Recyclable plastics as substrata for settlement and growth of bryozoans *Bugula neritina* and barnacles *Amphibalanus amphitrite*

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**Abstract**

Plastics are common and pervasive anthropogenic debris in marine environments. Floating plastics provide opportunities to alter the abundance, distribution and invasion potential of sessile organisms that colonize them. We selected plastics from seven recycle categories and quantified settlement of (i) bryozoans *Bugula neritina* (Linnaeus, 1758) in the lab and in the field, and of (ii) barnacles *Amphibalanus (= Balanus) amphitrite* (Darwin, 1854) in the field. In the laboratory we cultured barnacles on the plastics for 8 weeks and quantified growth, mortality, and breaking strength of the side plates. In the field all recyclable plastics were settlement substrata for bryozoans and barnacles. Settlement depended on the type of plastic. Fewer barnacles settled on plastic surfaces compared to glass. In the lab and in the field, bryozoan settlement was higher on plastics than on glass. In static laboratory rearing, barnacles growing on plastics were initially significantly smaller than on glass. This suggested juvenile barnacles were adversely impacted by materials leaching from the plastics. Barnacle mortality was not significantly different between plastic and glass surfaces, but breaking strength of side plates of barnacles on polyvinyl chloride (PVC) and polycarbonate (PC) were significantly lower than breakage strength on glass. Plastics impact marine ecosystems directly by providing new surfaces for colonization with fouling organisms and by contaminants shown previously to leach out of plastics and impact biological processes.

**1. Introduction**

We live in the Plastic Age of the Anthropocene. Plastics are ubiquitous and contribute to all aspects of modern society (Andrady and Neal, 2009; Thompson et al., 2009). Inexpensive plastics with lightweight, strong, and durable properties are molded into a vast array of products for commercial, industrial, medicinal and municipal applications (Thompson et al., 2009). Annual global plastic production increased more than 60 fold from 5 million tonnes in 1960 to 311 million tonnes in 2014 (PlasticsEurope, 2015). Globally, poor management practices result in visible accumulation of discarded plastic waste (Barnes et al., 2009; Andrady, 2011; Jambeck et al., 2015).

Plastic pollution in oceans is a major and growing environmental problem (Moore, 2008; Ryan et al., 2009; Cózar et al., 2014; Jambeck et al., 2015). Eriksen et al. (2014) estimated that there are more than 5 trillion plastic pieces weighing over 250,000 tons floating in the global oceans. Plastic pollution poses numerous hazards to marine environments and wildlife, including biological impacts of ingestion, entanglement, smothering and invasion (Barnes, 2002; Gregory, 2009), leaching of toxic and steroidogenic compounds, and adsorption of persistent organic pollutants (POPs) from surrounding environments (Teuten et al., 2007, 2009; Cole et al., 2011; Rochman et al., 2013b; Gall and Thompson, 2015). Moreover, marine plastics are ubiquitous and unprecedented substrata for rafting organisms (Barnes, 2002; Barnes and Milner, 2005; Bravo et al., 2011; Gall and Thompson, 2015; Kiessling et al., 2015). Assemblages associated with plastics exceed those on other floating substrates such as algae, pumice and wood (Thiel and Gutow, 2005; Goldstein et al., 2014; Gall and Thompson, 2015). Microorganisms, hydrozoans, bryozoans, and barnacles are the most common taxa on floating plastics (Winston, 1982; Aliani and Molcard, 2003; Barnes and Milner, 2005; Zettler et al., 2013; Gall and Thompson, 2015; Kiessling et al., 2015). Goldstein et al. (2014) reported 95 taxa in rafting communities from 11 phyla on
plastics from the North Pacific. Settlement and development of organisms can alter the buoyancy and degradation of plastics (Ye and Andrady, 1991; Bravo et al., 2011; Muthukumar et al., 2011) and consequently the fate and ecological impacts of marine plastics (Barnes, 2002; do Sul and Costa, 2014). Floating plastics transport their assemblages to new ecosystems like the Pacific and Atlantic subtropical gyres (Carson et al., 2013; Zettler et al., 2013; Goldstein et al., 2014), or the Antarctic and Arctic oceans (Barnes and Milner, 2005). Organisms colonizing plastics threaten global biodiversity (Barnes, 2002). Thus, interaction of fouling organisms and marine plastics is a growing environmental problem.

