A solid phase extraction based non-disruptive sampling technique to investigate the surface chemistry of macroalgae

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ABSTRACT
The surface chemistry of aquatic organisms determines their biotic interactions. Metabolites in the spatially limited laminar boundary layer mediate processes, such as antifouling, allelopathy and chemical defense against herbivores. However, very few methods are available for the investigation of such surface metabolites. An approach is described in which surfaces are extracted by means of C18 solid phase material. By powdering wet algal surfaces with this material, organic compounds are adsorbed and can be easily recovered for subsequent liquid chromatography/mass spectrometry (LC/MS) and gas chromatography/mass spectrometry (GC/MS) investigations. The method is robust, picks up metabolites of a broad polarity range and is easy to handle. It is more universal compared to established solvent dipping protocols and it does not cause damage to the test organisms. A protocol is introduced for the macroalgae Fucus vesiculosus, Caulerpa taxifolia and Gracilaria vermiculophylla, but it can be easily transferred to other aquatic organisms.

Introduction
Natural products play a fundamental role in ecological interactions on biotic surfaces under water. Surface metabolites can for example act on the interface of water and macroalgae, corals or sponges. Such compounds control settling processes, regulate predator/prey relationships and mediate infection processes (Wahl 2009; Dobretsov et al. 2013; da Gama et al. 2014), and may also control competitors by means of allelochemical activity (Gross 2003; Lu et al. 2011; Rasher et al. 2011). Furthermore, regulation of fouling is influenced by these natural products (Dobretsov et al. 2013; da Gama et al. 2014). Simple mechanistic considerations suggest that surface metabolites are highly concentrated and thus most active in a very narrow diffusion-limited laminar-boundary layer of water in the immediate vicinity of the producing organism (Hurd 2000; Grosser et al. 2012). Knowledge about surface concentrations is thus relevant for the investigation of the ecological role of metabolites (Dworjanyn et al. 1999, 2006). Nevertheless, until now most investigations on the effect of surface metabolites have been based on bioassays with extracts of whole organisms, or with compounds applied in concentrations found in whole tissue extracts (e.g. see Hellio et al. 2000). Such experiments do not reflect the real ecological relevance of surface active substances, because only metabolites at the surface or in the immediate vicinity of a producer should be considered (Nylund et al. 2007). The determination of metabolites within the laminar boundary layer around an aquatic organism, a thin film of about 100–200 μm that determines the transition between the surface and the surrounding water, is thus crucial for planning and evaluation of experiments.

Studies performed with resonance Raman microspectroscopy allowed visualization of the gradient of carotenoids in the boundary layer around the macroalgae Fucus vesiculosus and Ulva mutabilis. A pronounced decline in concentration from up to millimolar values in the immediate vicinity of the algal surface to concentrations below the detection limit at a 100 μm distance was observed (Grosser et al. 2012). However, this elaborate method is limited to a few Raman active metabolites. Most investigations of algal surface chemistry rely on the extraction of secondary metabolites by so-called ‘dipping’ methods (de Nys et al. 1998; Lachnit et al. 2010). Here, algae are immersed in a solvent for a brief period, during which the metabolites are partially extracted from the surface. After concentration in vacuum, the extracts can be analyzed by analytical methods such as gas chromatography-mass spectrometry (GC/MS) and liquid chromatography-mass spectrometry (LC/MS) and mass spectrometry (MS). However, most of these methods are time-consuming and require large amounts of algal material. Furthermore, they do not allow the extraction of metabolites at the surface or in the immediate vicinity of producers.
spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). Although useful, dipping methods are rather problematic since solvent exposure can cause cell lysis and thereby contamination of the surface extract with intracellular metabolites. Algae only tolerate exposure to rather non-polar solvents such as hexane for a few seconds. However, these solvents only extract a very limited range of non-polar metabolites and do not penetrate surface associated water. If solvent mixtures containing methanol are employed, massive damage of the algae can be observed, thereby questioning the validity of results. To overcome these limitations, a non-destructive solvent-free and universal method for extracting secondary metabolites from marine macroorganisms is introduced. The method is based on the adsorption of organic metabolites onto a C18 extraction sorbent and has been optimized in terms of recovery, reproducibility and ease of use with the brown macroalga *Fucus vesiculosus* as a model organism. *F. vesiculosus* is a common, well-studied brown alga that can be found at the coasts of the North Sea, the western Baltic Sea, and the Atlantic and Pacific Oceans. Due to its important ecological role, this alga has been the subject of numerous investigations of its chemical defense and antifouling capacity (Lachnit et al. 2010, 2013; Saha et al. 2011, 2012; Rickert et al. 2015). In addition, the green alga *Caulerpa taxifolia* and the red alga *Gracilaria vermiculophylla* were extracted for proof of concept.

