Biomass Estimation of Marine Biofilms on Plastic Surfaces

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Abstract

Plastics have become a major source of marine pollution, which threatens food safety and quality, human health, and marine ecosystems. Due to the drastic negative effects of plastics in a marine environment, alternative biodegradable plastics are being generated that are more eco-friendly and have less environmental impact. Though some of these plastics are known to biodegrade, the process of degradation for bioplastics has not been heavily studied in a marine environment. This pilot project sought to both quantify the process of biodegradation and compare across different methods for effectiveness of biomass estimation, which serves as an indicator of biodegradation. Plastics were provided by Danimer Scientific®, with polyhydroxybutyrate (PHB) as the main ingredient in the plastic formulations. PHB is known to serve as a substrate for bacterial growth, so DNA quantification, plate counts, and crystal violet staining were employed as methods of biomass estimation for the resulting biofilm. The marine biofilm was involved in the degradation of PHB samples, as each method demonstrated that PHB samples contained greater biofilm growth and degradation when compared to the negative control. Though each method had benefits and drawbacks, crystal violet staining was the most useful and consistent estimate for biomass while plate counts and DNA quantification were variable and inconsistent. Crystal violet staining was also the most straightforward assay, while plating and DNA extraction were more time-consuming, involved, and expensive in comparison. For these reasons, crystal violet assay is recommended as a consistent and cost-effective method for measuring biofilm growth over time. Though crystal violet staining assesses biofilm bound to the plastics, plating and other DNA based studies can provide additional information about the biodegrading microbes. The methods developed and used in this investigation can be adapted for future research in biofilm removal, biomass quantification, and testing for evidence of bacteria-mediated degradation in a variety of bioplastic formulations and environments.

Introduction

Plastic pollution in the ocean has major impacts on both marine and human ecosystems. The accumulation of these plastics can persist for decades in the ocean. Notably, human plastic accumulation is visible from space in the Great Pacific garbage patch that swirls in the north pacific gyre, which is the largest accumulation of ocean plastic in the world [1]. Most of this plastic contains toxic chemicals [2], and is becoming a ubiquitous substance in the ocean. Animals confuse the plastic for food, causing malnutrition, and these toxic chemicals affect humans through the food chain. Motivated by environmental issues and the increase in oil prices, many companies look to biodegradable plastics as an enticing alternative. These options are more viable with manufacturing costs decreasing as new production methods are being developed. While there are many methods to produce biodegradable plastics, some of the best understood and useful plastics are synthesized by microbes [3].

Poly-hydroxybutyrate (PHB) is a biodegradable plastic found in the environment as a reserve carbon source for microbes, which accumulate this polymer in their cells. The production of PHB is non-toxic, renewable, and has a low environmental impact especially when compared to petroleum-based polymers. When PHB is exposed to the environment, the process of its degradation begins via surface attachments and biofilm formation [4].

Danimer Scientific produces biodegradable plastic using polyhydroxyalkanoates (PHAs) like PHB, specifically two formulations tested named 303 and 205. These PHB plastics degrade in seawater, and the company enlisted our help in a pilot project to determine if genuine biological degradation is occurring, not just degradation due to UV or exposure to seawater. As there are few studies published measuring biodegradation and biofilm growth in a marine environment, this pilot project tests the efficacy of various methods of biofilm biomass estimation on plastic surfaces with the goal of providing method recommendations. To study the process of degradation, PHB and PE plastics were submerged in seawater for 18 weeks at the Cal Poly Pier. The investigation of bioplastic degradation by marine microbes allowed for comparison between methods of biomass estimation that provide useful evidence for further research.

Our investigation of bacterial degradation employed multiple approaches to provide quantitative and qualitative evidence that plastic degradation was mediated by microorganisms. Biofilm biomass was quantified with crystal violet staining, plating, and DNA extraction. Each method provides unique information that is potentially useful for both reproducing this study or concentrating on more specific molecular approaches.

Crystal violet is a positively charged, basic dye which binds to the negatively charged peptidoglycan and proteins in the bacterial cell wall. Gram positive and Gram negative bacteria differ in their level of peptidoglycan, but both contain some level of it and will therefore be stained by the dye. Crystal violet staining has been used to provide a quantitative measure of bacterial growth over time in a way that is relatively quick, with little preparation and materials required [7]. Some downsides include the staining of extracellular biofilm material that does not

represent the live community of organisms and the inability to differentiate PHB degrading bacteria from the rest of the population.

Compared to crystal violet staining, plate counts require more effort to conduct but provide information of the physiology of bacterial colony characteristics and growth in specific media. Plating is used to distinguish individual bacterial colonies by using specific media to grow them. In addition, colonies can be isolated for further morphological studies or genetic analysis. PHB degrading bacteria were quantified by observing the presence of a “halo” surrounding individual bacterial colonies on media containing PHB (Figure 4). A downside of growing bacteria on specific media at room temperature is that it may inhibit the enumeration of some groups of marine microbes while promoting others. So while plating provides information on some bacterial colonies from biofilm, bacterial species in the biofilm may be omitted on plates due to sample variation and trophic requirements.

DNA quantification is a way of measuring biofilm biomass, and once extracted, DNA can be further studied through other molecular approaches such as PCR and genetic analysis. Downsides for DNA extraction are that it requires expensive kits and is more time-consuming than crystal violet staining and plating. DNA measured may be from any living organism so these results may include other temporary microbes and algal residents which do not directly contribute to degradation.

Over the 18 week submersion of the PHB and PE plastics, these methods were used to quantify biomass over time. We hypothesized that bacterial growth would occur due to the breakdown of PHB by biofilm bacteria. This would result in higher levels of biomass on PHB plastics in each assay compared to a non-biodegradable Polyethylene (PE) plastic control.

Materials and Methods

Testing Sodium Pyrophosphate as a Biofilm Removal Agent

In a preliminary test before the samples arrived for the pilot study, nine samples 303 chips (0.5 cm x 2 cm) were incubated at 27°C for 10 days with shaking at 150 rpm in 3 ml of Difco™ Marine Broth 2216 with 500 µl seawater to promote biofilm formation for use in optimization assays.

After biofilm formation was observed, chips were removed, lightly rinsed with sterile water, and chips were placed in triplicate in 1 ml sterile seawater containing different concentrations of pyrophosphate (0 mM, 1 mM, and 5 mM). Each sample was vortexed for 1 minute, placed in a Fisherbrand™ FS6 Ultrasonic Cleaner water bath sonicator at 5 minutes, and then vortexed again for 1 minute (Waltham, MA). Treated chips were subjected to a crystal violet assay (see below). When testing for biofilm removal, the level of bound crystal violet remaining (µg/cm²) correlates to the amount of biofilm remaining after each removal treatment. This crystal violet measurement was only used to compare biofilm removal methods. In all other cases, crystal violet was used to measure biofilm not removed from plastics.

Crystal Violet Staining

Each sample type was stained using triplicates. After samples were rinsed with sterile seawater, sterile scissors were used to cut out a 0.5 cm x 2 cm sized rectangle (for a staining area of 1 cm x 2 cm). The samples were then placed in a Petri dish and covered with 150 µl of crystal violet dye (Figure 1B). After 1 minute, the sample was turned over with tweezers and stained on the other side for another minute. These samples were then rinsed thoroughly with DI water until the runoff was clear. The chips and films were added to 1.5 ml Eppendorf tubes with 1 ml of 100% ethanol, vortexed and left for at least 15 minutes in order for more crystal violet to enter the solution. The sample was then removed with tweezers and the absorbance of the crystal violet was measured in a Molecular Devices Spectrophotometer (San Jose, CA) at 595 nm with the 100% ethanol as the standard. To obtain a more accurate representation of the biofilm growth, the results were recorded as extrapolated values. Due to absorbance readings over 1 being considered inaccurate, this extrapolation was achieved through dilution of the ethanol solution after staining and multiplying the recorded values by the dilution factor (Figure 5C).

To convert absorbance values from the spectrophotometer to units of µg/cm², a molar absorptivity coefficient of 87,000 M^{-1} cm^{-1} and a molar mass of crystal violet 407.979 g/mol were used: $\mu g/cm^2 = Abs \times [(407.979\ g/mol \times 1\ cm \times 0.001\ L) / (87{,}000\ M^{-1}cm^{-1} \times 10^6) / (2\ cm^2)$.