Plastics are formed from long chain polymeric molecules and during plastic manufacture, plasticizers (e.g., Phthalates), catalysts (e.g., Sb, Cd, Organotins), sacrificial oxidants (e.g., Phosphites), flame retardants (e.g., PBDEs), and other chemical compounds are added to maintain and improve plastic properties (Andrady and Neal, 2009; Talsness et al., 2009; Halden, 2010). Chemicals leach from plastics into the environment (e.g., Oehlmann et al., 2009; Teuten et al., 2009; Halden, 2010), and may impact on aquatic animals.

Using laboratory-reared barnacle larvae, Li et al. (2016) reported measurable toxicity and inhibition of settlement in leachates from new plastics from all 7 recycle categories. Between 50 and 150 different chemicals were found in 24 h seawater leachates of new plastics from all 7 recycle categories. Between 50 and 150 measurable toxicity and inhibition of settlement in leachates from plastics into the environment (e.g., Oehlmann et al., 2009; Teuten et al., 2009; Halden, 2010), and may impact on aquatic organisms colonizing plastics threaten global biodiversity (Barnes, 2002). Thus, interaction of fouling organisms and marine plastics is a growing environmental problem.

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Using laboratory-reared barnacle larvae, Li et al. (2016) reported measurable toxicity and inhibition of settlement in leachates from new plastics from all 7 recycle categories. Between 50 and 150 different chemicals were found in 24 h seawater leachates of new recycle plastics. Most of these compounds could not be identified because they were absent from existing databases. Toxicity was measurable in leachates for the survival of barnacle nauplii and strongly related to the critical surface energy. Settlement inhibition was even detected at the lowest concentration (0.004 m2/L) in leachates. As plastic leachates caused adverse impacts on barnacle larvae in the lab, here we extend the study of Li et al. (2016) to compare barnacle settlement on plastics in the field, and the growth and development of barnacles on plastics in static conditions in the laboratory. We quantify the growth, mortality and breakage strength of barnacles on the 7 standard categories of recyclable plastics. Furthermore, we used bryozoan Bugula neritina to compare their larval settlement in the lab and in the field, with the aim to assess the impacts of plastics on these globally common marine foulers. We cannot culture bryozoans in the quantities required for this experiment and they grow as bushy colonies with one attachment point. Thus we did not attempt to culture bryozoans for growth, mortality and adhesion.

2. Materials and methods

2.1. Material preparation

All 7 recyclable categories of new plastic products were purchased in common, commercially available forms: polyethylene terephthalate (PET), high density polyethylene (HDPE), polyvinyl chloride (PVC), low density polyethylene (LDPE), polypropylene (PP), polystyrene (PS), and polycarbonate (PC). The commercial products had the embossed recycle identification code designed by the Society of Plastics Industry (SPI). Plastic products were cut into 8 × 10 cm panels for settlement in the field and 7 × 15 cm panels for barnacle growth in the laboratory. In experiments each plastic where: W100 = drop spread at 100% HPLC grade water (mm), W80 = drop spread at 80% HPLC grade water in HPLC grade methanol (mm), etc; 8 = number of solvent concentrations used; 4 = minimum possible drop measurement in millimeters; 16 = maximum range of drop sizes in millimeters.

Adult barnacles of Amphibalanus ampitrite used for brood stock were collected from the dock of Duke Marine Laboratory (Beaufort, NC, USA). Larval rearing, settlement, and juvenile barnacle growth in the laboratory were done according to the methodology described in Rittschof et al. (1992a) and Holm et al. (2005). Laboratory settlement of Bugula neritina was done after Rittschof et al. (1988). Bryozoan colonies brooding larvae were collected in the morning of each trial day from floating docks. Colonies released larvae in the laboratory soon after collection.