**Materials and methods**

**Organisms**

*Fucus vesiculosus* was collected in February, April, May and June 2014 in the Kiel Fjord on an easy-to-reach beach (54°21′36.8″N, 10°10′44.0″E). The plants were transported in plastic bags with pulp paper moistened with Baltic seawater at a maximum of 18°C to the University of Jena where they were immediately cleaned with deionized water to reduce epibionts. Then each individual was put into a 7 l aquarium filled with Instant Ocean medium adjusted to the salinity of the Baltic Sea (14–16 psu). The aquaria were aerated with air pumps and kept at room temperature (20–25°C) with a day/night cycle of 12 h/12 h and light intensity at the water surface of 40 μmol m⁻² s⁻¹. *Gracilaria vermiculophylla* was collected in the Kiel Fjord (54°21′36.8″N, 10°10′44.0″E) at the end of April/beginning of May 2014 and transported to Jena in plastic bags with pulp paper moistened with Baltic Sea water. Once in the laboratory, the plants were washed carefully with medium and put into 7 l aquaria filled with Instant Ocean Medium, which was adjusted to the salinity of the Baltic Sea (14–16 psu). The aquaria were kept under comparable conditions to those of *C. taxifolia*.

**Materials**

All reagents used were of analytical grade or superior purity. The absorption material used was a fully end-capped silica gel 90 C18 material (pore size 90 Å, particle dimensions 40–63 μm, Sigma-Aldrich, Deisenhofen, Germany). For collection of absorption material, empty 6 ml polypropylene columns with PE (polyethylene) frits (Macherey-Nagel, Düren, Germany) were used. HPLC-grade methanol and ethanol (Sigma-Aldrich) were used for elution. Standards of fucoxanthin, canthaxanthin and FAME (fatty acid methyl esters) were purchased from Sigma-Aldrich.

**Method development**

Before extraction, the algae (number of replicates *n*=5) were taken out of the tanks and hung on clamps for ~2 min, to let most of the water drip off. This resulted in wet algal surfaces with comparable amounts of surface water. The algae were not blotted dry to avoid removal of the water in the laminar layer of the thalli. Meanwhile, the C18 absorbing material (0.51 g ± 0.01 g, *n*=5, weighed with a Kern ALJ 220-4 balance) was spread in 58 cm² Petri dishes. Then 36.5 ± 6.5 cm² fragments of *F. vesiculosus* were put into the Petri dishes and, after closing, the dishes were gently shaken for ~10 s, in order to obtain a full and uniform coverage of the entire algal surface with the absorption material (the entire procedure is illustrated in Figure 1). The extracted surface of the algae was determined by taking photographs after the treatment and analyzing the images with the software ImageJ (Rasband 1994-2014). Due to the humidity of the algal surfaces, C18 material that came into contact with it remained attached on the surface. The excess remaining material in the Petri dish (~0.4 g) did not contain any detectable surface metabolites (verified by ultra performance liquid chromatography-mass spectrometry (UPLC-MS); see below) and...
was discarded. After covering with C18 material, the algae were left for 60 s in the Petri dishes without movement. This incubation time was optimized for recovery of fucoxanthin in several experiments (20 to 300 s). Subsequently, the algae were rinsed with an excess of artificial seawater to wash off the C18 material. The material was directly collected, with the help of a glass funnel, into an empty solid phase extraction (SPE) cartridge to which a vacuum was applied (~550 Torr). The C18 absorption material settled at the bottom of the cartridge, care being taken not to dry the powder. The funnel and the C18 material were washed three times with 10 ml of deionized water (discarded after washing), in order to remove salts. Metabolites adsorbed on the C18 material were eluted with 3 × 0.5 ml of MeOH. The extracts were combined and split in two equal samples for UPLC-MS and GC-MS investigations. To UPLC-MS samples, 200 μl of canthaxanthin (500 nM in MeOH) were added as an internal standard. The extracts were then dried under a stream of nitrogen. UPLC-MS samples were taken up in 200 μl of methanol and GC-MS samples in 100 μl of methanol. At this stage samples were stored at −20°C until further measurements. After extraction, algae were observed under a confocal light microscope (BX40, Tokyo, Olympus, Japan) to verify cellular integrity.

