Title: Plastic alters biofilm quality as food resource of the freshwater gastropod *Radix balthica*

Authors: Alexander T. L. Vosshage[1,2], Thomas R. Neu[3], Friederike Gabel[1]

[1] Institute of Landscape Ecology, University of Münster, Heisenbergstraße 2, 48149 Münster

[2] present adress: Helmholtz Centre for Environmental Research GmbH – UFZ, Brückstraße 3a, 39114 Magdeburg

[3] Helmholtz Centre for Environmental Research GmbH – UFZ, Brückstraße 3a, 39114 Magdeburg

Abstract

High amounts of plastic debris enter and accumulate in freshwater systems across the globe. The plastic contamination of benthic habitats in lakes and running waters poses a potential threat to freshwater ecosystems. This study investigates the effects of plastic on two trophic levels of the aquatic food web: primary production, i.e. epiplastic biofilm, and primary consumption, i.e. a benthic invertebrate grazer. Two plastic types, Polymethyl methacrylate (PMMA) and Polycarbonate (PC), and glass (control) were used as substrata for natural biofilm establishment. PMMA and PC are e.g. intensively used in the automobile, construction and electronical industry, in cosmetics (PMMA) and CDs and DVDs (PC). These biofilms were fed to the freshwater gastropod *Radix balthica* (Linnaeus 1758) in a laboratory grazing experiment. Biofilm structure and composition were observed using confocal laser scanning microscopy before the grazing experiment. Sublethal effects on *R. balthica* were observed measuring consumption of biofilm and growth rates. The biofilm composition on PMMA significantly differed compared to PC and glass. The grazing experiments showed limited biofilm consumption and lower growth rates of *R. balthica* in both plastic treatments. Concluding, plastic in freshwaters has a direct effect on the primary production and an indirect effect on higher trophic levels.

Keywords: plastic, freshwater, benthic grazer, benthic biofilm, trophic interaction

Introduction

Plastic debris is present in all habitats of marine and freshwater ecosystems[1-4]. It is found across the globe,[5] even in remote areas,[6,7] either as macroscopic fragments or microplastic particles.[8,9] Plastic accumulates in these systems because of its persistence in the environment.[10,11] Negative effects of plastic debris on many marine organisms were shown in several studies, including reduced growth, reduced reproduction, effects on the endocrine system, or even higher mortality rates.[12,13] In contrast, effects on freshwater organisms have been investigated to a much lesser extend but negative effects are expected likewise.[9,14] One concern is the effect of plastic on freshwater food webs,[15,16] since studies have shown direct ingestion of plastic particles by freshwater fauna[7,17-19]. The effect of directly ingested microplastic particles on freshwater organisms has recently been investigated for taxa at all trophic levels, mostly with negative impacts on life history traits (reviewed in Eerkes-Medrano et al. 2015, Anderson et al. 2016[5,14]). Nevertheless, some studies did not find any impacts on organisms through direct ingestion of plastic particles.[20,21]

Apart from direct ingestion, indirect mechanisms might pose a threat to biota as well. Leaching of additives from plastics[22] or adhesion of persistent organic pollutants (POPs) to plastic surfaces[23] may result in higher exposure of toxic substances to freshwater organisms. Many of these plastic associated additives and pollutants, e.g. Bisphenol A, are prominent as endocrine disrupting substances.[24]

When a clean solid substratum enters aquatic habitats, a conditioning film covers the substratum surface within seconds.[25] In a second step a microbial biofilm establishes on its surface within minutes to days.[26] Typically the epilithic biofilm plays an important role in aquatic ecosystems. Aquatic nutrient cycling is influenced by biofilm processes[27] and biofilm builds up the habitat for benthic meiofauna.[28] Furthermore, the aquatic biofilm is the basis of the benthic food web and serves as an important food source for higher trophic levels, like invertebrates (e.g. grazers, scrapers and gatherers) and fish.[29-31] First

studies indicate that biofilms on plastic surfaces have a distinct microbial composition differing from natural substrata and surrounding waters.[32,33] Thus, alteration in biofilm composition caused by plastic as substratum may affect the nutrient availability for benthic primary consumers. However, effects of epiplastic biofilms on higher trophic levels are virtually unknown.[34]