2.2. Bryozoan and barnacle settlement in the field

Barnacle settlement assays were carried out in the field in October 2014 and May 2015, and bryozoan settlement was conducted in May 2015. Each assay was for four days on a floating dock and based on prior studies (Rittschof et al., 1989; Roberts et al., 1991). Three replicates were used for each recycling category of plastic and glass reference standards. Arrays were arranged randomly on a vexar screen (11 mm mesh size) and panels were secured to screen mesh with rubber grommets. The screen was tied to a 50 × 70 cm PVC frame. The frame was hung horizontally and exposed all panels at the same depth of 20 cm from the surface on the floating dock (Rittschof et al., 1992b). Bryozoan and barnacle settlement on panel surfaces were monitored after immersion for 24, 48, 72 and 96 h, respectively. For examination, each panel was removed carefully from the frame and the number of settled bryozoans and barnacles was counted under dissecting microscope. The panels were put back on the screen and hung on the raft until the end of test interval.

2.3. Bryozoan settlement assays

For bryozoan settlement assays in the laboratory, plastic
products were cut into $2 \times 2$ cm pieces. Clean glass slides were baked at 500 °C for 4 h as controls. Three pieces of each material were put on the bottom of a 10.5 cm diameter glass fish bowl. The bowl was lined with moist paper toweling and covered with another finger bowl to prevent evaporation of the water drops containing larvae. Bryozoan colonies from the dock were placed in a 4 L glass tank filled with aged filtered seawater. Under artificial light shock, colonies released bryozoan larvae quickly. The photo-positive larvae were collected at the rim of the holding tank, and immediately used in assays. Ten active bryozoan larvae in approximately 1 ml aged, filtered seawater were placed carefully on each testing surface. Settlement was confirmed not to be affected by the water drops as the spread areas of drops were similar on different plastics (Li et al., 2016). The bowls were incubated at 25 °C and settlement and metamorphosis of bryozoans were recorded after 4 h. The assays were repeated and data were combined for statistical analyses.

2.4. Barnacle culture

Barnacle growth, mortality and breakage force assays were conducted in the laboratory. Barnacle larvae from field-collected adults were reared in mass culture to cyprids with microalgae Skeletonema costatum at 28 °C (Rittschof et al., 1992a). Cyprids were collected by a sieve cascade after 4 days, and held at 6 °C for 3 days until use. Settlement was conducted by placing cyprids in water drops on the test surfaces, and then incubated at 28 °C and with a 12:12 light-dark cycle using fluorescent lamps for 3 days. Barnacles settled on each test panel were reared separately in static conditions in a container with 725 ml of aged filtered seawater. Barnacle spat were fed daily with 40 ml of microalgae Dunaliella tertiolecta; after 6 weeks 10 ml of concentrated newly hatched brine shrimp Artemia spp. were added to the diet. Seawater in the containers was changed twice per week. In order to prevent overgrowth, some barnacles were removed during rearing. The same density was maintained on each panel. Growth and mortality of barnacles was examined under an Olympus SZX7 dissection microscope every week. Photos were taken for each panel and the basal diameter of barnacles was measured with software Image J (versions 1.48). After examination, dead barnacles were removed with a dissecting needle and mortality per week determined. Growth was estimated on the mean basal diameter of total barnacle individuals each week on each type of substrata. Mortality was calculated as the proportion of dead barnacles each week on each type of substrata.

2.5. Breaking force of barnacle side plates

Forces for breaking barnacles were measured by quantifying the attachment tenacity in shear with a modification of the ASTM D 5618-94 method (Anonymous, 1997; Rittschof et al., 2008). After eight weeks of rearing, barnacles for measures were >6.0 mm in basal diameter. Panels with barnacles were fixed on a testing table. Using a hand-held force gage (Shimpo MF-5lb, #93953-10), the parallel force to remove the barnacle from the base was measured. However, the side plates of all barnacles tested broke before the base plates released. Breaking force was recorded for five barnacles, chosen randomly, on each panel. The basal diameter of the barnacle was measured and the basal area estimated with the standard methods (Anonymous, 1997). Breaking force was standardized to the size of the barnacle by dividing the measured force required to break barnacles by the basal area.