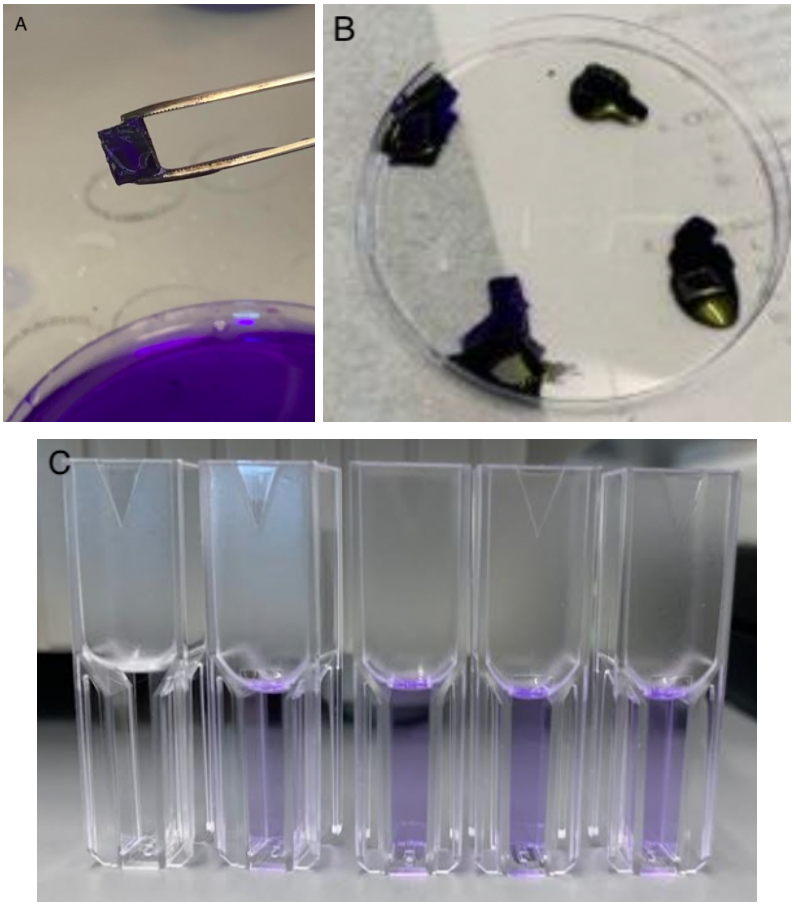

Figure 1. *A.* Crystal Violet stained PHB chip. *B.* Amount of crystal violet chips are submerged in. *C.* Various dilutions of crystal violet for absorbance measurement in a spectrophotometer.

Plastic submersion in seawater and experimental sampling

PHB plastic formulations 303 and 205 along with PE plastic provided by Danimer Scientific® were attached to plastic racks, and then submerged in a 64 gallon plastic bucket at the Cal Poly Pier for 18 weeks (Figure 2). This bucket had filtered seawater flowing through and was covered by a wooden lid. This was to keep debris and UV radiation from affecting the degradation process by bacteria. Each week, samples were collected at noon by Dr. Ray Fernando's team and then brought to the Center for Applications in Biotechnology (CAB) for biofilm extraction. Chips were collected on Wednesdays and film samples on Fridays.

Figure 2. Experimental layout for submersion of plastic formulations in flow-through seawater. Chip submersion is shown on the left and film submersion shown on the right.

Comparing sonication and vortexing to scraping for DNA extraction

For Week 1 chips, biofilm removal through scraping was compared to removal through sonication and vortexing by comparing levels of DNA biomass. In the first biofilm removal method, biofilm was scraped with a dental scaler, placed in a 1.5 ml Eppendorf tube, and sonicated and vortexed to break apart biofilm. In the second, biofilm was removed through sonication and vortexing in a 1.5 ml Eppendorf tube, and then the chip was removed. Both removal methods were followed by DNA extraction and DNA yields were measured by absorbance at 280 nm. DNA yields were compared to determine the preferred biofilm removal method.

Biofilm Collection and Processing

Each sample was removed from the ziploc transport bag, before being gently rinsed with 1 ml of sterile seawater on each side to remove excess non-biofilm residue. For DNA extraction and plating, plastic chip pieces were cut out using sterilized scissors, measuring 2 cm x 0.5 cm. This same size was measured without cutting for film samples. For the DNA quantification, both chip and film samples were then scraped with a sterilized 0.5 cm dental scaling tool to remove biofilm on two 1 cm^2 sections. This scraped biofilm was then transferred to a bead beating tube from the DNA extraction kit. For plating, the same procedure was used but the resulting biofilm was collected in 1.5 ml Eppendorf tubes, prefilled with 1 ml of sterile seawater. To further break apart the biofilm for plating, sonication and vortexing were performed on each biofilm in the Eppendorf tubes. Each tube was vortexed for 1 minute, placed in a Fisherbrand™ FS6 Ultrasonic Cleaner water bath sonicator (Waltham, MA) at 5 minutes, and then vortexed again for 1 minute.

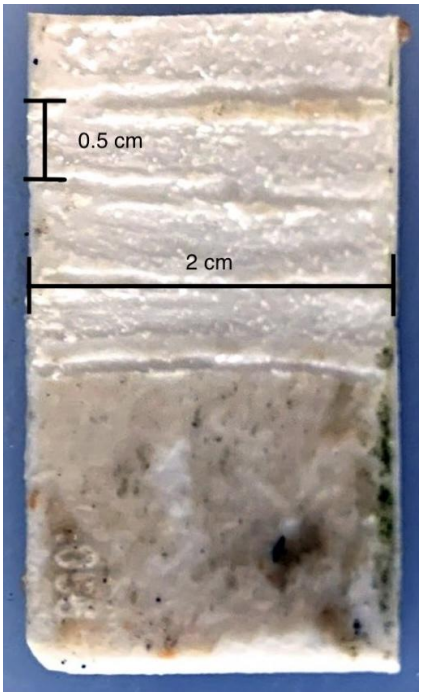

Figure 3. 0.5 cm x 2 cm scrape procedure for both film and chip. Two scrapes were performed for DNA extraction and plating each.

After week 3, scraping was no longer possible for the 303 film samples as they became too brittle and broke apart when using the dental scaler. At this point, we began cutting 1 cm^2 sections of the film followed by sonication and vortexing. After week 5, the 303 samples had completely degraded, and the film was no longer collected for further experimentation. 205 film samples fully degraded after week 11, so the data collection continued only with plastic chips until week 18.

DNA Extraction and Quantification as Biomass Estimation

To begin DNA extraction, the biofilm was scraped into a tube containing glass beads and lysis buffer from the QIAGEN Powersoil PowerLyzer DNA extraction kit (Germantown, MD). Each biofilm sample was homogenized in a Fast Prep homogenizer for 135 seconds at 6.5 m/s. DNA was then isolated and purified following the manufacturer's extraction protocol. Three different 2 µl aliquots from each DNA extraction were quantified (ng/µl) at 280 nm in the Molecular Devices Spectradrop (San Jose, CA). The average DNA concentration for each plastic formulation (303, 205, PE) was calculated by averaging the three spectrophotometric measurements from each of three replicate plastic formulations. Values were then converted to µg/cm^2 with the equation: µg/cm^2 = (1 ng/µl) × (1 µg / 1000 ng) × (100 µl / 2 cm^2).

Plate Counts

Media

Total aerobic heterotrophic bacteria (AHB) were estimated by plating on media containing 3.74% marine broth powder, 1.2% agar, and 0.4% PHB powder resuspended in distilled water. Colony forming unit (CFU) counts on agar plates were used to determine the frequency of PHB degraders and total AHBs on each sample of plastic (Figure 3). PHB degradation was represented as "halo" clearings in the media where the polymer is removed by bacterial colonies producing extracellular depolymerase enzymes. At week 7, new media was developed which selected for PHB degraders by limiting the carbon sources. This media contained 0.0025% yeast extract, 1.2% agar and 0.4% PHB powder resuspended in sterile seawater (to provide marine type environment); on this media it took less time for PHB degrading colonies to form halos on media.