The benthic invertebrate *Radix balthica* (Linnaeus 1758) grazes on biofilms present on various surfaces[35] and is considered as an important link for the functioning of benthic freshwater food webs.[36] *R. balthica* belongs to the pulmonates, has a broad environmental tolerance, is widely distributed in different waterbodies, prey for fish and leeches,[37] and it is an important component of aquatic ecosystems, and therefore an ecologically relevant test organism.[38]

This study investigates the effects of plastic on a freshwater food chain, covering two trophic levels: The primary production, i.e. 'epiplastic' biofilms, and the primary consumers, i.e. the benthic grazer *R. balthica*. Therefore, the biofilm composition of two different plastic types were analysed and compared with a control. In addition, sublethal effects (feeding rates and growth) of these biofilms on the grazer *R. balthica* were studied to determine differences in the food quality of the biofilms. We hypothesized that (1) biofilms differ in structure and composition according to substratum type, and that (2) biofilms grown on plastic substrata provide a lower food quality for the grazer *R. balthica*.

Material & Methods

Biofilm

Establishment of biofilms took place on substratum slides (colonisable area 28 x 48 mm) of different materials exposed for 49 days in a shallow, highly productive lake (Lake Aasee, Münster; N 51° 56.49'; E 7° 35.49'). Test substratum materials were polymethyl methacrylate (PMMA) and polycarbonate (PC), verified via FTIR-analyses by Wessling GmbH, Altenberge, Germany (FTIR-spectra: Supplemental material

S1). Glass served as control substratum, as recommended by Danilov & Eklund,[39] because it is inert in nature and proved its suitability as sample substratum.[40,41] Both plastic types were transparent and with a plain surface, excluding differences in physical properties of the substrata.

Sample slides were attached in two holding frames to realize vertically orientated exposure in the water column. Each frame contained seven rows with eleven sample slides, respectively. The frames were located at the northern shore of the lake, freely floating in the trophogenic layer facing southwards on August, 3rd 2015. The frames were transported to the laboratory in lake water one day prior to experiment start. Slides for subsequent application in the grazing experiment were stored in sterile filtered lake water at 4 °C in darkness. Biofilms were analysed before experiment start investigating five slides per substratum. Slides for biofilm analysis were fixed in paraformaldehyde (concentration: 3.5 %). Confocal laser scanning microscopy (CLSM) was applied to classify biofilms. The microscope TCS SP5X (Leica, Wetzlar, Germany) available was equipped with an upright microscope and a white light laser. The system was controlled by the software LASAF 2.7.3.0723 (Leica). Biofilm sample slides were stained with the fluorochromes SYBR®Green (ThermoFisher Scientific Inc.; Waltham, USA) and the fluor-labelled lectin AAL-A568 (Vector Laboratories, Burlingame, USA) to mark bacteria and the lectin-specific glycoconjugates of the extracellular polymeric substances (EPS), respectively.[42] Four channels were recorded using a 25x NA 0.95 water immersible objective lens: reflection (excitation 500 nm, emission 495 – 505 nm), bacteria (ex. 500 nm, em. 515 – 560 nm), glycoconjugates (ex. 575 nm, em. 590 – 650 nm) and algae (ex. 633 nm, em. 620 – 720 nm). The intensity of the emitted signals is received and translated into 8-bit data. Three of the four recorded channels were used for data interpretation, excluding the reflection channel. Each biofilm slide was scanned at five randomly chosen locations. The dimension of a scanned area was 620 x 620 µm (xy-dimensions). Scan steps in z-direction were either 1 or 2 µm, according to biofilm thickness. By stacking each scanned layer a 3D data set is created which can be used for volume calculations and visualizations. Volumes of the biofilm components were

quantified via digital image analysis using JImageAnalyzer 1.4 (UFZ Leipzig-Halle, TU Braunschweig, TU München). For each treatment, manually chosen thresholds were adjusted to turn 8-bit information into binary data to filter out signal detection noise. Subsequently, pixels per scanned layer are counted for volume calculations. Via calculation of voxel size and normalization to scanned area, biofilm volume per area ($\mu m^3\ \mu m^{-2}$) was derived for each biofilm component separately. Biofilm thickness was derived by the height of the scanned spots. Visualizations were generated with Imaris 8.1.2 (Bitplane AG, Zürich, Switzerland).

