, ceramic, PET, or PHA). Significance was determined using ANOVA (*** p-value < 0.001, n=3).

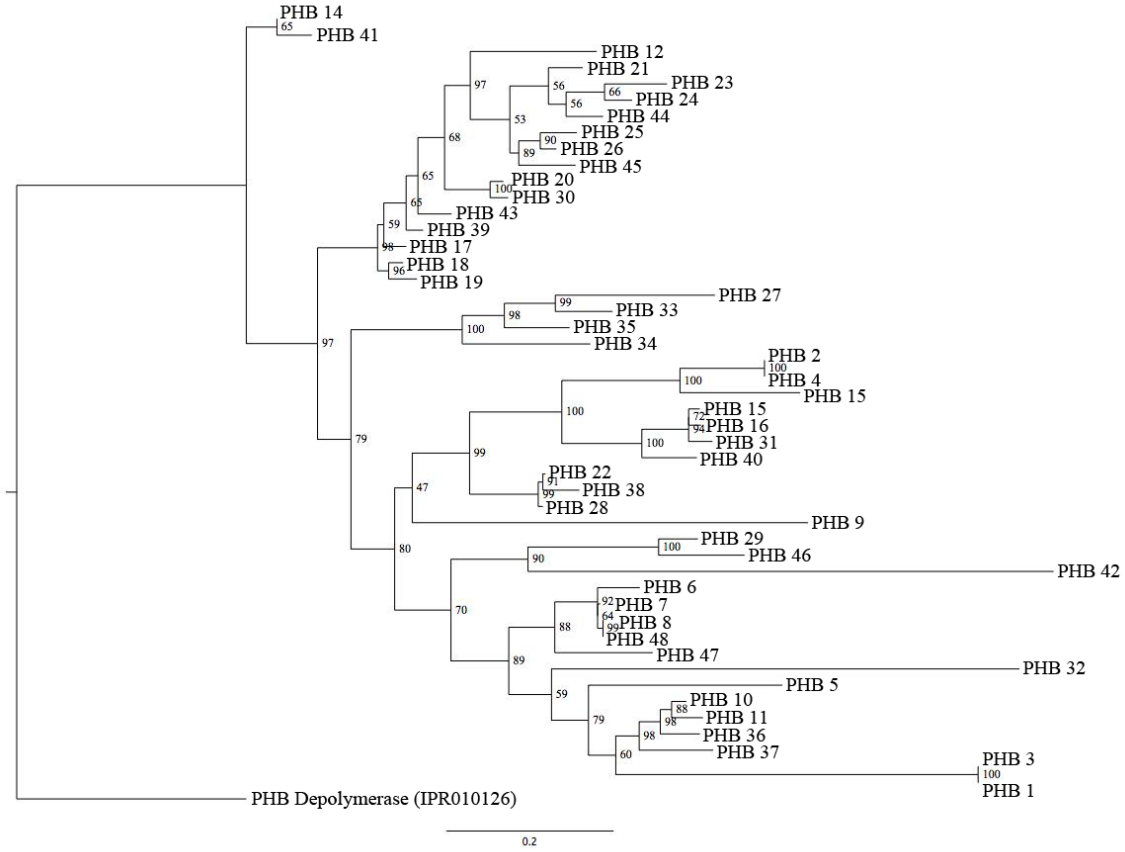

Figure 15. Maximum-likelihood tree showing the diversity of the 46 enriched PHB depolymerase gene sequences. Node labels show the bootstrap support values. Branch lengths represent the average number of substitutions per site.

Figure 16. The Coastal Bend community and student populations learning about the issue of plastic pollution through TAMU-CC's Center for Coastal Studies Aquatic Education Program (AEP). The program reached over 1,300 participants.