2.6. Statistical analyses

Data from the plastics and glass controls in each assay were compared. Normality and homogeneity of variances were tested with Shapiro-Wilk and Levene median, respectively. When data complied with the requirements, one-way analyses of variance (ANOVA) were conducted, and if significant effects were found, Tukey’s post-hoc test was used to determine the differences between plastics and glass controls. Otherwise, non-parametric Kruskal-Wallis tests were applied. Relationships between surface energy (SHM) of plastics and settlement of barnacles/bryozoans were analyzed using Spearman’s correlation test. Statistical analyses used the SPSS statistical package. In all cases, significant difference was set at $p < 0.05$.

3. Results

3.1. Bryozoan settlement

_Bugula neritina_ settled in high numbers on most of the plastic surfaces in the field (Fig. 1A). Settlement increased gradually over the four-day interval. Significant differences compared to glass were found for PVC, PP, PC, PET and PS. PVC surfaces had more than 600 individuals of _B. neritina_ on the $8 \times 10$ cm panels after 96 h immersion. However, Glass and HDPE surfaces had less than 100 individuals on $8 \times 10$ cm panels. In lab assays (Fig. 1B), bryozoan settlement in water drops on different substrata was similar to the results in the field (Fig. 1A), exhibiting higher settlement on plastics than on glass. PP surface had highest settlement in the lab and PVC
had highest settlement in the field. Bryozoan settlement was not correlated with the surface energy (SHM) of plastics in field or lab assays (Fig. 2).

3.2. Barnacle settlement

Barnacle settlement in the field was highest on glass and significant differences were observed between all plastics and glass controls after 24 and 48 h. In October 2014, no barnacles settled on plastic surfaces after 24 and 48 h, and after 72 h a small amount of barnacles settled on HDPE, LDPE and PVC surfaces (Fig. 3A). After 96 h, barnacles settled on all plastics except PC and there was higher settlement on LDPE and HDPE than on the other plastics.

In contrast, more barnacles settled on plastic surfaces in May 2015 (Fig. 3). Barnacles settled on PET and PVC after 24 h and on six of seven categories of plastics after 48 and 72 h (Fig. 3B). After 96 h, all of plastics had barnacle settlement and PVC surfaces had the largest number of barnacles. Significant differences compared to glass controls were found for all plastics after 24, 48, and 72 h. Barnacle settlement was not correlated with surface energy (SHM) of plastics (Fig. 4).

3.3. Barnacle growth

Barnacles grew on all plastic surfaces (Fig. 5A). After the first two weeks, barnacles on all plastics except HDPE were significantly smaller than on glass controls. However, barnacles on plastics grew as well as on glass after 4 weeks and 8 weeks. At the end of assays, although all barnacles were statistically similar in sizes, barnacles on PVC surfaces were the largest and barnacles on PS surfaces were the smallest. Barnacles reached sexual maturity at about 5 mm in basal diameter.

3.4. Barnacle mortality

Mortality per week increased gradually from week-1 to week-4, and declined for all plastics and glass controls in week-8 (Fig. 5B). After one week, mortality was <4% on all plastics and glass. After four weeks, weekly mortality was the highest (10.9%) on LDPE and the lowest (5.6%) on PP surfaces. After eight weeks, barnacles continued to die in low percentage on PVC and PC surfaces. No significant differences in mortality were found between plastic and glass control surfaces (Fig. 5B).

3.5. Barnacle breakage

Barnacles were firmly attached to surfaces and broke with attempted push off. Forces for breakage of barnacles after 8-weeks of rearing on PVC and PC were significantly lower than breakage forces on glass substrata (Fig. 6). Additional differences were significant between the following plastics: PVC and PET, PVC and PS, PC and PET, PC and PS (Kruskal-Wallis tests, p < 0.05).

