LIMNOLOGY
and
OCEANOGRAPHY: METHODS

ASLO

Limnol. Oceanogr.: Methods 18, 2020, 1–7

doi: 10.1002/lom3.10339

Biofilm pads—an easy method to manufacture artificial biofilms embedded in an alginate polymer matrix

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Abstract

Periphytic biofilms are the major resource for many herbivorous invertebrates in both marine and freshwater benthos. They are of crucial importance for benthic food webs, substrate stability, and biogeochemical processes in littoral zones. While the importance of invertebrate grazing on biofilms has been studied extensively using natural, mixed algal communities grown on artificial substrates, there is so far no method available to create defined periphyton communities for these grazing studies. The reason for this is that many benthic algae interact with co-occurring species within the extracellular polymeric substances (EPSs) that form the nonorganismic part of the biofilm. Here, we present a novel method that allows the manufacturing of defined monoculture and multispecies biofilms by using an alginate polymer as artificial EPS structure, into which algal cultures can be embedded. Using confocal laser scanning microscopy, we show that alginate effectively embeds various algal taxa in an EPS matrix that is very similar to natural biofilms. In a grazing experiment, we demonstrate that several common freshwater herbivorous invertebrates can graze these alginate biofilms efficiently. As the method is easy to handle, it allows for highly controlled feeding experiments with benthic herbivores to assess, for example, the role of algal biodiversity on the efficiency of top-down control, the effects of environmental drivers such as nutrients, salinity, or seawater acidification on biofilm community structure, and the impacts of herbivory in benthic communities.

Biofilms are complex communities of bacteria, algae, and various other unicellular and colonial organisms and will form on almost any submerged aqueous surface (Costerton et al. 1995; Donlan 2002). They are ubiquitous in aquatic systems from rivers and lakes to oceans and drainage pipes (Stevenson et al. 1996). Littoral algae communities are among the most productive assemblages in aquatic ecosystems (Pinckney and Zingmark 1993). Benthic algae are the basis of many aquatic food webs (Stevenson et al. 1996) and usually form biofilms (also called periphyton). As such, periphyton biofilms and their trophic interactions with various grazers are widely studied (e.g., Lawrence et al. 2002; Weitere et al. 2018). Yet, due to the complex nature of the biofilm communities and their diverse composition, it is particularly difficult to approach biofilms in an experimentally standardized manner (Stevenson et al. 1996). Studies investigating trophic interactions between biofilms and their primary consumers have to rely on biofilms cultured in the lab (e.g., Wimpenny 1996; Franklin et al. 2015; Groendahl and Fink 2017) or under seminatural conditions in the field on various surfaces (often tiles or glass slides, e.g., Siboni et al. 2007; Norf et al. 2009; Witt et al. 2011). Furthermore, these techniques produce complex multitaxa biofilms that can only be controlled for species composition and nutrient loading with great difficulties (Norf et al. 2009; Wey et al. 2009). The preculturing of biofilms is also often time consuming and at the same time yields little biomass of appropriate quality. Other studies use sedimented, precultured algae as a substitute for substrate-associated biofilms (Moelzner and Fink 2014; Groendahl and Fink 2017). Yet, using what is de facto seston rather than actual periphyton is only possible understanding-water conditions without current that would resuspend the food source. Moreover, in trophic ecology studies, this approach is only applicable for grazers capable of collecting sedimented particles, which can lead to erroneous estimates of grazing efficiency and food intake.

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In order to provide a biofilm that is both attached to a substrate and controlled for its composition, we developed a simple approach to manufacture synthetic biofilms that can mimic numerous natural biofilm structures and properties. Natural biofilms consist not only of sessile organisms (commonly bacteria and algae), but they are typically surrounded by an organic extracellular polymeric substance (EPS) matrix (Flemming et al. 2007). EPS is largely responsible for the structural attributes of a biofilm and is an important substance for many interactions between organisms co-occurring in biofilms (Bruckner et al. 2008; Flemming and Wingender 2010; Neu and Lawrence 2017). Additionally, EPS can also attract consumers of the biofilm or deter their grazing activity (Wotton 2004; Skov et al. 2010).