Grazing experiment

Individuals of *Radix balthica* (L.; 1758) were hand collected from the stream Hessel upstream of a modified weir with marginal flow velocities at the shores (N 51° 58.25'; E 7° 55.04') on September, 3rd 2015. After immediate and careful transportation to the stocking aquarium in the laboratory, gradual acclimatization to laboratory conditions lasted for two weeks. Lettuce was offered ad libitum as food. Individuals with approximately equal shell lengths of 8.96 (± 1.18) mm were isolated from the stocking aquarium and starved for two days prior to experiment start.

The snail grazing experiment lasted for five weeks. Low form glass beakers (200 mL) filled with 150 mL dechlorinated tap water served as experiment aquaria. Five aquaria were set up per tested substratum. Each aquarium contained three snails at experiment start, so that a total of 45 *R. balthica* (15 per substratum) were tested. Dead snails were removed immediately from the aquaria. Single biofilm slides were placed at the bottom of the aquaria to allow grazing. After five days of grazing, biofilm slides were replaced by slides from storing. Every three days, half of aquarium water was renewed to maintain semi-continuous water exchange. Ambient conditions were set to 18.3 ± 0.4 °C water temperature with an artificial lighting (wavelength spectrum of natural sun light) of 16 h light/8 h dark cycle. Shell size was

measured with a digital slide calliper at experiment start, and every third of experiment duration. Growth rates were separately determined for three phases to account for the case that sub-lethal effects may not occur immediately, which is prominent in ecotoxicological studies.[43,44] Snail faeces were sampled with a disposable pipette every fifth day of the experiment. Faeces were dried for 24 h at 95 °C and dry mass was weighed (± 0.01 mg) afterwards.

Statistics

Biofilm volumes were compared using ANOVA with Tukey's 'Honest Significant Difference (HSD)' method as post-hoc procedure if both the assumption of normality (tested with the Shapiro-Wilk Normality Test) and homogeneity of variances (Brown-Forsythe Levene test) were assumed. If the assumption of normality was violated, Kruskal Wallis test was used, followed by Dunn's post hoc test with Bonferroni's correction. If homogeneity of variances between groups was not given, a one-way ANOVA followed by the max-*t* test for multiple comparisons of means was applied. This test uses the heteroscedastic consistent covariance estimation, which helps avoiding false positive results.[45] Data of biofilm volumes were $\log_{10}$-transformed to achieve normal distribution of data, with exception of bacteria volumes.

Faeces dry weight per aquarium was converted to individual faeces mass. Effect of treatment was computed with Kruskal Wallis test followed by Dunn's post hoc procedure with Bonferroni's correction for multiple comparisons. Shell length data was averaged per aquarium and converted to individual growth per day. Differences of growth rates were compared among treatments with Kruskal Wallis test. Multiple comparisons of treatments by means were performed with LSD test and Hochberg's p-value adjustment. This was done for each third of experiment duration separately. All individual based calculations were corrected for individual number changes over time. For significant results effect sizes are provided as η^2 (ANOVA) or ε^2 (Kruskal Wallis test). All tests were performed in R[46] with the external packages 'agricolate' (LSD post hoc test with Hochberg's correction)[47], 'car' (Brown-Forsythe Levene test)[48], 'multcomp' (max-*t* test)[49], 'PMCMR' (Kruskal Wallis test, Dunn's post hoc test, Bonferroni

144 correction)[50], 'rcompanion' (ε^2 calculation)[51], 'sandwich' (heteroscedastic covariance estimation)[52] and
145 'sjstats' (η^2 calculation)[53].

146

147 **Results**

148 Analysis of biofilm height and volumes of single biofilm components showed differences among the
149 substrata. Representative CLSM images are shown in Figure 1. The volume of algae was significantly
150 lower on PMMA compared to PC and control algae volumes ($p < 0.05$, $\eta^2 = 0.29$, ANOVA with max-*t* post
151 hoc test: $n_{Control} = n_{PC} = n_{PMMA} = 25$, Fig. 2). In contrast, the volume of lectin-specific glycoconjugates was
152 significantly higher on PMMA compared to the other two substrates ($p < 0.05$, $\eta^2 = 0.10$, ANOVA with
153 Tukey 'HSD' post-hoc: $n_{Control} = n_{PC} = n_{PMMA} = 25$, Fig. 2). Bacteria were the least voluminous component of
154 the biofilm on all substrata and did not differ among substrates ($p = 0.10$, Kruskal Wallis test with Dunn's
155 post hoc test: $n_{Control} = n_{PC} = n_{PMMA} = 25$, Fig. 2). PMMA biofilm built up significantly higher structures than
156 biofilm of PC and control ($p < 0.01$, $\eta^2 = 0.14$, ANOVA with Tukey 'HSD' post hoc:
157 $n_{Control} = n_{PC} = n_{PMMA} = 25$, Fig. 3A). After the grazing experiment, on both plastic substrates patches of
158 unconsumed biofilm were left over, while on the control substrate the biofilms were entirely grazed.