Plating

Serial dilutions were performed using the treated biofilm in 1.5 ml Eppendorf tubes, with 100 µl of the solution added to 900 µl of sterile seawater. These dilutions resulted in a total of 1 ml and were repeated to obtain the desired dilutions for plates. Appropriate dilutions were plated (100 µl) onto a separate plate of both media formulations, and then spread using sterile glass beads. All plates were incubated at room temperature for one week, at which point they were counted and allowed to grow for another week to observe any additional PHB degradation or colony formation. The final colony count after two weeks was included in the results. Countable numbers were defined as 30-300 colonies and each count was based on 2 cm^2 of biofilm scraped from each plastic. The values were divided by two in order to get counts per cm^2 of plastic surface. In some cases PHB degraders were below countable numbers, so if all three replicates were too few to count but not zero, then an average of values was included to provide a form of data. If only one or two plates were countable then only those values were used and no standard deviation was recorded. When all 3 replicates were too many to count no data was recorded. These methods of counting were used for both media types.

For AHBs, a broad range of dilutions were plated initially to determine which would provide the most consistent number of countable colonies. The final dilutions were determined to be 10^{-1} to 10^{-3} for PE samples, and 10^{-3} to 10^{-5} for 303 and 205. Total bacterial colonies were counted as one metric, with PHB degraders consisting of another.

After week 7, new media was used to to promote PHB degrader growth while limiting the growth of certain AHBs. Dilutions with this media were lower, with PE samples being 10^{0} to 10^{-1} and the PHB formulations being 10^{0} to 10^{-2}. The small size of all colonies on this media required true PHB degraders to have both a halo and identifiable colony within the zone in order to be counted, as the modified media sometimes displayed gaps in PHB cover (Figure 5).

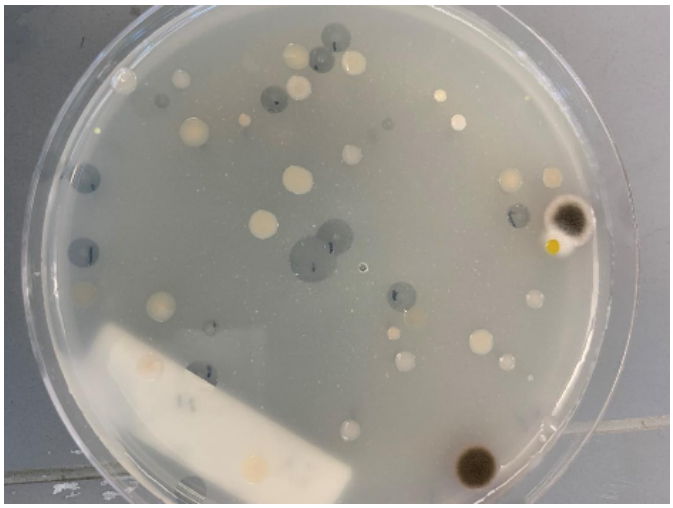

Figure 4. Marine broth plate containing PHB showing a mixed culture of degraders and other marine bacteria, where PHB degraders produce clear halos. This example demonstrates a higher percentage of PHB degraders than what was commonly observed.

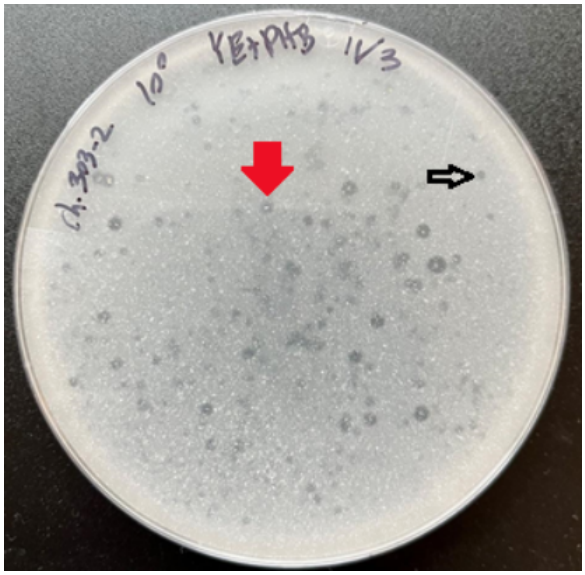

Figure 5. Minimal yeast and PHB plate with halo-forming degraders, with higher selectivity than seen on nutrient-rich media. The red arrow points to a bacterial colony with a degradation halo surrounding it, while the empty black arrow points towards a gap in PHB cover with no visible colony inside and was therefore not counted.

Results and Discussion

Pyrophosphate Testing

Pyrophosphate was considered as a cell-detachment pre-treatment option for biofilm removal based on methods described in Dussed *et al.* [5], in which they applied 1 mM pyrophosphate during 30 min at room temperature in the dark, followed by a sonication to

optimize cell detachment. Crystal violet staining was used to test the efficacy of removal, and we expected more bound crystal violet to correlate to worse biofilm removal. On average, the chips that were treated with both 1 mM and 5 mM pyrophosphate had a more remaining crystal violet bound when compared to those that were only sonicated and vortexed (Figure 6).

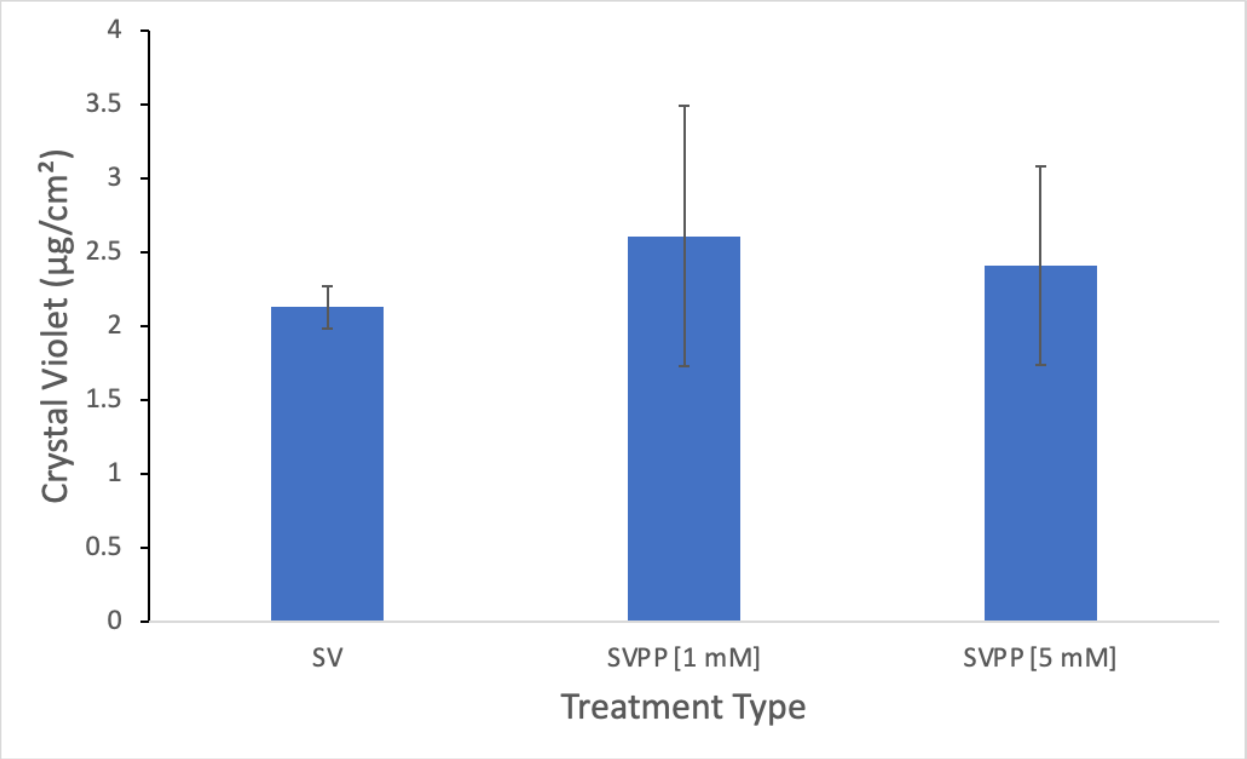

Figure 6. Optimization procedure 1 results: Average amount of crystal violet (µg/cm²) remaining bound to 303 chips with increasing concentrations of pyrophosphate. SV is chip treatment without pyrophosphate, and SVPP includes pyrophosphate. Treatment type and concentration of pyrophosphate are on the x-axis. Error bars show the standard deviation from three replicates.

SV chips appeared to have less CV bound after treatment compared to SV + pyrophosphate chips, indicating that there is less biofilm remaining (Figure 6). However, there was no significant difference in biofilm removal for each treatment according to the one-way ANOVA where $p = 0.673$, $\alpha = 0.05$, so pyrophosphate was discontinued as a removal agent in the full timecourse experiment.

