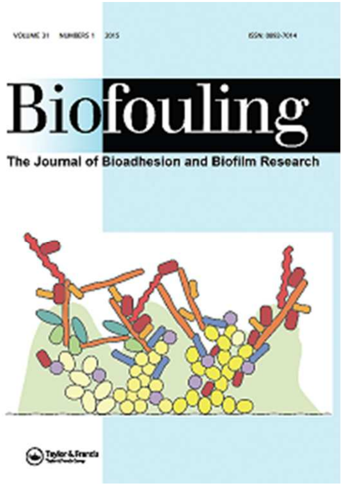

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Journal:	*Biofouling*
Manuscript ID	GBIF-2015-0072.R1
Manuscript Type:	Original Paper
Keywords:	Surface chemistry, extraction protocol, macroalgae, natural products chemistry, non-disruptive, chemical ecology

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 is decisive for their biotic interactions. Metabolites in the spatially limited laminar boundary layer mediate processes, such as anti-fouling, allelopathy and chemical defense against herbivores. However, very few methods are available for the investigation of such surface metabolites. Here we introduce an approach 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 superior to established solvent dipping protocols since it does not cause damage to the test organisms. The method was developed for the macroalgae *Fucus vesiculosus*, *Caulerpa taxifolia* and *Gracilaria vermiculophylla*, but can be easily transferred to other aquatic organisms.

Key words

Surface chemistry, extraction protocol, macroalgae, natural products chemistry, non-disruptive, chemical ecology

Introduction

Natural products play a fundamental role in ecological interactions on biotic surfaces under water. Surface metabolites can e.g. act on the interface of water and macroalgae, corals or sponges. Such compounds control settling processes, regulate predator / prey relationships and mediate infection processes (da Gama et al. 2014, Dobretsov et al. 2013, Wahl 2009). But also control of competitors by means of allelochemical activity (Gross 2003, Lu et al. 2011, Rasher et al. 2011) and regulation of fouling is influenced by these natural products (da Gama et al. 2014, Dobretsov et al. 2013). A hallmark of such interactions is the locally much focused action of the compounds in question. Indeed, 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 (Grosser et al. 2012, Hurd 2000). The actual surface concentrations are highly important if ecologically relevant effects are under consideration (Dworjanyn et al. 1999, Dworjanyn et al. 2006). Nevertheless, until now most investigations on the effect of surface metabolites were based on bioassays with extracts of whole organisms, or with compounds applied in concentrations found whole tissue extracts (see e.g. 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 experiment planning and evaluation. Studies performed with resonance Raman micro spectroscopy allowed to visualize the gradient of carotenoids in this boundary layer around the macroalgae *Fucus vesiculosus* and *Ulva mutabilis*. A pronounced decline of concentration from up to millimolar values in the immediate vicinity of the algal surface to concentrations below the detection limit in 100 µm distance was observed (Grosser, et al. 2012). Besides this elaborate method that is limited to very few Raman active metabolites, only relatively few approaches have been reported to determine surface concentrations on surfaces of marine organisms. Most investigations of algal surface chemistry rely on 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 submitted to analytical methods, such as GC-MS and LC-MS. Although useful, dipping methods are rather problematic since solvent exposure can cause cell lysis and thereby contamination of surface extract with intracellular metabolites. Algae only tolerate exposure to rather unpolar solvents such as hexane for few seconds. However these solvents only cover a very limited range of unpolar 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, we developed a new, non-destructive solvent free and universal method for extracting secondary metabolites from marine macroorganisms. The method is based on the adsorption of organic metabolites onto C18 extraction sorbent and has been optimized in terms of recovery, reproducibility and ease of use with the brown macro alga *Fucus vesiculosus* as 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 anti-fouling capacity (Lachnit et al. 2013, Lachnit, et al. 2010, Saha et al. 2012, Saha et al. 2011). But also 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 on 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 algae were transported in plastic bags with pulp paper moistened with Baltic sea water at maximum 18°C to the University of Jena. Algae were immediately cleaned with deionized water to reduce epibionts. Then each individual was put into a 7 L aquarium filled with Instant Ocean Medium (Instant Ocean, Blacksburg, Virginia, USA), which was adjusted to the salinity of the Baltic Sea (14-16 PSU). The aquaria were kept in a temperature controlled (15 °C) climate chamber under a constant 14 h / 10 h light / dark regime (light intensity of 65 µmol m^{-2} s^{-1}) with aquarium pumps guaranteeing constant ventilation. In the first week, it is necessary to change water every two or three days in order to keep the algae clean, afterwards weekly change of water is required. Under these conditions algae survived in good shape for three weeks or up to a month. *Caulerpa taxifolia* was obtained by a tropical fish store (Aqua-Reptil-World, Jena, Germany) and transported to the lab in a plastic bag. Algae were washed carefully with deionized water and put into 7 L aquaria filled with Instant Ocean medium adjusted to Mediterranean salinity. 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 by 40 µmol m^{-2} s^{-1}. *Gracilaria vermiculophylla* was collected in the Kiel Fjord (54° 21'36.8" N, 10° 10' 44.0" E) during the last days 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, algae were washed carefully with medium and put into 7 L aquaria filled with Instant Ocean Medium (Instant Ocean, Blacksburg, Virginia, USA), which was adjusted to the salinity of the Baltic Sea (14-16 PSU). The aquaria were kept under comparable conditions as those of *C. taxifolia*.