Several macroalgal species such as the kelp *Laminaria* and other brown seaweeds produce large amounts of alginic acid, also called alginate (McHugh 2003); a substance present in the EPS of natural biofilms (Flemming et al. 2017). This anionic polysaccharide is used, for example, by the food industry as thickening agent or from the pharmaceutical industry for various applications (McHugh 2003). When reacting with Ca^{2+}, alginate forms a hydrogel-like mucous polymer (Liu et al. 2003) that shows properties similar to the EPS of microalgae in natural biofilms. Here, we use commercially available sodium alginate as an artificial EPS replacement to compose biofilms of precultured algae on glass fiber filter pads. Monoculture and multispecies biofilms-pads can be produced in the same way, which allows for control over the entire biofilm composition. At the same time, the manufactured biofilms are fixed to a substrate and thus, have structural properties similar to naturally occurring biofilms. This allows for a more natural study of the interactions between algal biofilms and their consumers. The properties and applicability of these artificially manufactured biofilms (here named biofilm pads) are explored and described herein.

Materials and procedures

Cultures

The filamentous green alga *Uronema fimbriata* (strain SAG/36.86 from the Göttingen Algal Culture Collection, SAG, formerly classified as *Ulothrix fimbriata*) was cultivated in batch cultures on Cyano medium (von Elert and Jüttner 1997) on a rotary shaker with a light (photosynthetically active radiation (PAR)) intensity of 80 μmol photons s^{-1} m^{-2}. The filamentous chlorophycean *Klebsormidium flaccidum* (strain CCAC/2007B from the Cologne Algal Culture Collection), the unicellular conjugatophycean *Roya obtusa* (strain CCAC/0219B from the Cologne Algal Culture Collection), and the stalked, unicellular benthic diatom *Achnanthes biasolettiana* (originally isolated from Lake Constance (Fink et al. 2006*b*) were cultivated in batch cultures on a modified WC medium (Guillard 1975) as described above.

For the ingestion experiment, five different exemplary species of freshwater herbivores/omnivores with different feeding types were used: The unselective grazers *Lymnaea stagnalis* (LINNAEUS 1758, Gastropoda, Pulmonata), and *Planorbarius corneus* (LINNAEUS, 1758, Gastropoda, Pulmonata), and the selective grazers *Gammarus pulex* (LINNAEUS, 1758, Crustacea, Amphipoda), *Neocaridina davidi* (BOUVIER, 1904, Crustacea, Decapoda), and *Asellus aquaticus* (LINNAEUS, 1758, Crustacea, Isopoda). *G. pulex* were obtained from a pet shop, where they are sold as live fish food (Fischfutter Etzbach FEE, Schleiden, Germany). All other organisms used in this study were collected from ponds on the campus of the University of Cologne, Germany. Grazers were acclimated to laboratory conditions in tap water in a climate chamber at 20 ± 0.5°C under constant dim light from a 40 W light bulb. Tap water aerated with an aquarium pump. Grazers were fed with Tetra PlecoMin™ fish food pellets (Tetra, Melle, Germany) ad libitum.