Scraping with a dental scaler was also considered as an effective method for biofilm removal from the plastic samples, so during week 1 of the 18 week time course, scraping was compared to sonication and vortexing. The dental scalers were 0.5 cm in length, which allowed precise 1 cm² scrapes to be performed (Figure 2).

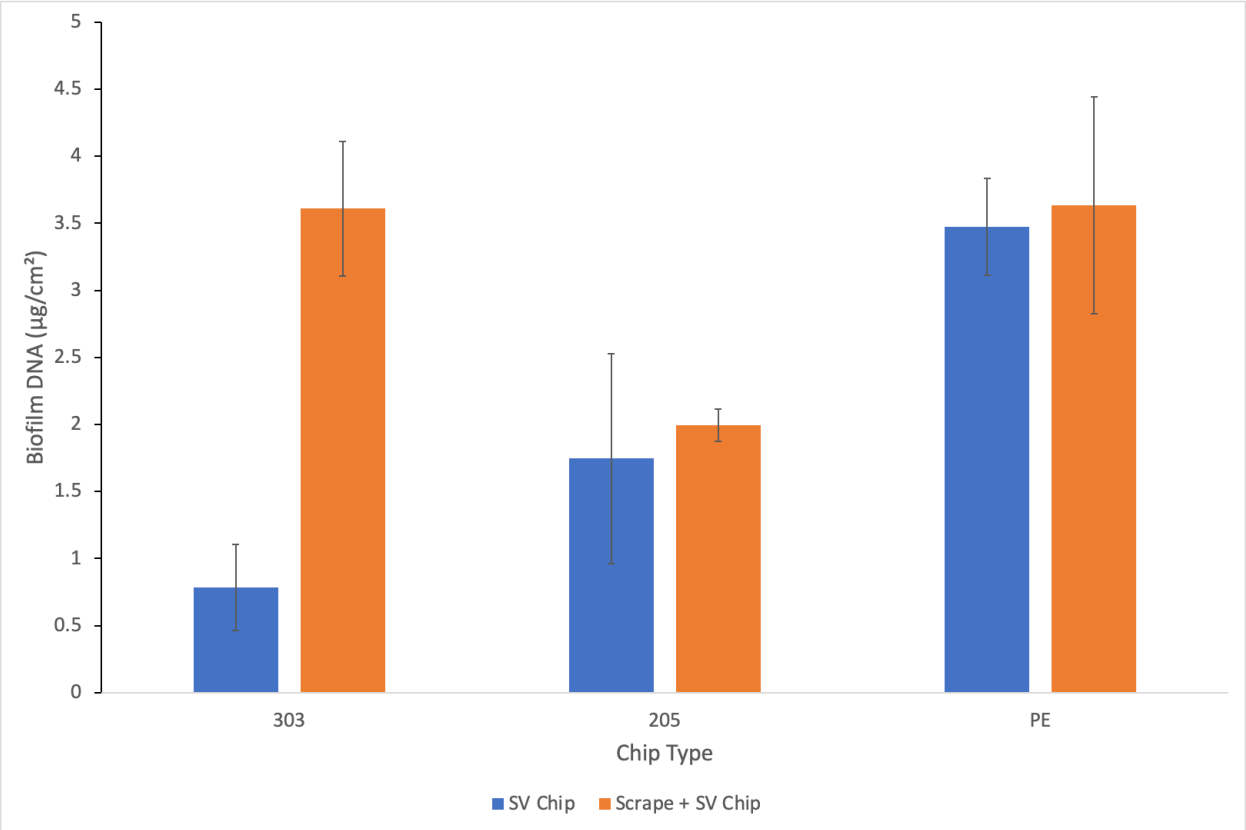

Figure 7. Biofilm DNA removed from whole chip through sonication and vortexing (SV) versus biofilms scraped off followed by sonication and vortexing (Scrape). Error bars represent standard deviation of triplicate samples.

In each treatment, scraped chips showed DNA collected was greater on scraped chips than SV chips, which suggests that scraping with a dental tool is preferable for biofilm removal (Figure 7). For the 303 chips, biofilm that was scraped showed higher DNA yield (3.61 µg/cm²) than chips with biofilm removed through sonicating and vortexing alone (0.79 µg/cm²), and a t-test suggested that there was a significant difference in these treatments ($p = .05$). Thus scraping followed by sonication and vortexing was chosen as the preferred method for biofilm removal throughout the timecourse experiment.

Quantification of bacterial biomass by DNA concentration analysis

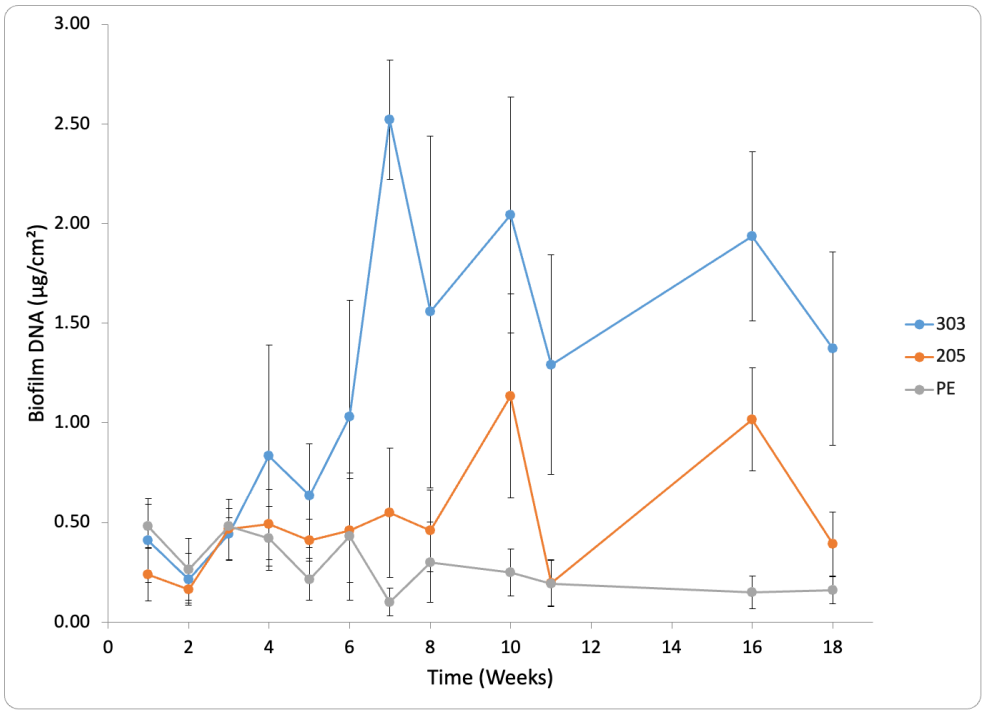

Figure 8. Average changes in Chip DNA concentration (µg/cm²) over 18 weeks for formulations 303, 205, and PE. Error bars represent standard deviation of triplicate samples per timepoint and triplicate assays per sample.

DNA (µg/cm²) increased for the 303 chips during the first six weeks from 0.41 µg/cm² to 1.03 µg/cm² until leveling off after week 7 (2.52 µg/cm²). DNA for 205 chips increased from 0.24 µg/cm² until it leveled off from week 4 at 0.49 µg/cm² until week 8, and the average DNA ranged widely in measurements after week 10. DNA on PE chips increased in a similar trend as 303 initially until it dropped off after week 3 (0.48 µg/cm²) and remained low for the remainder of the timecourse (Figure 8). Though there is large variation and a drop in week 11 for both 303 and 205 samples, the overall trends suggest preferred biofilm growth on the PHB containing plastic chips when compared to the PE control.