Materials

All reagents used were of analytical grade or superior purity. The absorption material used was a fully encapped silica Gel 90 C18 material (pore size 90 Å, particle's dimensions 40-63 µm, Sigma-Aldrich, Germany). For collection of absorption material, empty 6 mL polypropylene columns with PE frits (CHROMABOND, 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 algae (number of replicates n=5) were taken out of the tanks and hanged on clamps for ca. 2 minutes, in order to let most of the water drip off. This resulted in wet algal surfaces with comparable amounts of surface water. Algae should not be 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, weighted with a Kern ALJ 220-4 balance) was spread in 58 cm^2 Petri dishes. Then 36.5 ± 6.5cm^2 fragments of *F. vesiculosus* were put into the petri dishes and, after closing, the dishes were gently shaken for ca 10 seconds, in order to obtain a full and uniform coverage of the algal surface with the absorption material (the entire procedure is illustrated in Figure 1). The extracted alga's surface was determined by taking photos of the algae 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 got into contact with it remained attached on the surface. The excess remaining material in the petri dish (ca. 0.4 g) did not contain any detectable surface metabolites (verified by UPLC/MS see below) and could be discarded. After covering with C18 material, the algae were left for 60 s in the Petri dishes without moving them. This incubation time was optimized for recovery of fucoxanthin in several experiments (20 to 300 s). Subsequently, the alga was rinsed with an excess of artificial sea water to wash of 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 vacuum was applied (ca. 550 Torr). The C18 absorption material settles at the bottom of the cartridge and attention must be paid not to dry the powder. The funnel and the C18 material were washed three times with 10 mL deionized water, in order to remove salts. Metabolites adsorbed on the C18 material were eluted with 3 x 0.5 mL of MeOH. The extracts were combined and splitted 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 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 can be stored at -20°C until further measurements.

After extraction algae were observed under a confocal light microscope (BX40, 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® (Massachusetts, USA) ACQUITY UPLC-MS system with a Micromass ® Q-ToF micro ESI-TOF mass spectrometer. 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, Biosolv) 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 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 wash step the polarity of the eluent was increased till 6 min (5 % B) and held till 6.5 min. Till 7.5 min the solvent was re-adjusted to 50 % B. The injection volume was of 10 µL, by means of an autosampler. 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 Thermo Scientific® (Massachussets, USA) Trace GC-ULTRA system coupled to a Thermo Scientific® ISQ EI-mass spectrometer, equipped with a quadrupole analyzer. The column used was an Agilent® 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 gas carrier 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).

Results and Discussion

Development of the extraction procedure

The optimum extraction method for surface metabolites is the one which maximizes 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 unipolar solvent allows only extraction of unpolar 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). We therefore developed an extraction method 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-encapped silica Gel 100 C18 reversed phase material (100 Å pore size, 40-63 µm particle's dimensions) 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 encapped silica Gel 100 C18 reversed phase material (100 Å pore size, 15-25 µm particle's dimensions) was suitable for extraction of surface metabolites exhibiting low background but the very fine powdered material proved to be problematic in the handling (Supplemental information). The small particles attached poorly to the algal surface and could only be transferred incompletely into the extraction cartridges. encappedFully encapped silica Gel 90 C18 material (90 Å pore size, particle's dimensions 40-63 µm, Sigma-Aldrich, Germany) proved to be superior with respect to the low background and the ease of handling (Supplemental Material, Figure 4) .

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. Even if application using a sieve consumed less material, coverage was higher using the "Petri-dish method" that was further pursued. For *F. vesiculosus* a thallus piece of 36.5 ± 6.5 cm^2 proved to be sufficient for the generation of GC-MS and LC-MS samples. However, smaller sample sizes could be envisaged in cases of limitation of biological material since of the generated 200 µL UPLC and 100 µL GC samples only few microliters were required for analysis. The amount of C18 material recovered in the cartridge, weighted at the end of the experiment after the complete evaporation of the remaining elution solvent, was 0.13 ± 0.01 g. Again, in case 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 gives the most uniform coverage of the alga and allows an easy handling. The incubation time of 60 s represents 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 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 seconds as indicated above (Figure 2a, Supplemental material 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 C18 material, visual inspection revealed that surface cells were not damaged or otherwise altered. In strong contrast, even after only 5 seconds of extraction with the "dipping" method, the colour of the *F. vesiculosus* surface changes from the typical yellow-brown to green, which is indicative for damage and pigment loss of the surface cells. Also fronds of *C. taxifolia* did not show any signs of damage after C18 treatment as examined by light microscopy (Supplemental material Figure S1). To visualize dead cells, they were stained with Evans Blue (Weinberger et al. 2005) and RGB (red, green, blue) colours of the recorded microscopic images were analysed. This test confirmed that already after 5 seconds of hexane / methanol dipping, cells were damaged as indicated by a substantial change in red/green ratio, while values did not differ significantly from controls when using the C18 method (Figure 2b). It has to be noted that, despite its broad application, the Evans Blue staining method is not unproblematic 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 step. In initial experiments we monitored for the presence of the carotenoid fucoxanthin, a dominant pigment of brown algae such as *Fucus*. This metabolite is ideally suitable for method development and comparison since earlier studies using the hexane / methanol dipping method as well as Raman-imaging already indicated that this compound is released into the surface environment of *Fucus* (Grosser, et al.