**UPLC-MS and GC-MS**

After solid phase extraction, the samples were analyzed with UPLC-MS and GC-MS. UPLC-MS measurements were performed on a Waters (Milford, MA, USA) ACQUITY UPLC-MS system with a Micromass Q-ToF micro ESI-TOF mass spectrometer (Micromass, Waters, Manchester, UK). For the separation a BEH C18 column from Waters (2.1 mm × 50 mm, particle size 1.7 μm) was used. The eluents were A: water (UPLC-MS grade, Biosolve, Dieuze, France) with 0.1% formic acid (v/v) and B: acetonitrile (UPLC-MS grade, Biosolv) with 0.1% formic acid (v/v). The flow rate was 0.6 ml min⁻¹ and the equilibration time 1 min. The gradient started with 50% B and was ramped within 4 min to 100% B and held till 5.5 min. As a wash step, the polarity of the eluent was increased up to 6 min (5% B) and held until 6.5 min. At 7.5 min, the solvent was re-adjusted to 50% B. The injection volume was adjusted to 10 μl. A commercial fucoxanthin standard (5 μM in 100 μl of MeOH) was used for identification of the algal metabolite. GC-MS measurements were performed on a ThermoScientific (Waltham, MA, USA) Trace GC-ULTRA system coupled to a ThermoScientific ISQ EI-mass spectrometer, equipped with a quadrupole analyzer. The column used was an Agilent (Santa Clara, Figure 1. Schematic workflow of the C18 method. (1) Algal fronds are removed from the water and left for 2 min to remove excess water by dripping; (2) fronds are transferred to Petri dishes and covered with absorption material; (3) the C18 material is washed off with excess seawater and collected in an empty solid phase extraction cartridge equipped with a frit; (4) the material is washed with deionized water to remove salts; (5) elution with organic solvents finalizes sample preparation.
CA, USA) Durabond DB5MS (30 m length, 0.250 mm diameter, 0.25 μm internal film). The volume injected was 1 μl in splitless mode. The inlet was heated to 250°C and the carrier gas was He with a flow of 1.2 ml min⁻¹. The temperature program started at 60°C (held for 4 min) and was ramped at 15°C min⁻¹ to 300°C (held for 5 min). To prepare the external calibration curve using the ratio of peak areas of fucoxanthin and cantaxanthin, 0.66 mg of fucoxanthin (purity ≥ 95%, Sigma Aldrich) were weighed and dissolved in 10 ml of methanol in order to obtain a 100 μM stock solution. From this stock, several dilutions to different concentration were prepared (1 μM, 750 nM, 500 nM, 250 nM, 100 nM). Ten μl of a 20 μM cantaxanthin (analytical standard grade, Sigma Aldrich) solution in methanol were added to 190 μl of each sample in order to give a final concentration of 1 μM cantaxanthin. After this, every point of the curve was measured three times using the UPLC-MS method described above.

Results and discussion

Development of the extraction procedure

The optimum extraction method for surface metabolites will maximize extraction efficiency while minimizing damage to the alga. The available solvent dipping methods exhibit shortcomings in both respects. Dipping of algae in hexane causes little to no damage of cells, but this highly unpolar solvent allows only extraction of non-polar metabolites (de Nys et al. 1998). Dipping in a mixture of hexane and methanol extracts metabolites with a broader polarity range, but causes significant stress (Lachnit et al. 2010; Saha et al. 2011). Thus, an extraction method was developed that covers a broad range of metabolites, but causes no damage to the algae. This method relies on the absorption capabilities of solid-phase extraction material. By powdering the algal surface with this material, metabolites in the boundary layer around the alga and on its surface can be absorbed (Figure 1). To cover a broad range of metabolites C18-material was selected for method development. Initially, different materials were tested for recovery, purity and ease of handling. Non-endcapped silica gel 100 C18 reversed phase material (100 Å pore size, 40–63 μm particles) could be easily handled and was suitable for the extraction of surface metabolites. However, substantial impurities that could not be removed by conditioning interfered with the detection of algal metabolites (Supplemental material Figure S2). Fully endcapped silica gel 100 C18 reversed phase material (100 Å pore size, 15–25 μm particles) was suitable for extraction of surface metabolites exhibiting low background, but the very fine powdered material proved to be problematic in handling (Supplemental material). The small particles attached poorly to the algal surface and could only be transferred incompletely into the extraction cartridges.