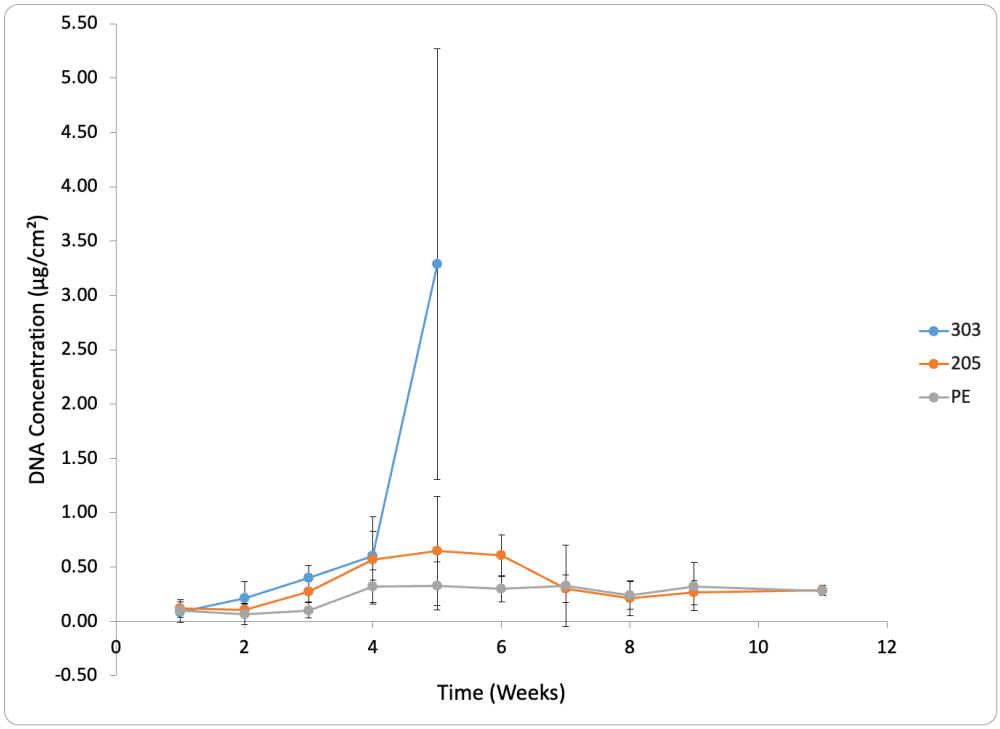

Figure 9. Average changes in Film DNA concentration (µg/cm²) over time (weeks) for formulations 303, 205, and PE. Error bars represent standard deviation triplicate samples per timepoint and triplicate assays per sample.

Biofilm DNA on 303 film increased until week 5 (3.29 µg/cm²) when the film was fully degraded, while biofilm DNA on 205 film steadily increased until week 6 (0.61 µg/cm²), after which it remained at similar levels of DNA as PE film (Figure 9). Though it had similar levels of DNA, 205 film fully degraded by week 11, while PE remained fully intact throughout the timecourse. The overall trend suggests that the biofilm on the biodegradable plastics was increasing to a greater extent than the PE control, and the degradation timeline of 303 followed by 205 is also consistent with the rates of biofilm growth on the plastic. For both films and chips, the order of apparent rate matched the order of degradation.

Though DNA quantification followed the same trends as crystal violet staining and plate counts throughout the timecourse, the large standard deviation in triplicate assays of triplicate samples shows that biomass estimation with DNA is not consistent across measurements (Figures 8 & 9). This inconsistency may be a result of the measuring process in the Molecular Devices Spectradrop, into which 2 µl aliquots of sample DNA were measured for absorbance at 280 nm. Because this device showed different absorbance values for each sample measurement, the absorbance assays were performed in triplicate and averaged to account for this inconsistency. However, even with triplicate readings of the same samples, lots of variance remained in the observed data around the mean (Figures 8 & 9). This variance may be a result of just measuring 2 µl of DNA having a large error, whereas measuring the whole sample may be

more accurate. Due to this high variance, DNA is not recommended as a method for biomass estimation.

Plate Counts

Plate counts for AHBs increased every week until leveling out at 10^7 CFU/cm² in week 6 for both 303 and 205 chips, while PE chips remained fairly consistent at 10^4 CFU/cm² after week 2 (Figure 10). The missing data point for week 18 PE chip was a result of no countable plates available and this general lack of countable AHBs on PE was observed through the various weeks as points with no standard deviation. The combination of fewer viable plates and lower levels of AHBs indicate a smaller bacterial population in the PE chip biofilm, when compared to both PHB chips.

PHB degrader counts on chips were fewer in number than AHBs. When available, standard deviations were larger and the great majority of PE plates did not contain any observable degraders (Figure 11). For the first 6 weeks, there were multiple missing data points for the PHB samples and some weeks contained only one countable plate. Initially, it was difficult to count the PHB degraders on media as the halo-forming colonies would often be hidden or overwhelmed by other AHBs. The media switch at week 7 mitigated some of these issues as the remainder of weeks had no missing data points, with at least one countable plate for all PHBs. Within about three weeks the number of bacteria in the PHB degrading communities remained fairly stable on 303 and 205 samples at 10^4 CFU/cm² and 10^3 CFU/cm², respectively. When PE chips contained PHB degraders, they were under 10^1 CFU/cm².

AHB CFU/cm² continued to increase about 4 weeks longer than PHB degraders, although both eventually leveled out. PHB-containing chips resulted in greater counts of AHBs and PHB degraders in comparison to PE, with 303 being about 10 times more numerous than 205 (Figures 10-11). AHBs and PHB degraders were observed at a greater level on PHB chips when compared to the PE chips, at an order of magnitude of 3. This same order of magnitude is the difference between average AHBs compared to PHB degraders, with AHBs being about 1000 times more numerous in plate counts of all chips. The proportion of PHB degraders to AHBs did not increase over time. For example, week 8 chip samples showed PHB degraders as 0.358% of the AHB population on 303 and as 0.565% on 205. At week 16, the 303 samples had PHB degrading bacteria as 0.302% and 205 as 0.137% of the total AHB grown. For PE chips, the week 8 degraders were 0.022% of AHB population, while at week 16 they were 0.258%. Although this was an order of magnitude increase, there were only two points in which these degraders were observed making it difficult to observe any trends in growth. These frequencies demonstrate that while PHB is crucial for the presence of degraders, their niche role is also influenced by other factors in the marine biofilm environment.

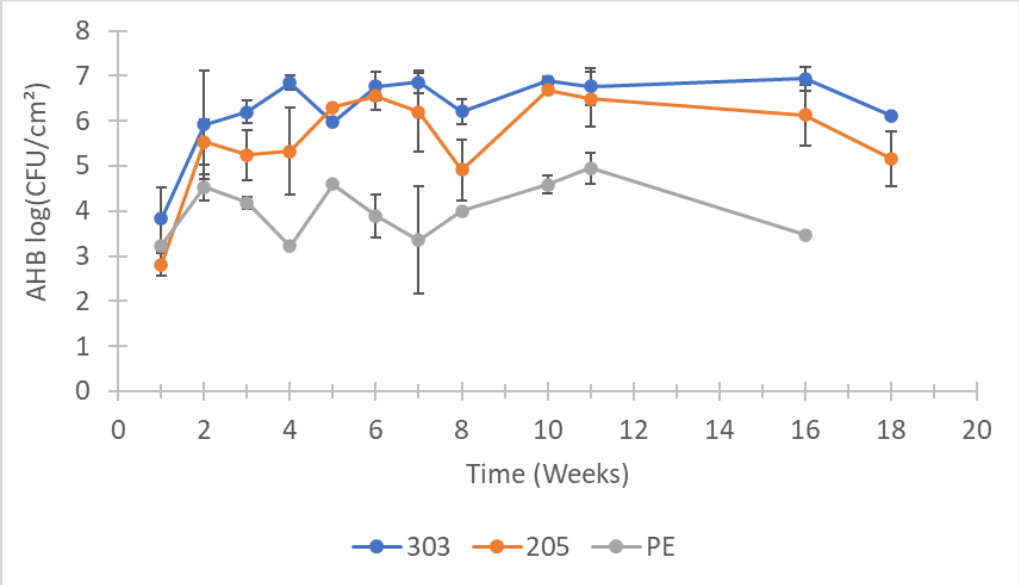

Figure 10. Total aerobic heterotrophic bacteria counted on marine broth + PHB plates, showing the log(CFU/cm²) for each plastic chip type over time. Missing data points are when no plates contained a countable number of colonies. Standard deviation utilized with triplicate results when available.