2012, Saha, et al. 2011). 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 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 5 specimens, with an average extracted surface of 36.5 ± 6.4 cm^2. Since extracts were split into two equal parts for GC-MS and LC-MS determination the values determined correspond to a 18.25 cm^2 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 a very good reproducibility of the extraction procedure. Only minor additional variability in comparison to sample drying, re-dissolution and measurement is introduced by the C18 extraction procedure. We compared the C18 method to the established hexane / MeOH dipping. Recovered fucoxanthin in dipping experiments is significantly higher compared to C18 experiments (Figure 3b). This can be due to overall better extraction success, but most likely also 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 (Supplemental Figure S3) allowed estimating the extracted fucoxanthin. Even if quantification is problematic since no reference method for the determination of absolute amounts of surface chemicals is available the procedure allows relating extraction success of this study to studies in the literature. C18 extraction gives ca. 1.2 µg fucoxanthin cm^{-2} while the hexane / methanol dipping recovers absolute amounts of ca. 14 µg fucoxanthin cm^{-2}. Previous studies using the dipping method gave similar values (0.7-9 µg fucoxanthin cm^{-2}) and it can be concluded that algae in our study and the analytical work-flow are matching those in the literature within the margins of natural and experimental variability (Saha et al. 2011). It can however not be finally answered if the C18 method quantitatively extracts surface metabolites and if hexane / methanol overestimates the content due to cell lysis or if 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 facts that the C18-method is by far better 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. Extraction and measurement can be easily carried out without additional concentration steps using routine instrumentation. Experiments using hexane as extraction solvent did not result in detectable amounts of extracted fucoxanthin (data not shown). This solvent that causes minimum damage of algal surfaces is thus not suitable for the extraction of the metabolite using a sample size sufficient for the C18-method and hexane / methanol dipping.

To test the universal applicability of the C18-method we also investigated two macroalgae with hitherto unknown surface chemistry. No adaptation in the protocol was required for surface extraction of the green alga *Caulerpa taxifolia* and the red alga *Gracilaria vermiculophylla*. In the case of *C. taxifolia* 45.8±4.0 cm^2 (n = 3) algal surface was extracted without causing damage (Supplemental material Figure S1) and LC-MS revealed the presence of caulerpenyne (identified by comparison to authentic material (Jung and Pohnert 2001)). Again, elevated amounts were detected using the hexane / methanol dipping protocol. Since caulerpenyne is a very dominant intracellular metabolite, this elevated value can 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) of the alga. This is the first report demonstrating that caulerpenyne is also present at the surface of the alga (Figure 3c) motivatin further investigation of its potential role as surface defense compound or natural anti-fouling metabolite. As with *F. vesiculosus* extracts, hexane / methanol dipping resulted in overall higher caulerpenyne recovery, but also in substantial cell damage (data not shown) and a very high standard deviation (Figure 3d). Initial experiments with *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 to extract a broad range of non-polar and medium polar compounds, we tested its capability to extract structurally diverse surface metabolites. Since LC-MS techniques do not allow for easy compound identification we used the exploratory power of GC-MS supported by library identification of metabolites to test for additional compound classes picked up by the C18 method. The major metabolites that were extracted from *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* ($[C_6H_7]^+$ indicative for polyunsaturated fatty acids) and 74 *m/z* (a McLafferty ion indicative for saturated fatty acids) were detected and structure elucidation was performed by comparison with authentic standards (FAME, Sigma- Aldrich©, Germany). 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 and Stengel 2015), but were previously never detected as surface metabolites. Their presence in surface extracts can be explained with an active release mechanism of free fatty acids or alternatively with a partial hydrolysis of lipids on the surface of the alga. The fact that fatty acids as surface metabolites were overlooked till now might be due to limitations of previous experimental approaches. In accordance, samples that were generated with the hexane / methanol dipping method contained only few fatty acids and only in trace quantities (GC/MS after derivatisation, data not shown). In addition to free fatty acids we could identify substantial amounts of phytol and a not further identified steroid, confirming the broad extraction potential of the C18-method.

Conclusions

Investigation of the algal surface chemistry is central for the understanding of ecological interactions on and around these 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 in a very limited polarity range and can cause substantial damage to the algal tissue. Alternatively, expensive instrumentation required for desorption electrospray MS (Lane et al. 2009) or Raman techniques (Grosser, et al. 2012) is needed for surface investigations. The introduced method that is based on covering the algal surface with C18 extraction sorbent and collecting the material for subsequent extraction does not cause surface damage in 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 reliable results reflected by low standard deviations make 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.