159 Consumed biofilm calculated as faeces dry mass per individual (mg Ind^{-1}) differed significantly among
160 treatments. The effect of treatment was significant, with lower faeces dry mass in the PC treatment
161 compared to faeces dry masses in both control and PMMA treatments where no significant differences
162 were found ($p < 0.05$, $\varepsilon^2 = 0.14$, Kruskal Wallis test with Dunn's post hoc test: $n_{Control, PC, PMMA} = 35$, Fig. 3B).

163 Growth rates of snails (mm d^{-1} Ind^{-1}) did not differ among treatments in the first two thirds of the
164 experimental duration ($p = 0.89$ & $p = 0.76$, Kruskal Wallis test, $n_{Control, PC, PMMA} = 5$). In the last third of the
165 experiment, growth rates were significantly lower in the two plastic treatments compared to control
166 ($p < 0.05$, $\varepsilon^2 = 0.45$, Kruskal Wallis test with LSD post hoc, $n_{Control, PC, PMMA} = 5$, Fig. 3C). In the plastic

167 treatments some snails stopped growing completely, while in the control treatment all snails continued
168 growing until the end of the experiment. At the end of the experiment mortality of snails was below
169 50 % in all treatments, without differences among treatments ($p = 0.86$, ANOVA with Tukey 'HSD' post
170 hoc: $n_{Control, PC, PMMA} = 25$).

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172

173 **Discussion**

174 Effects of different plastic types on biofilms

175 The biofilms of this study generally contained low bacteria volumes, intermediate algae volumes and
176 high lectin-specific glycoconjugate volumes on all exposed substrata. Comparable proportions of these
177 biofilm components grown in natural freshwaters have been reported in other studies.[54-56] However, the
178 proportion of these compartments differed significantly among the different substrates in this study.

179 Immediately after immersion in water, a substratum is covered by a layer of macromolecules, described
180 as conditioning film, which is distinct among different substrata.[25,57] The characteristics of the initial
181 coating affect the subsequent establishment of a microbial conditioning film.[58,59] During maturation the
182 biofilm development on top of this initial biofilm layer is affected to a much greater extend by external
183 factors, resulting in equally composed biofilms independent of underlying substrata.[60] Contrarily to these
184 assumptions, the share of the three compounds of the biofilms and their structures of this study differs
185 according to substrate type, even though analyses were done after initial growth phase. In comparison
186 to control and PC slides, biofilm on PMMA slides contained lower volumes of algae but higher volumes of
187 lectin-specific glycoconjugates. EPS are produced by prokaryotic and eukaryotic microorganisms and
188 consist of polysaccharides, proteins, nucleic acids, lipids and other biological macromolecules.[61,62] Scott
189 et al.[63] indicate that EPS release is higher, when stressful conditions for primary producers are present.

Since biofilm on PMMA slides contains lower algae volumes at experiment start (indicating low primary production) but high volumes of lectin-specific glycoconjugates, PMMA may present more stress for primary producers than PC or glass as a substrate. Furthermore, lectin-specific glycoconjugates are crucial for biofilm structure and stability.[64] This might explain why biofilm on PMMA slides also formed significantly higher structures.