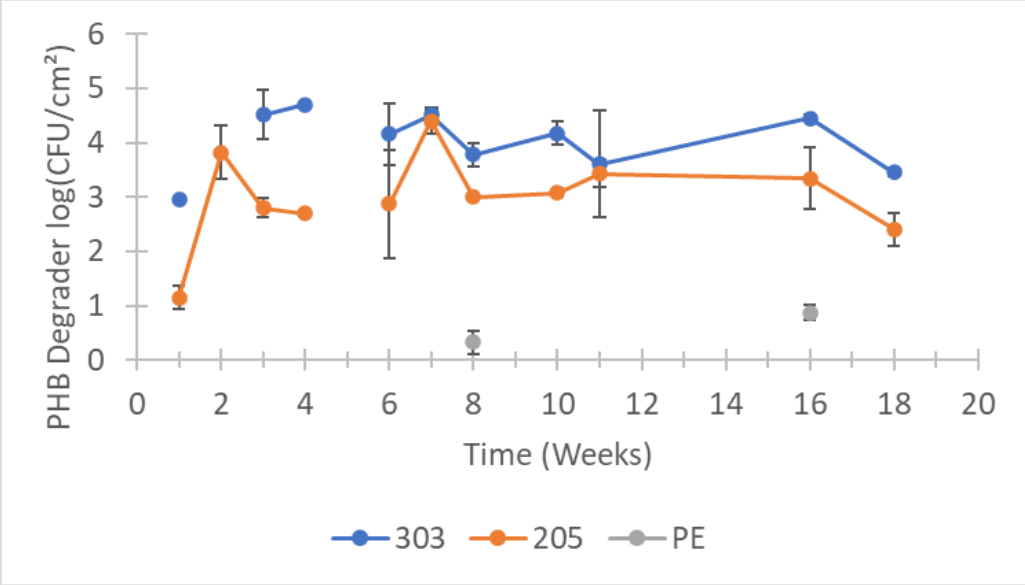

Figure 11. The amount of PHB degrading bacteria counted on either MB +PHB or limiting PHB plates, showing the log(CFU/cm²) for each plastic chip type over time. Media switch to better differentiate degraders was started in week 7. Missing data points are when no plates contained a countable number of colonies. Standard deviation utilized with triplicate results when available.

Film sample plate counts on AHBs were started on week 2, as the first week's sterile seawater was contaminated and rendered the plates uncountable. With this missing week, the 205 and PE films appeared to have leveled out by week 2 at 10^6 CFU/cm² and 10^3 CFU/cm², while 303 continued to rise steadily (Figure 12). The 303 film only lasted until week 5 and reached counts of 10^7 CFU/cm² before it was completely degraded and not available for sampling, while both PE and 205 remained stable until week 11. The standard deviations remained small throughout the weeks, and total AHBs stabilized fairly quickly on the film surface.

PHB degrader data was missing week 2 as well as week 1, as no plates of the triplicates showed any marked halo-forming colonies. Week 3 only contained one 205 plate with visible degraders, but with no other information the standard deviation could not be calculated, while 303 continued to show growth from weeks 3 to 5 before degrading completely (Figure 13). In these three weeks, 303 chip levels increased from 10^1 CFU/cm² to 10^5 CFU/cm². After week 5, 303 PHB degrader counts on new media could not be observed due to the film's complete degradation. These 303 film degraders reached levels ten times higher than 205 did throughout the time course. After week 7, degraders on 205 film appeared consistently and a couple low counts were also observed on PE with no more than 10^2 CFU/cm². The 205 degrader population appears to begin falling at around week 7, although the larger standard deviation values make decrease in degrader values more difficult to interpret.

The 303 film samples demonstrate the highest degrader proportion to AHBs, at around 10% at week 5 (Figures 12 and 13). It is also noteworthy that 205 film samples experienced a decrease in both AHB and degrader populations at around week 6 and onward. On the 303 and 205 films, PHB degraders were 10^5 CFU/cm² and 10^4 CFU/cm², which is only about two orders of magnitude less than film AHBs. Meanwhile, PE film AHBs were around 10^3 CFU/cm² and PHB degraders did not reach much higher than 10^1 CFU/cm². Without additional degrader and AHB data for 303 film it is difficult to make further comparisons or observe the same trends as 205 film.

Comparing AHBs and PHB degraders on film samples provides similar trends to those seen on chips. PHB degrading bacteria were a very small percentage of total AHBs, and were rarely ever observed on PE chips and films. The peak PE AHB counts were about the same on film and chip at about 10^4 CFU/cm². The chips with PHB generally had a slightly larger population of AHBs at 10^7 CFU/cm², while on film these values were mostly at 10^6 CFU/cm². Across both film and chip formats for 205 and 303 plates, AHBs followed similar trends, with 303 populations of AHBs being more numerous. On both film and chips, PHB degraders followed a similar trend, with 10^4 CFU/cm² on film, while chip degraders were a bit higher at around a 10^5 CFU/cm². The proportion of degraders to AHBs was about the same on film as on chip, with around two orders of magnitude difference. PHB chips generally hosted more

bacteria when compared to films, and PHB films had a decrease in CFU/cm² after week 6 while chips remained stable.

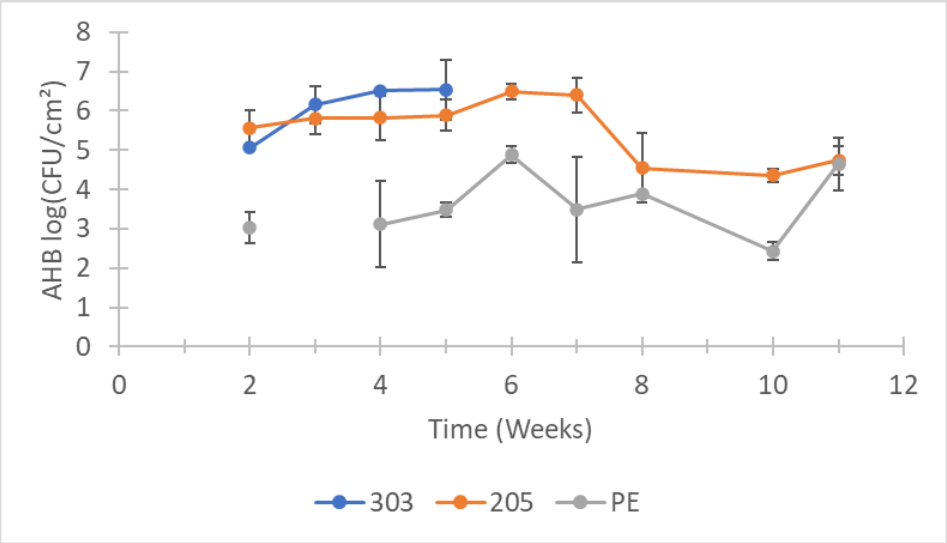

Figure 12. The total aerobic heterotrophic bacteria counted on marine broth + PHB plates, showing the log(CFU/cm²) for each plastic film type over time. Missing data points are when no plates contained a countable number of colonies. Standard deviation utilized with triplicate results when available. 303 film was completely degraded after week 5.

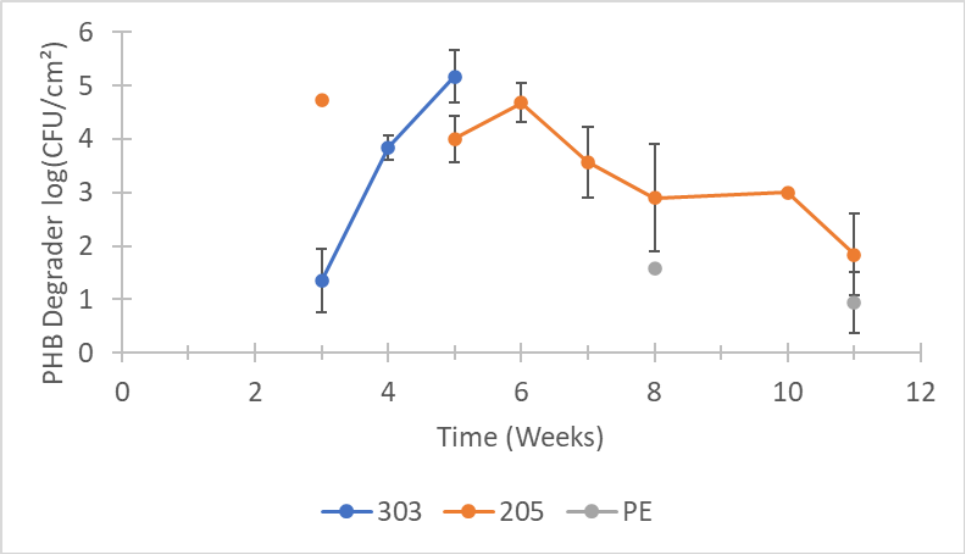

Figure 13. The amount of PHB degrading bacteria counted on either MB +PHB or limiting PHB plates, showing the log(CFU/cm²) for each plastic film type over time. Media switch to better differentiate degraders was started in week 7. Missing data points are when no plates contained a countable number of colonies. Standard deviation utilized with triplicate results when available. 303 film was completely degraded after week 5.