Fully endcapped silica gel 90 C18 material (90 Å pore size, 40–63 μm particles, Sigma-Aldrich) proved to be superior with respect to the low background and the ease of handling (Figure S4).

The recovery of fucoxanthin was tested over 5, 30, 60, 120, 300 and 600 s incubation with C18 material. Since no substantial differences were noticed in three repeated measurements (data not shown), an extraction period of 60 s was chosen due to ease of handling. The lack of time dependence can be explained by the fact that C18 powder takes up metabolites by adsorption, not extraction as in the dipping methods, and causes no damage that would result in additional release of metabolites from the cells under prolonged incubation.

The different methods for application tested included distribution of the powder with a sieve, dusting the material on the alga and shaking the alga gently in a Petri dish with silica gel. Although application using a sieve consumed less material, coverage was higher using the ‘Petri dish method’. For F. vesiculosus a thallus piece of 36.5 ± 6.5 cm² proved to be sufficient for the generation of GC-MS and LC-MS samples. However, smaller sample sizes can be used in cases where biological material is limited, since, of the generated 200 μl UPLC and 100 μl GC samples, only a few microliters were required for analysis. The amount of C18 material recovered in the cartridge, weighed at the end of the experiment after the complete evaporation of the remaining elution solvent, was 0.13 ± 0.01 g. Again, in cases of limitation of biological material, sensitivity could be increased by more quantitative washing off and recovery of the material. Even if the loss of absorbing material is high, this method gave the most uniform coverage of the alga and allowed easy handling. The excess material did not contain any detectable amounts of metabolites and therefore did not contribute to a loss of extract yield. The incubation time of 60 s represented the best compromise between a good interaction and absorption of metabolites with C18 material and an easy recovery of the powder from the alga.

Microscopic observation

It is essential for a method focused on the determination of surface metabolites that cellular integrity is maintained throughout the entire procedure. Otherwise the overlaying effects of metabolites released from lysed cells would be detected and no estimation of surface concentrations would be possible. To monitor for surface cell integrity, the algal surfaces were documented in microscopic pictures after applying the C18 extraction method for 5, 60 and 300 s as indicated above (Figure 2a, Figure S1). For comparison, surfaces of algae from the same batch were
investigated after applying the ‘hexane/methanol dipping’ treatment performed as described in Lachnit et al. (2010). Additionally a control group that was taken out of the aquarium for the same time without being extracted was evaluated. Independent of the incubation time with the C18 material, visual inspection revealed that surface cells were not damaged or otherwise altered. In strong contrast, even after extraction for only 5 s with the ‘dipping’ method, the color of the *F. vesiculosus* surface changes from the typical yellow-brown to green, which is indicative of damage and pigment loss from the surface cells. Also fronds of *C. taxifolia* did not show any signs of damage after C18 treatment as examined by light microscopy (Figure S1). To visualize dead cells, staining with Evans Blue (Weinberger et al. 2005) and RGB (red, green, blue) evaluation of the microscopic images was carried out. This test confirmed that even after hexane/methanol dipping for 5 s, cells were damaged as indicated by a substantial change in the red/green ratio, while values did not differ significantly from controls when using the C18 method (Figure 2b). Despite its broad application, the Evans Blue staining method is not without problems for the investigation of algal surfaces, since oxidation of the algal pigment after chloroplast rupture cannot be clearly distinguished from the effects of Evans Blue staining.

**UPLC-MS measurements**

The methanolic surface extracts resulting from the C18 method can be directly submitted to HPLC or UPLC-MS analysis without any further concentration steps. In initial experiments the presence of the carotenoid fucoxanthin was monitored. Fucoxanthin is a dominant pigment of brown algae such as *Fucus* spp. This metabolite is ideally suitable for method development and comparison since earlier studies using the hexane/methanol dipping method as well as Raman-imaging indicated that this compound is released into the surface environment of *Fucus* (Saha et al. 2011; Grosser et al. 2012). Fucoxanthin was identified in UPLC-MS measurements by comparing its retention time and ESI mass spectrum with a commercial standard (both 681 m/z ([M+Na]+ at 2.43 min). Fucoxanthin can be clearly detected in the samples extracted with the C18 absorbing material.