Acknowledgments

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

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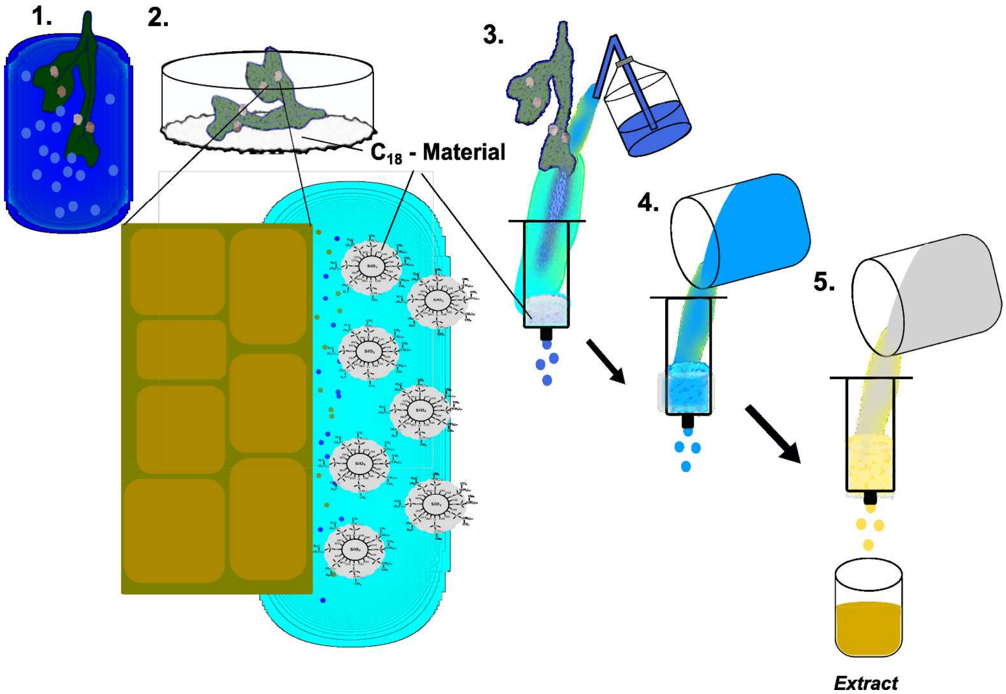
Figure legends

Figure 1 Schematic work flow of the C18 method. 1) Algae are removed from the water and left for 2 min to remove excess water by dripping; 2) algae are transferred to Petri dishes and covered with absorption material; 3) the C18 material is washed off with excess sea water 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.

Figure 2 Evaluation of surface damage by different extraction methods. A) Photographs of *F. vesiculosus* surfaces (Scale bars 100 µm). Top row control after removal from water for 5, 60 and 600 seconds. Middle row algae after C18 extraction and lowest row after hexane / methanol dipping for the same time spans. B) Evaluation of cell damage after Eavns blue staining by red / green ratio analysis at 5, 30, 60, 120, 300, 600 seconds exposure to C18 material (grey), hexane / methanol dipping (white) and control (black), (n = 5 ± SD)

Figure 3 a,b) Average chromatographic areas of fucoxanthin extracted from *Fucus vesiculosus* surfaces in relation to cantaxanthin as internal standard (Std.): a) C18 method, n = 5, surface extracted = 36.5 ± 6.4 cm^2 ; b) hexane / methanol dipping method, n=5, surface extracted = 29.3 ± 3.0 cm^2. c, d) Average chromatographic areas of caulerpenyne extracted from *Caulerpa taxifolia* surfaces and cantaxanthin as internal standard. c) C18 method, n=3, mean surface extracted= 45.8 ± 4.0 cm^2 ; d) hexane / methanol dipping method, n=3, mean surface extracted= 36.0 ± 7.3 cm^2.

Figure 4 GC-MS run of a C18 extract of *F. vesiculosus* performed with 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 (α-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.



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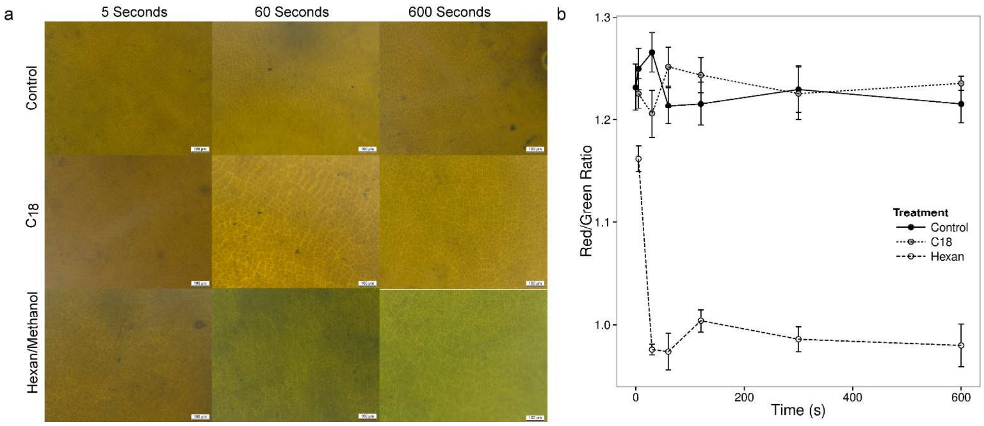

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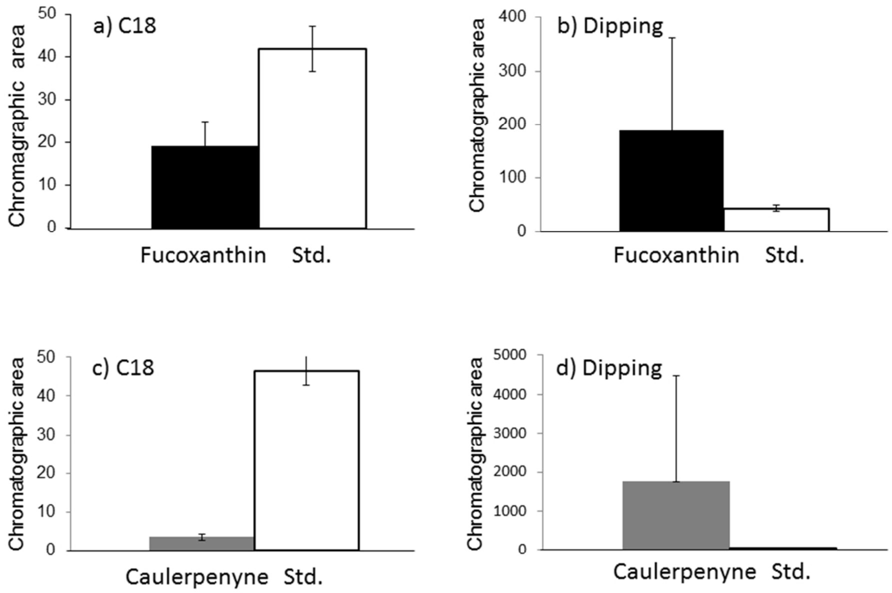