4. Discussion

In this study, plastics from seven recycling categories were documented as substrata for barnacle and bryozoan settlement. Barnacle and bryozoan settlement in the field were consistent with laboratory tests (Li et al., 2016), showing significant differences between plastics and glass controls. Barnacle settlement was different than bryozoan settlement on all plastic surfaces in the...
Barnacle settlement varied in season and substrata in the field. Compared to May 2015, barnacle settlement in October 2014 was relatively low. This result is related to larval availability in Beaufort, NC, as larvae are more abundant in spring and summer than in late autumn and winter. For different substrata, few barnacle larvae settled initially on plastics, but after 96 h settlement was similar on all surfaces. This implies that barnacle larvae could discriminate the newly immersed plastics, but lost this capability after 4-days of exposure of the plastic in the field. This observation is in line with a variety of studies on critical surface energy over time (e.g., Baier, 1970; Rittschof et al., 1988; Rittschof and Costlow, 1989; Roberts et al., 1991; Gerhart et al., 1992). Factors that affect larval settlement are very complex. In this study for example settlement was statistically related to surface energy and as in previous studies barnacles settled on more hydrophilic surfaces (e.g., Rittschof and Costlow, 1989; Roberts et al., 1991; Gerhart et al., 1992). In contrast, bryozoans settled in very high numbers on most plastics in the field with much less settlement on glass and high density polyethylene (HDPE). The result for glass is consistent with previous observations of settlement in relation to critical surface energy (e.g., Rittschof et al., 1988; Rittschof and Costlow, 1989; Roberts et al., 1991; Gerhart et al., 1992). However, the HDPE result is one that we cannot explain at this time. We speculate that something leaching from the HDPE interferes with bryozoan sensory capabilities. Bryozoan settlement was not related to surface energy of plastics. All plastics were similar but very different than glass (Rittschof and Costlow, 1989; Roberts et al., 1991; Gerhart et al., 1992). The commercial plastics were of lower surface energy than expected for scientific grade plastics (Li et al., 2016). We attribute this to additives added to plastics during production. The rank orders of different categories of plastics for bryozoan and barnacle settlement were variable between the field assays and the lab tests (Li et al., 2016). To date, influences of plastic substrata on larval settlement have not been very clear. In the field, larval settlement is affected by physical, chemical and biological factors, such as water temperature, salinity, flow, substratum, biofilm, and larval recruitment (e.g., Rittschof et al., 1984; Holm, 1990; Clare et al., 1992a, 1992b; Roberts et al., 1991; Faimali et al., 2004; Hadfield, 2011). We speculate that the difference between laboratory and field tests for plastics may be caused by the biofilms. The development of biofilms is a critical factor for initial settlement of...
invertebrate larvae (Wieczorek and Todd, 1998; Faimali et al., 2004; Hadfield, 2011). For some macrofoulers such as tube worms bacterial films are required (Hadfield, 2011). For others such as barnacles biofilms are not required and either have minimal effect or inhibit settlement (Maki et al., 1988; Maki et al., 1990, 1992; Holstrøm et al., 1992). Recently, in other parts of the world specific bacteria have been implicated in the induction of barnacle settlement (Qian et al., 2003, 2007). The macrofoulers we studied conform more to the larval availability than to the successional model for both barnacle and bryozoan settlement (Maki et al., 1989; Clare et al., 1992b; Gerhart et al., 1992). The biofilm issue awaits future research on different plastic types. However, based on results from the prior observations (Li et al., 2016) and here, the main factors that affect barnacle and bryozoan settlement are the properties of plastic surfaces, such as toxicity, other chemical functionalities, and surface energy. This is important as the abundant and wide distribution of plastics in the oceans provides new surface habitats for fouling organisms.

Barnacle growth on plastics in laboratory conditions was initially slightly retarded but became similar after 4 weeks to growth on glass. Barnacles were cultured past the time they become sexually mature and this might explain why all barnacles were still attached after 8 weeks. When barnacles reach sexual maturity growth slows as energy is shunted toward development of gonads and gametes. At the beginning of assays barnacles were smaller on plastics and showed higher mortality in plastic treatments. We hypothesize the effects may be related to the toxicity from plastics which can be detected in static conditions. In our earlier findings, many compounds are released in the leachates of plastics after 24 h exposure in seawater and inhibit barnacle settlement (Li et al., 2016). Plastic leachates are documented to cause adverse biological effects on aquatic animals like crustaceans Daphnia magna (Lithner et al., 2009, 2012), gastropods Potamopyrgus antipodarum (Wagner and Oehlmann, 2009), and echnioids Lytechinus variegatus (Nobre et al., 2015). The toxicity may be from catalysts, additives and non-polymerized monomers used in manufacturing (Oehlmann et al., 2009; Teuten et al., 2009). Toxic effects increase concerns about plastic pollution in our oceans (e.g., Teuten et al., 2009; Cole et al., 2011; Rockman et al., 2013a).