Effects on snails

Individuals of *Radix balthica* showed lower growth rates (partially growth stopped) at experiment end in both plastic treatments compared to the control treatment. This effect occurred with delay and was not detectable after short exposure times, as seen in ecotoxicological studies.[43,44] Hence, future studies investigating sub-lethal indirect effects on snails should at least last for six weeks. In the PC treatment the lower growth rates could be explained by the lowest consumption of biofilm. Contrarily, in the PMMA treatment biofilm consumption was as high as in the control treatment, while growth rates were significantly lower. This indicates that equal amounts of consumed biofilm did not result in equal growth. Since biofilm composition differed among substrata (significantly more glycoconjugates and significantly less algae on PMMA than on glass) the nutrient supply of PMMA biofilms was probably lower compared to glass biofilms. It was shown that periphyton with low food quality (high N:P and C:P ratios) can lead to lower growth rates of benthic grazers.[65] Likewise, Fink & Von Elert[36] reported lower growth of *Radix sp.* when low nutrition supply was available. Hence, the lower amount of algae in PMMA biofilms may represent lower nutrition supply for *R. balthica* leading to lower growth rates.

In the two plastic treatments, the snails did not graze on the entire biofilm resulting in patches of remaining biofilm on plastic slides after the grazing experiment. This means that in both plastic treatments the consumption of biofilm was restricted compared to the control treatment. Remaining

biofilm patches may have occurred because the attachment of biofilm components was stronger to plastic surface and grazing was not effective enough to remove the entire biofilm. Surface characteristics decide on the strength of initial biofilm attachment,[66-68] which could have prevented scraping off through grazing. However, the presence of bare areas on plastic slides after grazing indicate, that this is not the only explanation, because the snails were physically able to scrape off biofilm without leaving residues but a bare surface. Thus, the behaviour of the snails in the plastic treatments might have changed to lower grazing activity during the experiment. Influences on behaviour might be caused by endocrine disrupting chemicals (EDCs), including feeding behaviour and growth.[69-71] Different plastic types have been shown to directly leach EDCs.[72] Also plastic was shown to function as a carrier of EDCs and other pollutants.[23,73,74] PC is known to leach bisphenol A, an EDC into the environment.[72,75,76] For PMMA no environmental leaching of pollutants have been reported so far, probably because it is considered as material with good weather resistance.[77] Remarkably, the effect of reduced growth in the PMMA treatment was similar to that of the PC treatment. Presumably, the surface characteristics of PMMA might facilitate sorption of pollutants, since it is hydrophobic[78]and some hydrophobic pollutants easily adhere to plastic surfaces.[22,79]

Plastic as an artificial substratum may directly affect the primary production and subsequently indirectly affect primary consumers. Further effects on higher trophic levels can be expected, since lower growth rates of grazing invertebrates lead to a lower biomass, i.e. lower food supply for secondary consumers, respectively. Nevertheless, it has to be considered that benthic food webs are complex systems where further (grazing) organisms are involved and many abiotic and biotic factors influence the processes within these systems. Furthermore, grazers may compensate the reduced feeding or lower food quality by using biofilms on other available substrates. However, when other hard substrates are extensively covered by plastics these biofilms provide lower food quality for grazers.

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425

426 Figure legend

427 Fig. 1: Representative CLSM images of biofilms (xz projections). A = Control, B = PC, C = PMMA. Bacteria
428 are plotted in green, algae in blue, and lectin-specific glycoconjugates in red. Scale bar = 100 µm.

429

430 Fig. 2: Volumes of biofilm components before grazing. Different letters indicate significant differences
431 (n.s. = no significance). Algae: ANOVA with max-*t* test post hoc; $p < 0.001$. Bacteria: Kruskal Wallis test;
432 $p = 0.1021$. Lectin-specific glycoconjugates: ANOVA with Tukey 'HSD' post hoc; $p < 0.05$. Note the
433 different scales on the y-axis.

434

435 Fig. 3: (A) Height of biofilms according to substratum before grazing. ANOVA with Tukey HSD' post hoc
436 test; $p < 0.01$, $n = 25$ for all groups. (B) Consumed biofilm as faeces dry mass per individual during grazing
437 experiment. Different letters indicate significant differences among treatments. Kruskal Wallis test with
438 Dunn's post hoc test, $p < 0.05$, $n = 35$ for all groups. (C) Average snail growth rates among treatments
439 during the last third of the experiment. Different letters indicate significant differences. Kruskal Wallis
440 test with LSD post hoc; $p < 0.05$, $n = 5$ for all groups.