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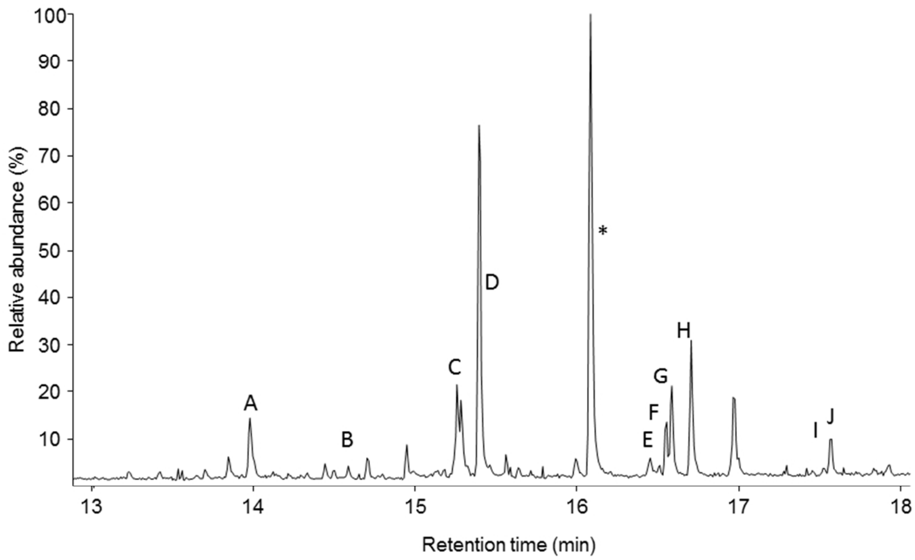

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Supplemental material

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

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1) Effect of *Caulerpa taxifolia* extraction

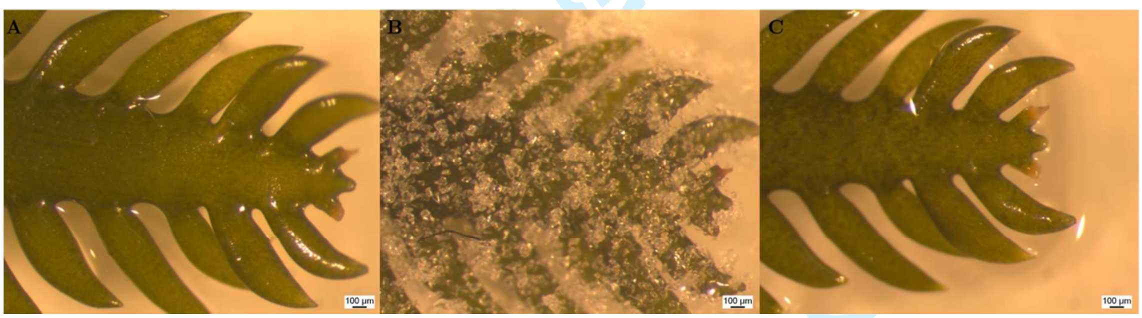

Figure S1: The pictures (light microscopy) show a *Caulerpa taxifolia* fronds before (left), during (center) and after a 1 min treatment with C18 material.

2) Evaluation of different C18 materials

Non-endcapped silica Gel 100 C18 reversed phase material (100 Å pore size, 40-63 µm particle's dimensions, Sigma-Aldrich©, Germany) was suitable for extracting fucoxanthin but was not suitable for GC-MS measurements. Some intense peaks of impurities between 14.5 and 18 min interfered with signals from the samples. These peaks cannot be completely removed even after excess conditioning of the powder with the extracting solvent.