Biofilm pads

Sodium alginate solution (5 g L^{-1}) and calcium chloride ($CaCl_2$) solution (20 g L^{-1}) were prepared with demineralized water. The alginate solution was stored at 7°C to minimize bacterial development and the $CaCl_2$ solution was stored at room temperature (21°C). Prior to the production of the biofilm pads, both solutions were adjusted to room temperature. All further steps were conducted at room temperature. A volume of 4 mL of the respective algal culture or mixture were mixed with 1 mL of the sodium alginate solution and filtered on preweighed glass fiber filters (GF/F, Ø 47 mm, VWR, Darmstadt, Germany). Care was taken to minimize the duration of the filtration and the pressure (max. –0.2 bar) to prevent algal cell lysis through shear stress and to retain a sufficiently moist (and alginate-saturated) filter surface. The filters were carefully submerged (algal side up) in the $CaCl_2$ solution in circular glass jars using forceps for 10 s to allow for hydrogel formation. Subsequently, the filters were rinsed by submersion in another dish filled with demineralized water for 30 s and transferred into circular glass jars (diameter: 12 cm), filled with 100 mL aerated tap water for the ingestion experiments. Control filters with alginate but without algae were manufactured to gravimetrically determine the approximate alginate dry mass (DM) per filter. The schematic workflow is presented in Fig. 1.

Confocal laser scanning microscopy

Biofilm pads were manufactured with the four algal taxa *Achnanthes biasolettiana* (Bacillariophyceae), *R. obtusa* (Conjugatophyceae), *Uronema fimbriata*, and *K. flaccidum* (Chlorophyceae) grown and applied as a mixture of all four species on the filters as described above and immediately subjected them to confocal laser scanning microscopy (CLSM) analysis. For CLSM, a TCS SP5X upright setup equipped with a super continuum light source was available. The system was controlled by the software LAS-AF version 2.4.1 (Leica Microsystems, Germany). Samples were mounted in a Petri dish

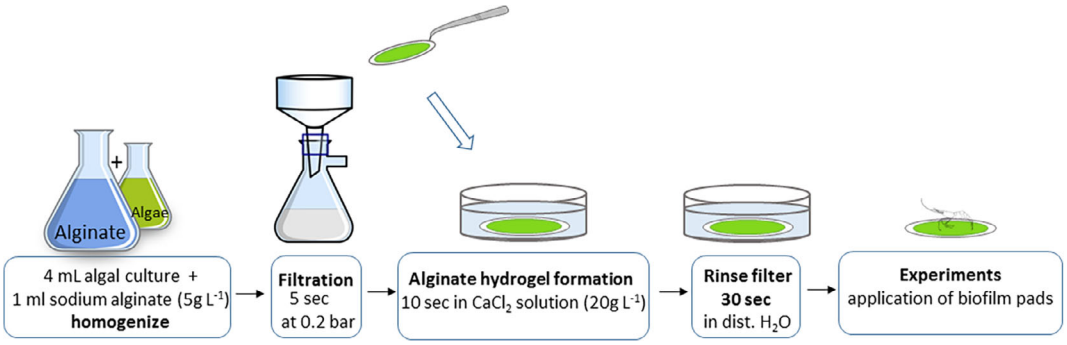

Fig. 1. Schematic workflow for the manufacturing of the biofilm pads.

and scanned with a 25x NA 0.95 water immersible objective lens. Three-dimensional image data sets were recorded in XY-Z and XZ-Y direction. To visualize the alginate layer above the algal cells on the filter's surface, we added 2 μm Fluoresbrite® microspheres (Polysciences Europe, Hirschberg an der Bergstrasse, Germany) at a dilution of 1:100. Excitation was at 483, 560, and 635 nm, emission signals were recorded from 473 to 493 nm (reflection), 500 to 550 nm (microspheres), 575 to 620 nm (phycobilins, but no signal was detected in this channel), and 660 to 720 nm (chlorophyll *a*). Data sets were projected with Imaris version 9.2.1 (Bitplane) and printed from Photoshop (Adobe).