**Quantification of surface metabolites**

For quantitative determination, canthaxanthin, another carotenoid pigment that is not found in *F. vesiculosus*, was used as internal standard. Figure 3a shows the average chromatographic area of fucoxanthin extracted from *F. vesiculosus* with the C18 method in relation to the average chromatographic area of the internal standard canthaxanthin. Values are given for extracts of five specimens, with an average extracted surface of 36.5 ± 6.4 cm². Since extracts were split into two equal parts for GC-MS and LC-MS determination the values determined correspond to an 18.25 cm² surface area. The standard deviations of fucoxanthin and canthaxanthin determinations are similar (5.66 and 5.26 of mean peak area). Since canthaxanthin was introduced after the extraction protocol was performed, this indicates very good reproducibility of the extraction.
became clear that the absolute values detected using dipping methods are highly dependent on the polarity of the solvent, thus a quantitative comparison of the methods is not advised. It also cannot be finally answered whether the C18 method quantitatively extracts surface metabolites and whether the hexane/methanol overestimates the content due to cell lysis, or whether the C18 method underestimates surface metabolites due to non-quantitative extraction. The reproducibility of the C18 method, however, suggests a highly reliable measurement. The standard deviation of signals in the C18 extract is substantially lower than that in hexane/methanol extracts. Given the fact that the C18 method is far more reproducible and that it does not introduce cell damage, this method has to be considered as superior. Lower recovery is not problematic, since even small thallus fragments provide sufficient extract for the entire analytical process.

To test the universal applicability of the C18 method two macroalgae with hitherto unknown surface chemistry were also investigated. No adaptation in the protocol was required for surface extraction of either the green alga *C. taxifolia* or the red alga *G. vermiculophylla*. In the case of *C. taxifolia*, 45.8 ± 4.0 cm$^2$ (*n* = 3) algal surface was extracted without causing damage (Figure S1) and LC-MS revealed the presence of caulerpenyne (identified by comparison with authentic material; Jung & Pohnert 2001). Again, elevated amounts were detected using the procedure. Only minor additional variability in comparison to sample drying, re-dissolution and measurement is introduced by the C18 extraction procedure. The C18 method was compared to the established hexane/MeOH dipping. Recovered fucoxanthin in dipping experiments was significantly higher compared to the C18 experiments (Figure 3b). This could be due to overall better extraction success, or to contributions of fucoxanthin released by the lysed cells in the hexane/MeOH treatment. An external calibration based on the evaluation of the peak areas of the standard canthaxanthin in relation to areas resulting from different amounts of fucoxanthin (Figure S5) allowed the extracted fucoxanthin to be estimated. Even if quantification was problematic, as no reference method for the determination of absolute amounts of surface chemicals was available, the procedure allowed the extraction success of this study to be compared with studies in the literature. C18 extraction gives ~1.4 ± 0.28 (SD) μg of fucoxanthin cm$^{-2}$ while the hexane/methanol dipping recovered absolute amounts of ~22 ± 18.95 (SD) μg of fucoxanthin cm$^{-2}$. Previous studies using the dipping method gave similar values (0.7–9 μg of fucoxanthin cm$^{-2}$) and it can be concluded that algae in the present study and the analytical work-flow match those in the literature within the margins of error and natural and experimental variability (Saha et al. 2011). The hexane dipping procedure did not result in any detectable fucoxanthin (data not shown).
hexane/methanol dipping protocol. Since caulerpenyne is a very dominant intracellular metabolite, this elevated value might be interpreted as a result of unwanted extraction of algal cells. Caulerpenyne is involved in chemical defense (Weissflog et al. 2008) and wound closure (Adolph et al. 2005) by the alga. This is the first report demonstrating that caulerpenyne is also present at the surface of the alga (Figure 3c) and motivates further investigation of its potential role as a surface defense compound or a natural antifouling metabolite. As with \textit{F. vesiculosus} extracts, hexane/methanol dipping resulted in overall higher caulerpenyne recovery, but with a very high standard deviation (Figure 3d). Quantitative differences might again be explained by substantial damage after hexane/methanol extraction, as was observed by visual inspection. Damage was not quantified since a substantial amount of algal material was physically degraded during the dipping procedure. However, a poor extraction efficiency with C18 powder could result in reduced recovery. Initial experiments with \textit{G. vermiculophylla} revealed ion traces corresponding to previously identified oxylipins from whole tissue extracts of the alga (data not shown) (Nylund et al. 2011; Rempt et al. 2012).