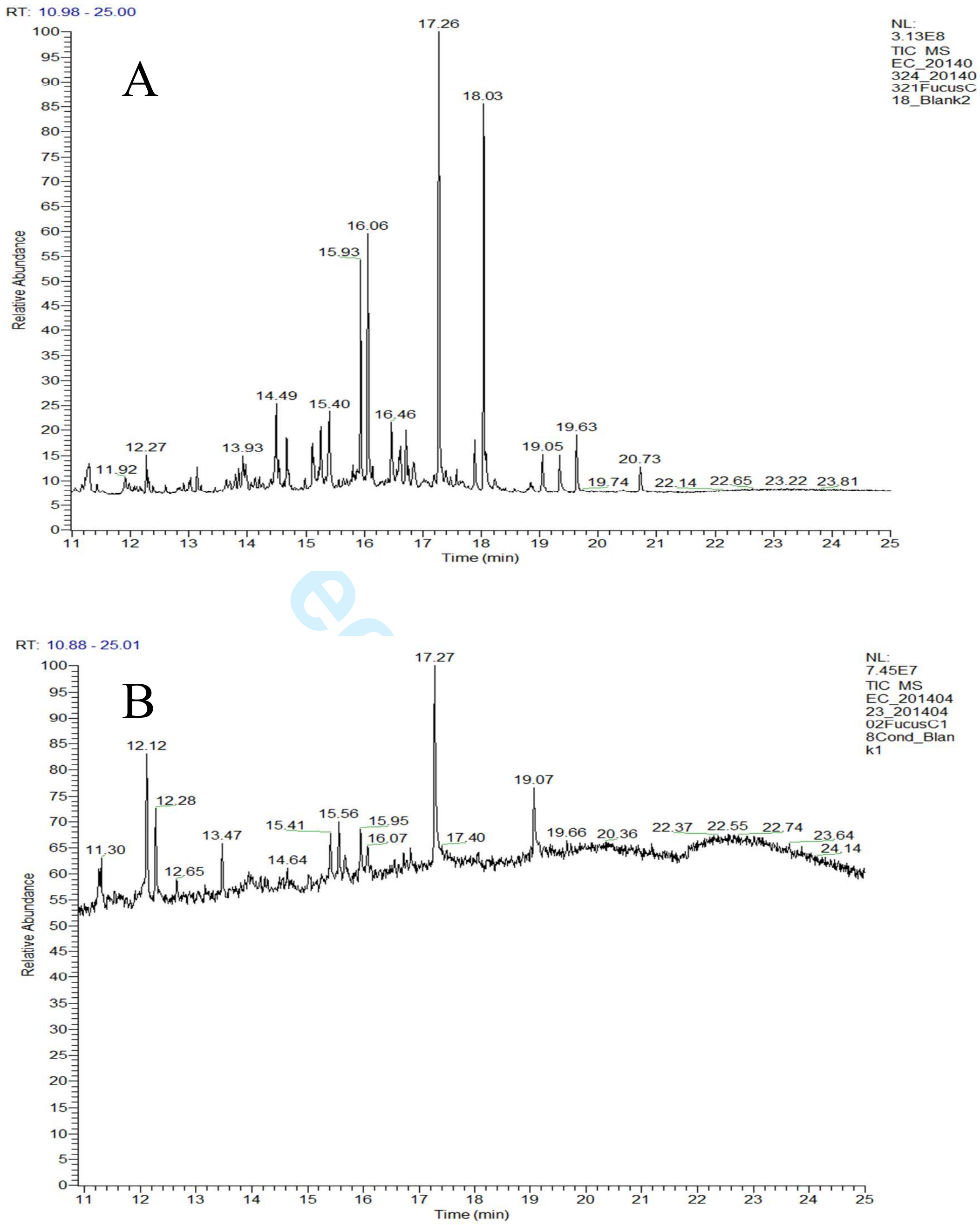

Figure S2: GC/MS profiles of C18 material (non endcapped) blank A) before and B) after conditioning with eluting solvent. All peaks did also show up in C18 surface extracts.

Fully endcapped silica Gel 100 C18 reversed material (100 Å pore size, 15-25 µm particle's dimensions, Sigma-Aldrich©, Germany) was suitable for extraction of surface metabolites (Figures S2 and S3). Contaminants did not substantially interfere with measurements, since the total ion current (TIC) of the blank was lower than the one of the samples and background subtraction was possible. Finally the silica Gel 100 was not further considered because of problems in the handling due to the very small size of the particles. The fine dust hardly stuck to the surface of the algae and could not be transferred quantitatively into the SPE cartridge.