Grazing assessment

Grazing experiments were conducted with monoculture biofilm pads covered with the filamentous alga *U. fimbriata*, as this species had previously been shown to be easy to cultivate and readily ingested by all our grazer species (Moelzner and Fink 2014; Groendahl and Fink 2017). These biofilm pads served as the sole food source for the five grazer species. Algal biofilm filters without grazers served as controls. Five replicates were prepared for grazing experiments and controls. Prior the ingestion experiment, the grazers were starved (i.e., without food supply) for 24 h in aerated tap water to increase their feeding motivation (Fink and von Elert 2006). Subsequently, grazers were placed individually in a circular glass jar filled with 100 mL aerated tap water and one algal filter each. During the experiment, the grazers were kept at 20 $\pm$ 0.5°C and dim light. After 3 h of grazing, grazers and fecal pellets (not quantified) were removed from the biofilm pads before drying for 24 h at 60°C to determine algal DM on a microbalance (Mettler UTM2, Giessen, Germany). The control pads were treated identically, that is, incubated for the same time under the same condition as the grazed biofilm pads to control, for example, for algal growth during the feeding trial. Since alginate is very hygroscopic, the weighing chamber of the microbalance was kept dry with silica gel to obtain stable weighing conditions.

At the end of the experiment, the soft-bodies of the snails (*L. stagnalis* and *P. corneus*) were removed from the shells under a dissecting microscope, frozen at −80°C and subsequently freeze-dried to determine the soft body DM. The bodies of *G. pulex*, *N. davidi*, and *Asellus aquaticus* were washed and dried for 24 h at 60°C without further treatment. The DM of all grazers was determined with a microbalance to the nearest microgram. Ingestion rates were calculated as the difference between the DM of food offered and the DM remaining at the end of the feeding trial, normalized to the experimental duration and grazers' body mass (for details, see Fink and von Elert 2006). The mean ingestion rate was measured for all grazers as ingested algal DM h^{-1} mg^{-1} soft body

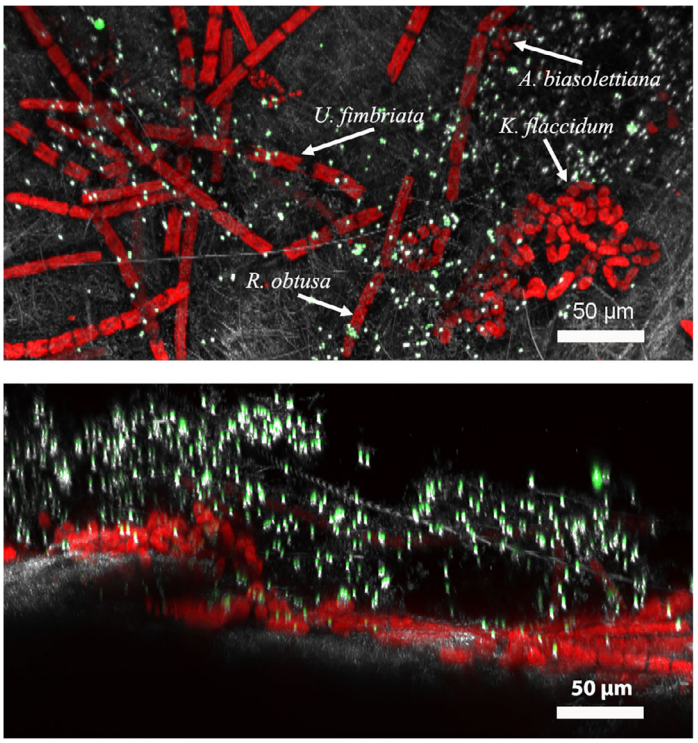

Fig. 2. Confocal laser scanning microscopy of the biofilm pads with a mixed algal community (algal species identity assigned with arrows); maximum intensity projections of XY-Z (top) and XZ-Y (bottom) data. Note the distance of the adhered microspheres from the algal layer in the XZ-Y projection indicating the thickness of the polymer matrix. Color allocation: reflection of filter—gray, fluorescent microspheres—light green, autofluorescence of algal layer—red.