**GC-MS measurements**

Since the C18 material is suitable for extracting a broad range of non-polar and medium polar compounds, its capability to extract structurally diverse surface metabolites was investigated. Since LC-MS techniques do not allow easy compound identification the exploratory power of GC-MS supported by library identification of metabolites was used to test for additional compound classes picked up by the C18 method. The major metabolites that were extracted from \textit{F. vesiculosus} surfaces were fatty acids that were transformed by the solvent MeOH to the corresponding methyl esters during elution. This transformation was verified by control measurements where EtOH instead of MeOH as elution solvent was used and where ethyl esters instead of methyl esters were detected in the extracts. Characteristic fragments of 79 \(m/z\) ([C\(_6\)H\(_7\)]\(^+\) indicative for polyunsaturated fatty acids) and 74 \(m/z\) (a McLafferty ion indicative of saturated fatty acids) were detected and elucidation of structure was performed by comparison with authentic standards (FAME, Sigma-Aldrich). A representative chromatogram and the assigned metabolites can be found in Figure 4. Fatty acids are common in brown algae (Pereira et al. 2012; Schmid & Stengel 2015), but were previously never detected as surface metabolites. Their presence in surface extracts can be explained by an active release mechanism of free fatty acids or alternatively by a partial hydrolysis of lipids on the surface of the alga. The fact that fatty acids as surface metabolites were overlooked until now might be due to the limitations of previous experimental approaches. In accordance, samples that were generated with the hexane/methanol dipping method contained only a few fatty acids and only in trace quantities (GC/MS after derivatization; data not shown). In addition to free fatty acids, substantial

![Figure 4. GC-MS run of a C18 extract of \textit{F. vesiculosus} performed with the C18 method and with MeOH as elution solvent. The range of elution of fatty acid methyl esters is shown. A, myristic acid methyl ester (C14:0); B, C15:0; C, C16:1 ((Z)9-hexadecenoic acid (palmitoleic acid) methyl ester); D, C16:0, *phthalate (contamination); E, C18:3 9,12,15-octadecatrienoic acid (\(\alpha\)-linolenic acid) methyl ester; F, C18:2 (9,12-octadecadienoic acid (linoleic acid) methyl ester); G, C18:1 (9-octadecenoic acid (oleic acid) methyl ester); H, C18:0 (octadecanoic acid (stearic acid) methyl ester); I, C20:4 (5,8,11,14-eicosatetraenoic acid (arachidonic acid) methyl ester); J, C20:5 (5,8,11,14,17-eicosapentaenoic acid methyl ester). All fatty acid methyl esters were confirmed with synthetic standards.](image-url)
amounts of phytol and an unidentified identified steroid were found, confirming the broad extraction potential of the C18 method.

Conclusions

Investigation of algal surface chemistry is central for the understanding of ecological interactions on and around the organisms. The most commonly used methods involve solvent dipping of the specimens and investigation of the resulting extracts (de Nys et al. 1998; Lachnit et al. 2010). However, these methods pick up compounds from a very limited polarity range and can cause substantial damage to the algal tissue. Alternatively, the expensive instrumentation required for desorption electrospray MS (Lane et al. 2009) or Raman techniques (Grosser et al. 2012) is needed for surface investigations. The method reported is based on covering the algal surface with C18 extraction sorbent and collecting the material for subsequent extraction, and did not cause surface damage to the investigated algae. It is universal and suitable for detecting a wide range of natural substances of different polarity. Its ease of handling and the reliability of the results reflected by low standard deviations makes it a universal tool for future investigations. The method that was validated for algal surface extraction in this study is potentially easily transferred to the investigation of other aquatic organisms and even submerged technical surfaces.

Acknowledgements

Florian Weinberger, Dominique Jacquemoud, Constanze Kuhlisch, Esther Rickert and Martin Rempt are acknowledged for assistance during algal collection.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The authors thank the Jena School for Microbial communication and the International Max Planck Research School on the Exploration of Ecological Interactions with Molecular and Chemical Techniques and the EU Marie Sklodowska-Curie Initial Training Network (ITN) program Algal Microbiome: Friends or Foes (ALFF) for funding.

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