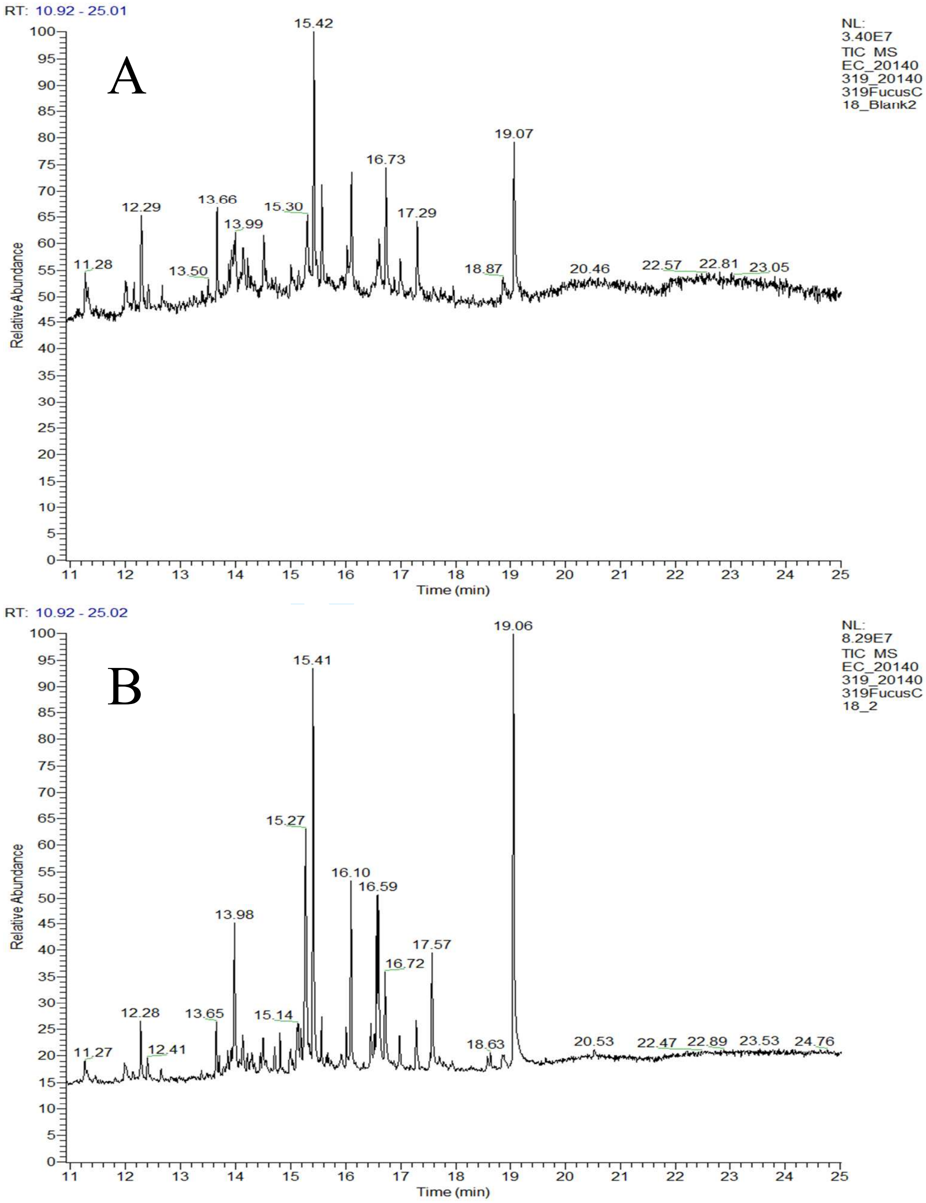

Figure S3: GC/MS profiles of silica Gel 100. A) Blank B) extract of *Fucus vesiculosus*.

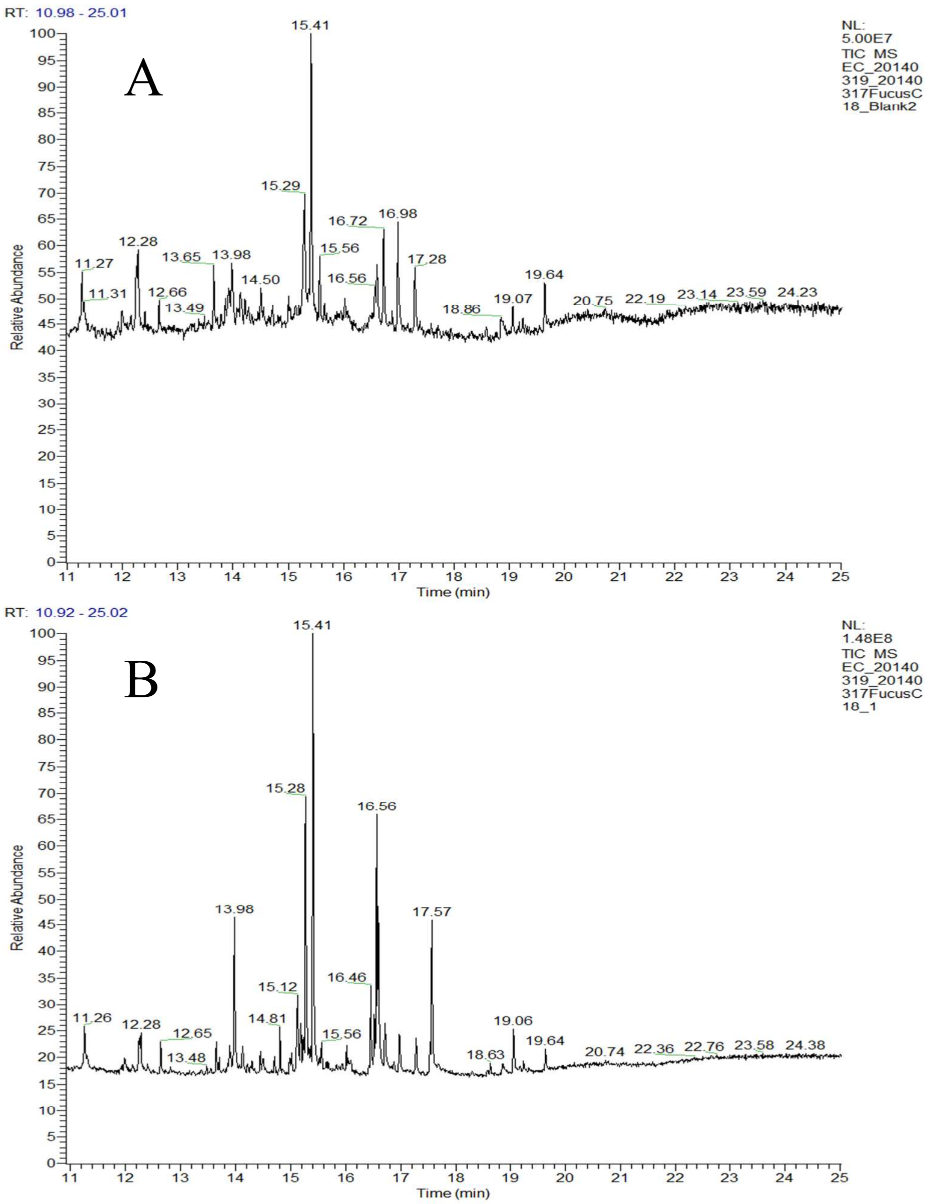

Figure S4: : GC/MS profiles of of silica Gel 90. A) blank B) extract of *Fucus vesiculosus*.