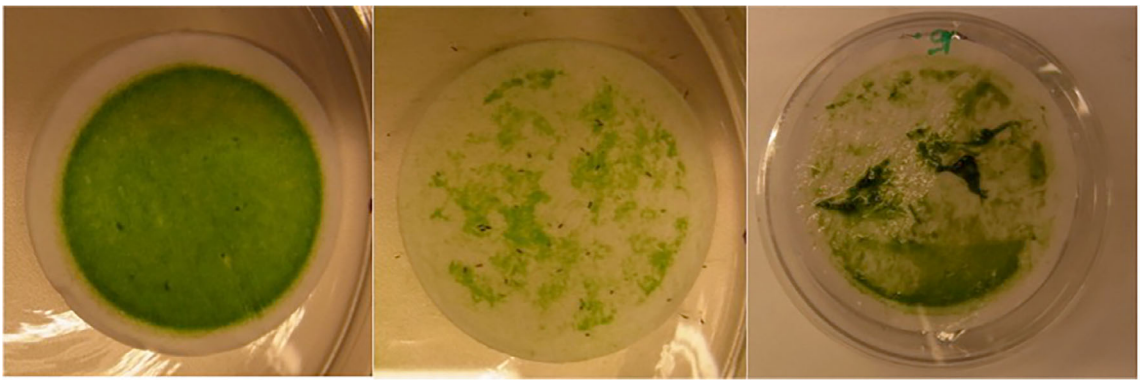

Fig. 3. Control biofilm pad (*Uronema fimbriata*, embedded in alginate), pad after grazing by *N. davidi* and after grazing by *L. stagnalis* (from left to right). Arrows indicate discarded biofilm by the snail grazing activity.

DM to enable a comparison of the different grazers despite their differences in body size and mass. Subsequently, ingestion rates were tested for homoscedasticity with Levene's test and a one-way ANOVA followed by Tukey's HSD post hoc comparisons in R version 3.5.1 (R Core Team 2018).

Results

Properties of artificial biofilms on biofilm pads

Multichannel confocal laser microscopy clearly showed the reflection signal of glass fiber filters, the autofluorescence of the algal layer, and the fluorescent microspheres associated with the alginate matrix. In the horizontal view (Fig. 2), the layer of beads could be visualized at a distance of up to 100 μm, thus demonstrating the three-dimensional structure of the alginate matrix and thus the model biofilm.

Our novel method allowed us to create artificial biofilms on glass fiber pads with very little time and material expense. The resulting layer of alginate-algae mixture adhered firmly to the filter carrier after polymerization. The algae in the alginate matrix stayed intact and remain green for at least 7 d despite alginate enclosure (T. Reinhardt pers. obs.). To avoid drifting or shifting of the filters, we placed the filter into petri dishes of the same diameter. Application of 1 mL alginate at 5 g L^{-1} concentration resulted in a mean amount of 6.83 mg ($\pm$ 0.56 SE of n = 5 replicates, determined gravimetrically) polymerized calcium alginate on each filter. Consequently, on average 42.3% of the DM of material on the filter was alginate when a mixture of alginate and only the filamentous alga *U. fimbriata* was used.