441

442 Figure 1

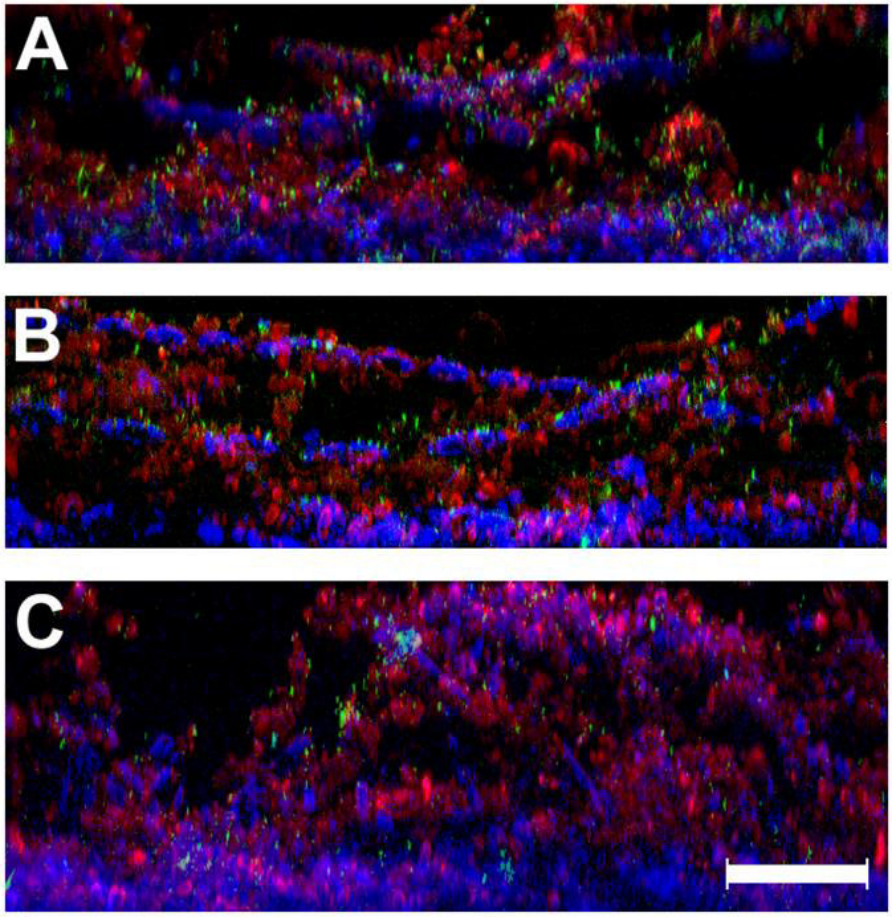

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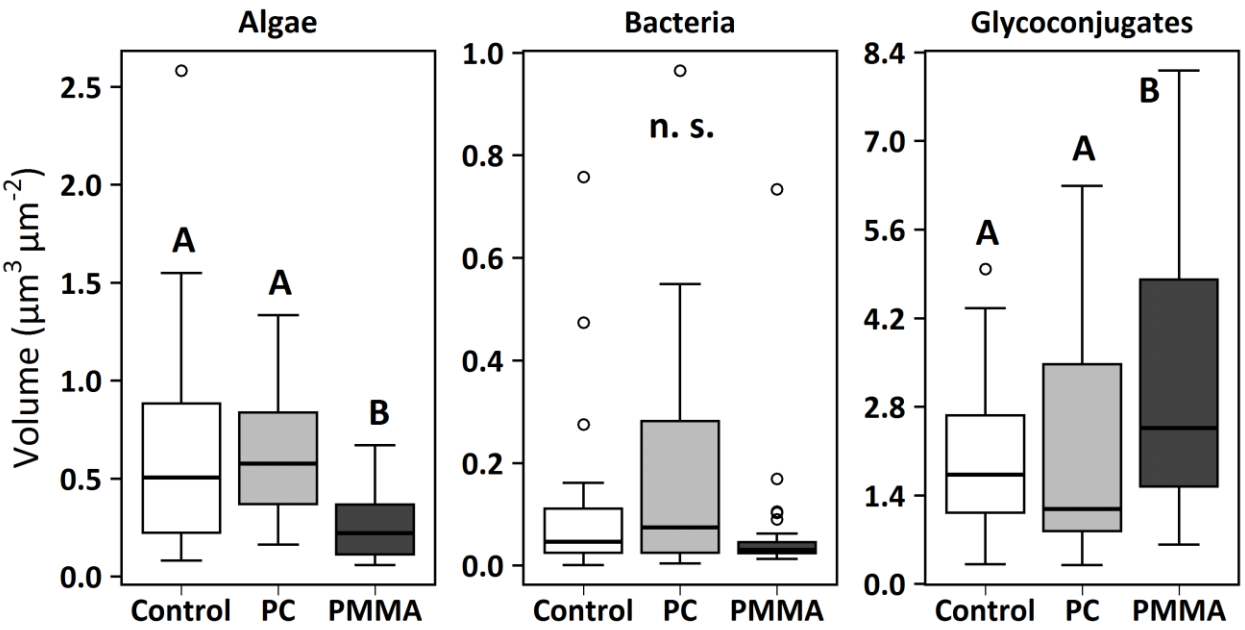
Figure 2