Crystal Violet Staining

Biomass on chip samples grew rapidly during the first 5 weeks, though increases in bound CV continued, there was a slight decrease in 303 at week 7 after reaching a peak of 70 µg/cm² (Figure 14). Biofilm on 205 chips continued to grow until reaching a peak of around 50 µg/cm². PE chip biofilm had very low growth, with small standard deviation values while never reaching above 10 µg/cm² of bound CV.

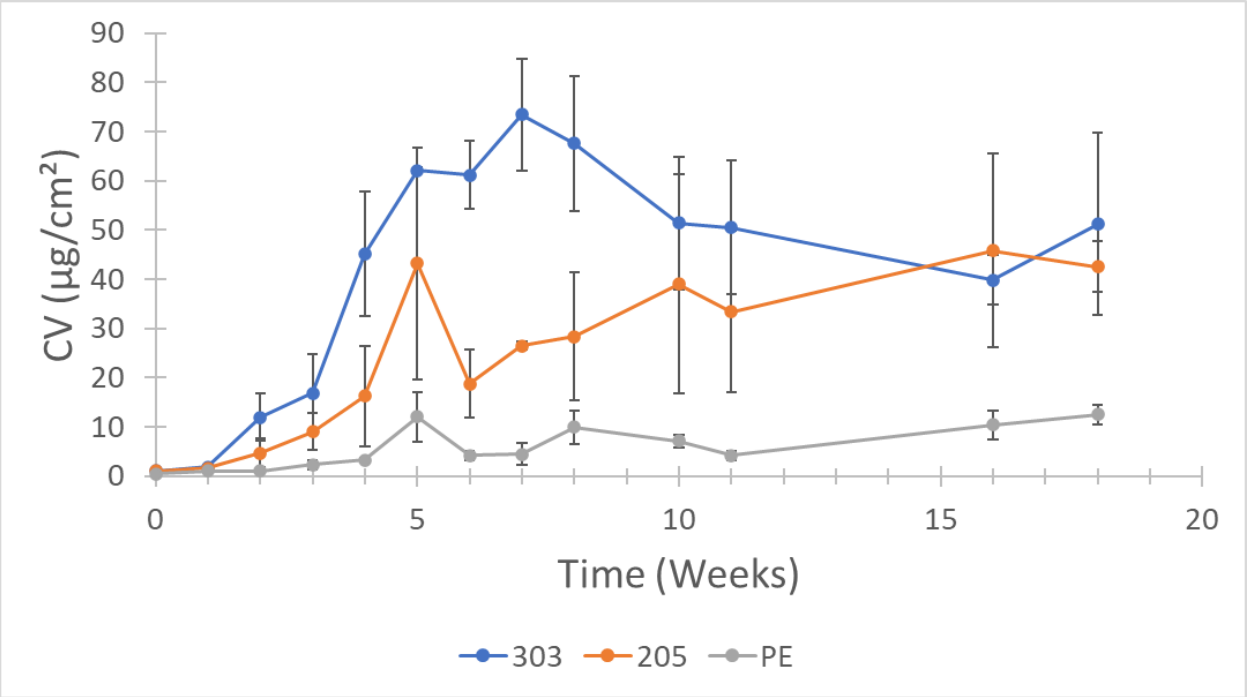

Figure 14. Bound crystal violet (µg/cm²) on chip samples over time, across 205, 303, and PE samples. Standard deviation utilized with triplicate results. Week 0 values indicate levels of CV binding to control film with no biofilm.

Bound CV on film samples followed similar trends to that of chips. The 303 chips had the highest bound CV, while 205 continued to steadily grow to surpass it (Figure 15). 303 chips at week 5 reached around 50 µg/cm² and 205 chip bound CV surpassed this at week 11 with about 60 µg/cm². At the final time point for PHB films there were large standard deviations as the film was nearly fully degraded, which may explain the great deal of variation. PE had the least amount of growth and remained close to baseline biomass levels throughout the 11 weeks at less than 10 µg/cm².

When comparing the bound CV between film and chip, the chip absorbance was consistently higher besides the final time point for 205 (Figures 14 and 15). The 205 film values did surpass 303, though this was not observed on chip values. The 303 films also did not

experience a decrease in bound CV values as seen in the chips. Both 303 and PE bound CV on chip were much greater when compared to the film counterparts, suggesting that perhaps the chips themselves provided a better scaffold for biofilm formation than the thinner films.

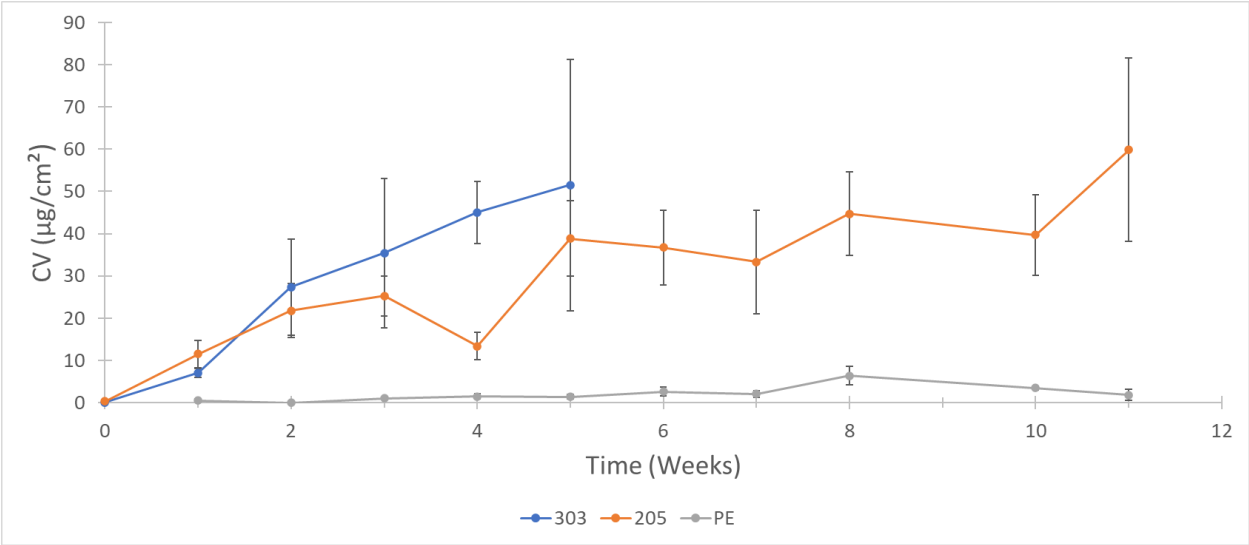

Figure 15. Bound crystal violet (µg/cm²) on film samples over time, across 205, 303 and PE samples. Standard deviation utilized with triplicate results. Week 0 values indicate levels of CV binding to control film with no biofilm.

Changes in Plastic Integrity

Though the methods for biofilm removal remained relatively consistent throughout collection, both the chip and film PHB-based plastic samples underwent significant changes throughout the sample collection. The film samples showed rapid degradation compared to the chips, with 303 films fully degraded by week 5 and 205 showing signs of wear and degradation over the time course and fully degraded by week 11 (Figure 16).