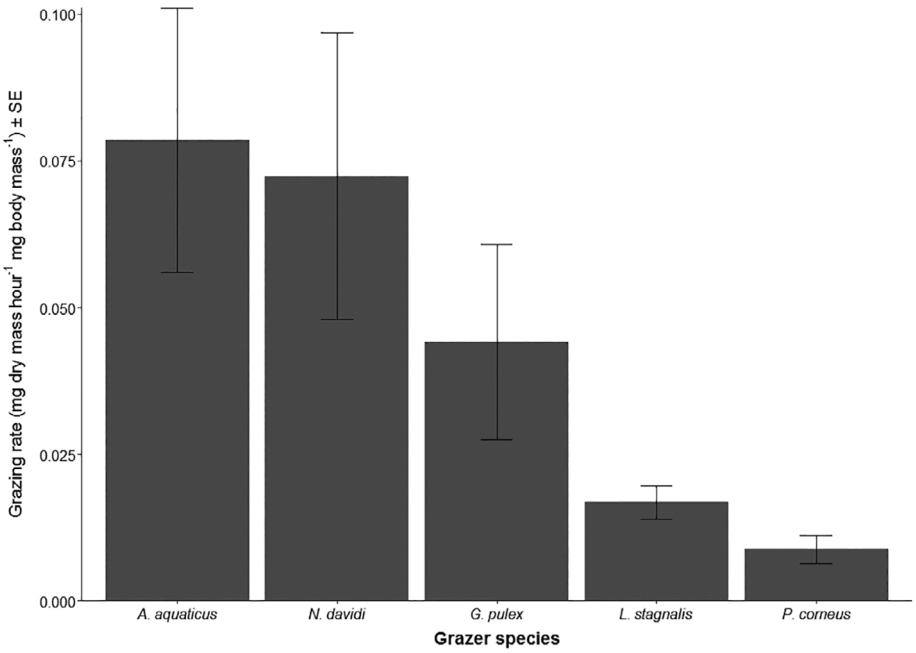

Fig. 4. Grazing rate (mean $\pm$ SE) mg algal DM h^{-1} mg soft body DM^{-1}) of different consumers (each n = 5) grazing the *U. fimbriata* biofilm-pads, rates differed significantly between grazer species (one way ANOVA, $p < 0.05$).

Grazing on biofilm pads

All grazer species left visible grazing tracs on the biofilm pads which offered artificial biofilms of *U. fimbriata* together with the alginate matrix (Fig. 3). After 3 h of grazing, the filters were removed and the remaining biofilm could be recovered and weighed.

Mean grazing rates on the biofilm pads ranged between 79 ($\pm$ 9 SE) μg mg^{-1} algal DM h^{-1} mg soft body DM^{-1} for *Asellus aquaticus* and 8 ($\pm$ 1 SE) μg mg^{-1} DM h^{-1} bodymass^{-1} for *P. corneus* (Fig. 4). In the same manner as the grazing intensity, the amount of feces remaining on the biofilm pads and the amount of artificial biofilm destroyed and discarded due to grazing and movements on the pads was grazer species-specific.

Discussion

The biofilm pad is a useful approximation for natural biofilms due to its simple production and utility. With established algal precultures, it can be manufactured rapidly and in a simple and highly reproducible workflow.

Using CLSM, we were able to demonstrate that the artificial algal community in the biofilm pads is embedded in an EPS made of alginate. The biofilm topography thus closely resembles natural biofilms with their ridged topography of valleys and towers of variable thickness. Similarly to natural biofilms (Suarez et al. 2018), the biofilm pads display a thickness between 10 and 100 μm. In fact, natural biofilms and particularly their EPS' vary greatly in terms of thickness and topography depending on their biotic properties, community composition, the presence of grazers, and abiotic conditions such as light, nutrients, or flow velocity (Costerton et al. 1995; Battin et al. 2016). With this, the biofilm pads are a good approximation to these natural conditions that can be specifically adjusted with the obvious benefits.

First, the biofilm pads can be assembled with various algal and bacterial species. For the grazing experiment, we used the filamentous epilithic alga *Uronema fimbriata*, as it is easy to cultivate and a common component of natural periphyton communities (Fink et al. 2006*a*). We further showed that we could assemble multispecies biofilm pads, which resemble diverse communities found in natural biofilms. In theory, the method can be applied to even more complex biofilms of algae, bacteria, and protists. For instance, we were able to attach the motile, planktonic chlorophyte *Chlamydomonas klinobasis* in a biofilm (J. Moelzner unpubl. data). The flagellate algae stayed intact and green for at least 3 d within the artificial biofilms.