Algae
Bacteria
Glycoconjugates
Volume (µm³ µm⁻²)
2.5
2.0
1.5
1.0
0.5
0.0
1.0
0.8
0.6
0.4
0.2
0.0
8.4
7.0
5.6
4.2
2.8
1.4
0.0
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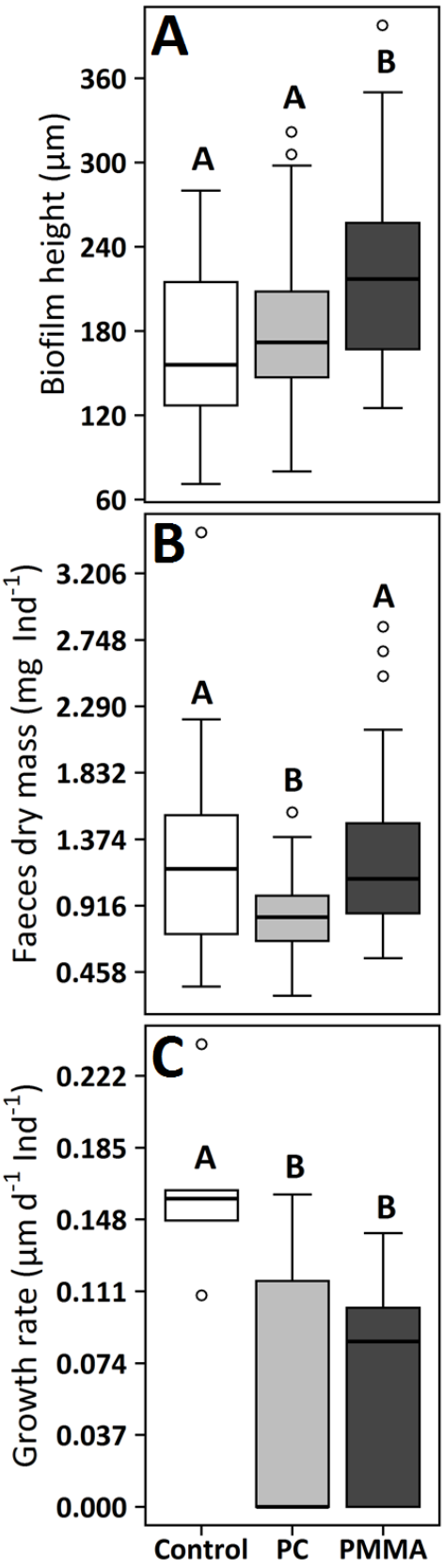
PMMA

458 Figure 3



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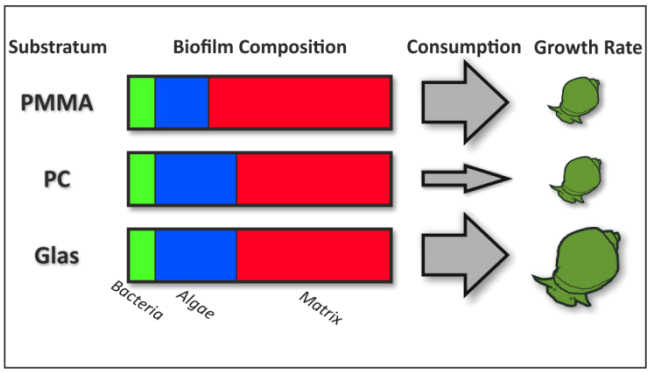

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