Adhesion may be a crucial factor for barnacle attachment and growth on plastics. In marine environments plastics are exposed to flow, waves, sunlight and sediment, resulting in oxidation, degradation, fragmentation and abrasion (Sudhakar et al., 2007; Artham and Doble, 2009; Bravo et al., 2011). Barnacle colonization on plastic surfaces may change the processes of degradation and sinking of marine plastics (Sudhakar et al., 2007; Muthukumar et al., 2011). In this study, breakage strength of barnacles varied on different substrata, ranging from 0.18 to 0.35 MPa. Holm et al. (2009) reported removal stress of barnacles was low on two silicone films, Veridian (0.09 MPa) and Silastic T-2 (0.14 MPa). Differences in barnacle adherence are mainly related to surface properties (Holm et al., 2005, 2009; Kamino, 2013; Raman et al., 2013) and compounds leaching out of the surfaces that impact the glue curing of barnacles (Rittschof et al., 2011). On silicone coatings the cement of many barnacles is a white, thick, and rubbery mass compared to a thin, transparent, and rigid film formed on other substrata (Kavanaugh et al., 2003; Wiegemann and Watermann, 2003; Wendt et al., 2006; Raman et al., 2013). A consequence of their weak adhesion on silicone coatings enable barnacles to be easily detached (Kavanaugh et al., 2003; Holm et al., 2005; Rittschof et al., 2008; Raman et al., 2013). In this study, results were quite different because all barnacles broke when we tried to push them off the surface. We interpret the differences in breakage strength due to completely cured glue as leachates from the plastics impacting barnacle physiology. Barnacles on PC and PVC need significantly lower breakage strength than those on glass. We speculate that in static rearing conditions, molecules that mimic mineralocorticoids in leachates enter barnacles, impact their physiology and alter calcification (Li et al., 2016). Polycarbonate, one of the two plastics resulting in weak shell plates are well known to leach estrogenic molecules (Talsness et al., 2009; Teuten et al., 2009). These molecules could mimic other steroids as well. PVC polymers are known with the presence of residual vinyl chloride monomers and other substances (e.g., Pb, Cd, dibutyltin dilaurate, phthalates), which have steroidogenic activity as well as toxicity for adhesion and growth of barnacles (Andrady, 2003; Teuten et al., 2009).

Here we studied the relationship between plastic substrata and macro-fouler settlement and growth. In marine environments, colonization by sessile organisms is variable on different categories of plastics (Winston, 1982; Bravo et al., 2011; Muthukumar et al., 2011), and plastics collected from different sites (Barnes and Milner, 2005) and different times (Sadhakar et al., 2007, Muthukumar et al., 2011). This phenomenon is partially explained by the theory provided in Clare et al. (1992b) in which settlement is dependent in a complex way on the availability of propagules in the water column and in part on critical surface energy which moves toward a common level for all surfaces after about 3 days in the water and in part on the chemistry of the surface. If settlement is rapid, the surface energy is a crucial factor when materials first are immersed in seawater, but if settlement is slow the factors for settlement will be complex. The oxidative chemistries of seawater combined with UV light further complicate the understanding of the processes impacting plastic surfaces in ocean environments.

An important consequence of plastics in marine environments is that they are settlement substrata for sessile organisms and due to their high persistence at the sea surface permit long distance dispersal by rafting (Winston, 1982; Barnes, 2002; Barnes and Milner, 2005; Kiesling et al., 2015). For example, the barnacle species Elminius modestus (Barnes and Milner, 2005), Dosisma fascicularis (Ryan and Branch, 2012), Solidobalanus fallax (Southward et al., 2004), and Perforatus perforatus (Rees and Southward, 2009), are documented as exotic invasive animals on floating plastics from global oceans. Invasive species are a threat to global biodiversity (Barnes, 2002). Therefore, the hazards of different types of plastic as substrata for marine organisms should be closely considered in assessment of ecological impacts of plastic waste at sea.

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