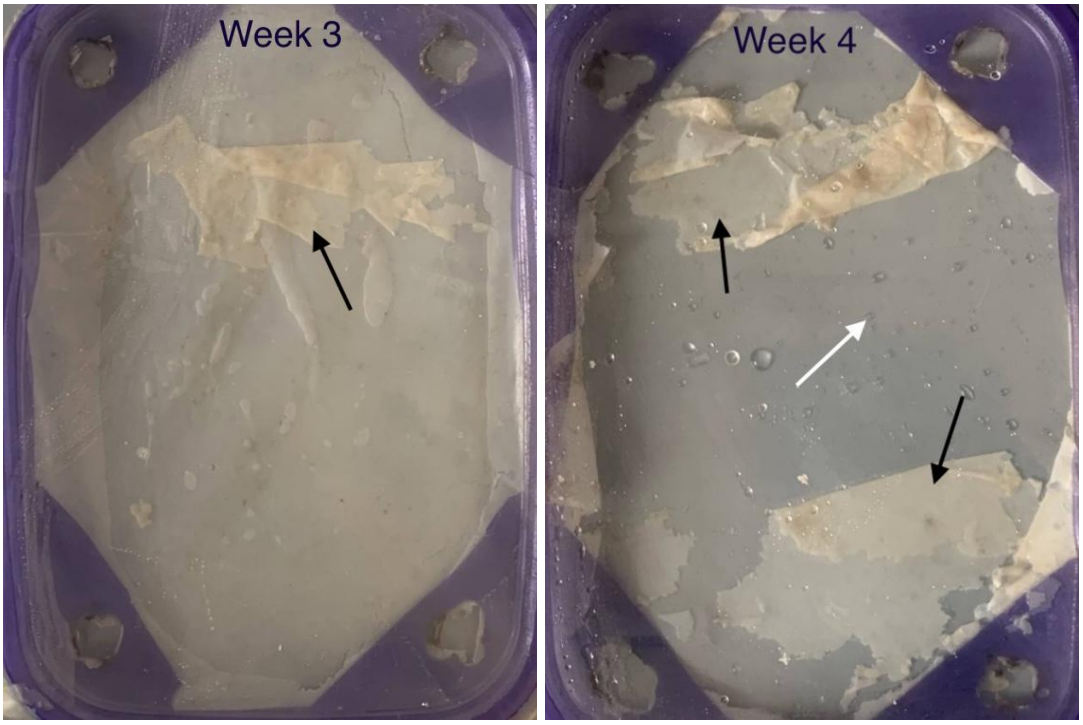

Figure 16. *Left* - Week 3, 303 film sample. Plastic shred on intact film indicated by the black arrow. *Right* - Week 4, 303 film sample. Image shows a degraded 303 sample that is almost at the point of unusability. The black arrows indicate plastic shreds and the white arrow indicates empty space.

Though the chips remained intact, there were significant observable differences in the biofilm growth and plastic integrity over time. Biofilm growth was visible with the naked eye on each plastic formulation, including PE. The biofilm scraped from 303 was dense and flaky, while biofilm from 205 chips developed into a slimy, mucoid-like layer (Figure 17, *Left*). The PE biofilm, on the other hand, remained consistently thin throughout. Over time, all the samples developed some algal growth (Figure 17, *Right*), which was gently rinsed off with 2 ml sterile seawater when processing the chips before cutting. In addition to changes in biofilm, changes in plastic consistency were also observed. Both the 205 and 303 chips became more flaky and hardened, but PE plastic consistency did not change over the entire time course.

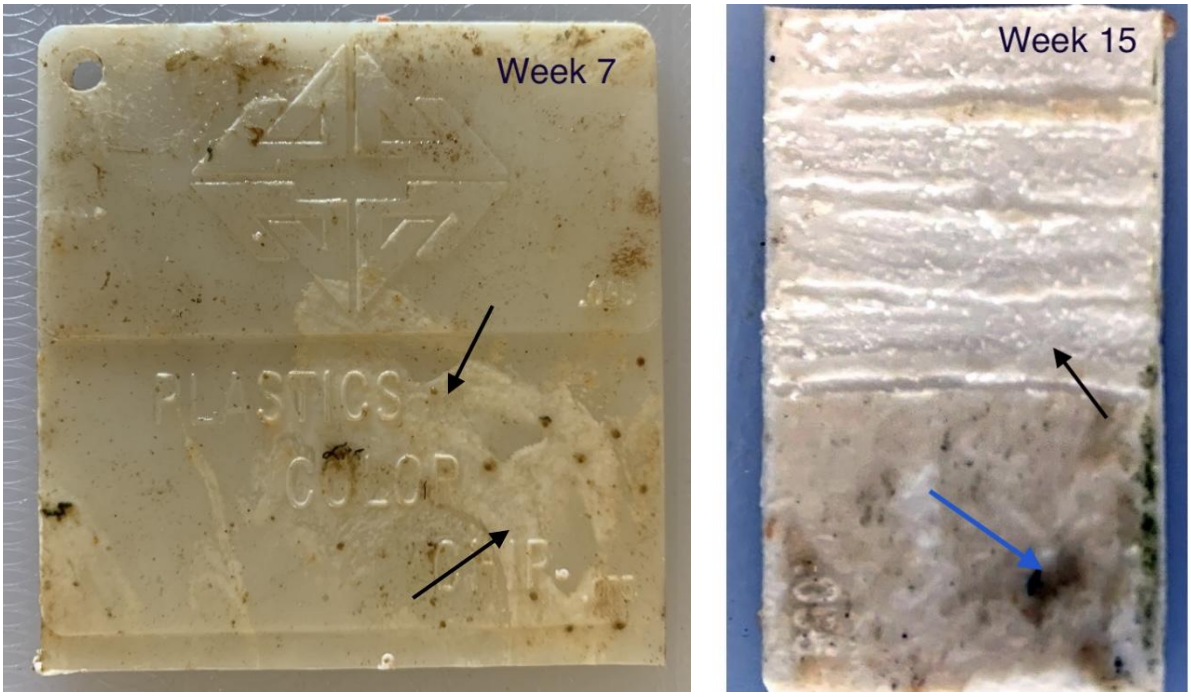

Figure 17. *Left* - 205 plastic formulation in week 7. Biofilm growth is shown as a gooey, mucoid substance covering the surface indicated by arrows. *Right* - Evidence of biodegradation when scraping biofilm off 303 formulation PHB-plastic during week 15. The rugged areas of the plastic are where the plastic was scraped off along with biofilm, as indicated by the black arrow. Algal growth is indicated by the blue arrow.

The 303 chips became so brittle that plastic began scraping off along with the biofilm (Figure 17, *Right*). These qualitative changes are representative of biofilm formation and evident plastic degradation for each of the PHB-based plastics, while PE plastic had no observable change over the entire time course. The observable process of deterioration for both 303 and 205 over 18 weeks in comparison to the decades-long degradation process for PE, demonstrates a huge reduction in the duration of breakdown for bioplastic degradation.

The breakdown of 303 film occurred within five weeks and 205 film within eleven weeks in a closed flow-through seawater system, and both showed an increase in biomass from each assay. Rapid degradation and the presence of PHB degrading microbes during plating confirm that degradation occurs in the ocean and that the PHB formulations serve as a substrate to support biofilm growth.

Conclusions

Crystal violet is recommended for biomass estimation as it is simple, time efficient, and cost effective in comparison to plating and DNA extraction. While crystal violet results reflect an increase in biofilm bound to dye over time, this may not directly correlate to the amount of

bacterial cells existing within the biofilm. Dead cells and a wide array of polysaccharide mixtures may be stained and influence the absorbance values that are gathered [9]. Therefore, crystal violet staining may not be useful for tracking cellular concentrations or specific organisms. Recommendations for further research using crystal violet would include staining larger areas to obtain better biofilm representation and using heat in the process of crystal violet removal could be beneficial for removing residual stain.

Plating for PHB degrader identification is useful to provide information on the number of degrading bacteria present and their prevalence in comparison to total aerobic bacteria, which can be estimated by plating for AHBs. Improvements for plating would include developing a low nutrient substrate to activate PHB degrading enzymes in bacteria that preferably use alternate carbon sources, this is because some bacteria would not express their PHB depolymerases in a nutrient-rich environment [5]. To better promote growth of seawater bacteria on plates, measuring the temperature of the seawater and incubating at that environmental temperature may be the most accurate representation of communities present in seawater.

DNA extraction from biofilm surfaces allows for further genetic analysis, but is not recommended for biomass estimation. The kits are expensive, the concentration measurements were variable, and the DNA extraction was the most time consuming process for each sample collection. We found inconsistencies when using the Molecular Devices Spectradrop for spectrophotometric measurements of DNA, so in future studies, a more consistent instrument would be necessary for obtaining these measurements.

Though each method had benefits and drawbacks, crystal violet staining was the most useful, consistent, and straightforward estimate for biomass. Plate counts and DNA quantification were variable, time-consuming, and expensive in comparison. Crystal violet staining assesses biofilm bound to the plastics, but plating and other DNA based studies can provide additional information about the biodegrading microbes. Improvements for this study would optimize crystal violet staining and plating assays to estimate biomass and PHB degraders without the use of DNA extraction.

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