Second, as the alginate can be mixed with different amounts and densities of algae, the biomass of the biofilm pad is flexible and measurable. As such, varying amounts of primary producer biomass can be supplied into experimental food chains. Yet, care must be taken when applying increasing algae to alginate biomass ratios, as the viscosity of the alginate carrier has to be increased to avoid an uncontrolled peeling of the biofilm pad's surface. An adjustment of the viscosity of the alginate layer is possible by changing the concentration of the alginate solution or altering the mixing ratio of algal culture and alginate solution. In a test series, mixing ratios of up to 10:1 (v/v, algal culture: 5 g L^{-1} alginate solution) yielded stable biofilms. For example, the alginate concentration chosen here (approximately 0.2%) is well in the range commonly used for the encapsulation of biocatalysts within hydrogels (Blandino et al. 1999). As such, deliberate variations in viscosity can also be used to create different biofilm quality and differently accessible algal resources for grazers in the experimental setup.

All tested benthivores readily consumed the artificial biofilms. Grazing rates of *N. davidi* and *L. stagnalis* on the pads match those previously reported on sedimented algal suspensions in other experiments (Groendahl and Fink 2017), indicating that the artificially created alginate EPS neither reduce the grazers' ability to ingest biofilm nor did it repel them. Importantly, biofilm was consumed by grazers which differ in feeding mode and behavioral patterns (Arens 1989), which further attests to the bioavailability of these biofilm pads. Both freshwater gastropods, *L. stagnalis* and *P. corneus*, graze on biofilms with their radulae, often consuming parts of the underlying surface in the process. *N. davidi* is a collection feeder that plucks particles from biofilms using their specialized first legs and maxillae; a feeding mode that discards some proportions of the biofilm (Fig. 2). The two other crustaceans, *G. pulex* and *Asellus aquaticus*, are mostly shredders that manipulate and dissemble biofilms with their mandibles.

Caution is advised before determining grazing rates. Grazers deposit fecal pellets onto the pads, which can make the separation of feces and biofilm challenging (Fink and von Elert 2006). Thus, it is essential to remove fecal pellets, particularly those with intact peritrophic membranes. Fecal pellets will, nonetheless, provide useful information on egestion rates. Furthermore, alginate contributed ~ 42% to the DM of biofilm pads, providing a significant food source for grazers. The addition of proper experimental controls is proposed if the differentiation between EPS and algae consumption is desired.

The use of biofilm pads mimics natural conditions and allows for experiments with natural grazer communities in which grazing strategies can be complementary to one another balancing the costs of competition and specialization (Barnese et al. 1990; Lawrence et al. 2002). The biofilm pads can further be used as a versatile tool to address a wide range of experimental questions and approaches. Various scenarios of biofilm nutrient stoichiometry can easily be tested by culturing algae on growth media with different dissolved nutrient levels before the incorporation into the alginate matrix (Cronin et al. 2002). Similarly, patchy biofilm environments can be generated by combining different biofilm pads to biofilm landscapes and observing the behavior of grazers in a more complex setup (Iannino et al. 2019). The alginate matrix can further be adjusted to the experimental conditions, thus, providing a carrier structure for testing the influence of other

components on the grazer community and their behavior. This includes the study of the influence of volatile organic compounds as attractors and feeding cues (Moelzner and Fink 2014), the influence of natural and artificial repellents, and the influence of toxins on grazers (Sabater et al. 2007; Rasmussen et al. 2008; Lundqvist et al. 2012).

Comments and recommendations

Overall, the biofilm pads are a novel approach with the potential to resolve a long-standing challenge in the experimental ecology of benthic trophic interactions. Due to their simple and versatile production, and the options to vary multiple parameters such as species composition, nutrient content, and spatial heterogeneity in a highly controlled and reproducible way, they allow for a multitude of experimental approaches. The biofilm pads could thus be a methodical advancement for benthic ecology, both in freshwater and marine environments.

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Acknowledgments

We thank D. Johannes and R. Parlow for help with the development and validation of the method, U. Kuhlicke for support with CLSM analyses, and E. von Elert for providing laboratory space and infrastructure. This study was partially funded by the Deutsche Forschungsgemeinschaft (DFG) through grant FI 1548/7-1 to P.F.

Conflict of Interest

None declared.

Submitted 02 July 2019
Revised 23 September 2019
Accepted 19 October 2019

Associate editor: Gordon Taylor