Calibration

To prepare the external calibration curve using the ration of peak areas of fucoxanthin and cantaxanthin, 0,66 mg of fucoxanthin (purity ≥95%, Sigma Aldrich©, Germany) were weighted and dissolved in 10 mL methanol in order to obtain a 100 μM stock solution. From this stock, several dilutions at different concentration were prepared (1 μM, 750 nM, 500 nM, 250 nM, 100 nM). 10 μL of a 20 μM canthaxanthin (analytical standard grade, Sigma Aldrich©, Germany) solution in methanol were added to 190 μL of each sample in order to have a final concentration of 1 μM of canthaxanthin. After this, every point of the curve was measured 3 times using the UPLC-MS method described in the main text.

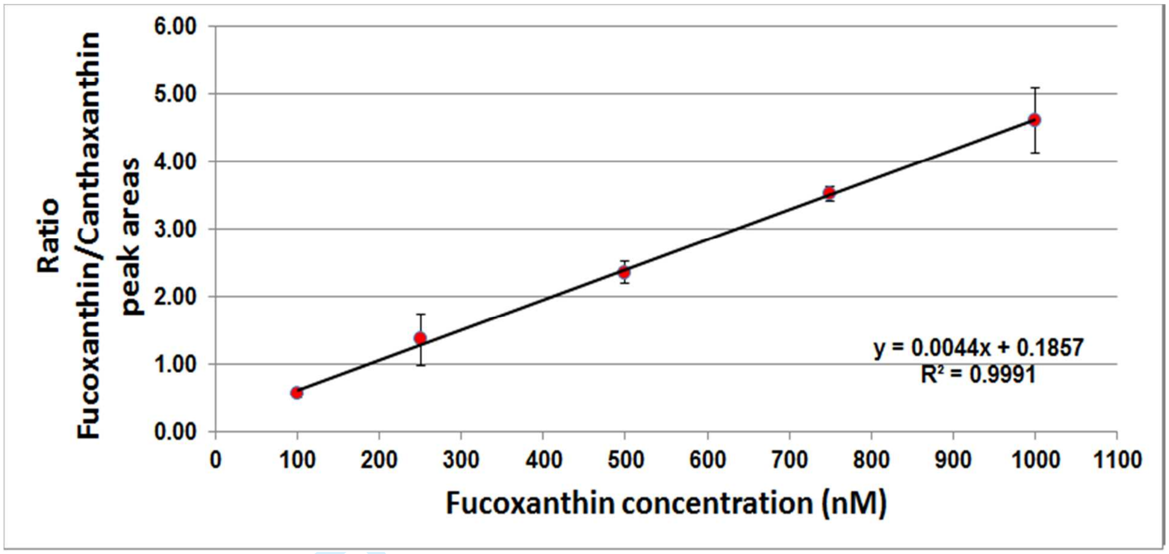

Fig S5: Calibration curve used for quantitative determination of the fucoxanthin content in extracts.

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 is decisive for their biotic interactions. Metabolites in the spatially limited laminar boundary layer mediate processes, such as anti-fouling, allelopathy and chemical defense against herbivores. However, very few methods are available for the investigation of such surface metabolites. Here we introduce an approach 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 (~~LC-MS and~~ GC~~-~~/MS) investigations. The method is robust, picks up metabolites of a broad polarity range and is easy to handle. It is superior to established solvent dipping protocols since it does not cause damage to the test organisms. The method was developed for the macroalgae *Fucus vesiculosus*, *Caulerpa taxifolia* and *Gracilaria vermiculophylla*, but can be easily transferred to other aquatic organisms.

Key words

Surface chemistry, extraction protocol, macroalgae, natural products chemistry, non-disruptive, chemical ecology

Introduction

Natural products play a fundamental role in ecological interactions on biotic surfaces under water. Surface metabolites can e.g. act on the interface of water and macroalgae, corals or sponges. Such compounds control settling processes, regulate predator / prey relationships and mediate infection processes (da Gama et al. 2014, Dobretsov et al. 2013, Wahl 2009). But also control of competitors by means of allelochemical activity (Gross 2003, Lu et al. 2011, Rasher et al. 2011) and regulation of fouling is influenced by these natural products (da Gama et al. 2014, Dobretsov et al. 2013). A hallmark of such interactions is the locally much focused action of the compounds in question. Indeed, 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 (Grosser et al. 2012, Hurd 2000). The actual surface concentrations are highly important if ecologically relevant effects are under consideration (Dworjanyn et al. 1999, Dworjanyn et al. 2006). Nevertheless, until now most investigations on the effect of surface metabolites were based on bioassays with extracts of whole organisms, or with compounds applied in concentrations found whole tissue extracts ~~(Dworjanyn et al. 1999, Dworjanyn et al. 2006~~(see e.g.~~,~~ Hellio et al. 2000). Such ~~samples~~ 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 ~~can affect ecological processes in this region~~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 experiment planning and evaluation. Studies performed with resonance Raman micro spectroscopy allowed to visualize the gradient of carotenoids in this boundary layer around the macroalgae *Fucus vesiculosus* and *Ulva mutabilis*. ~~There a~~A pronounced decline of concentration from up to millimolar values ~~close to~~in the immediate vicinity of the algal surface~~s~~ to concentrations below the detection limit in 100 µm distance was observed (Grosser, et al. 2012). Besides this elaborate method that is limited to very few Raman active metabolites, only relatively few approaches have been reported to determine surface concentrations ~~around~~ on surfaces of marine organisms. ~~In~~ Most ~~the~~ investigations of algal surface chemistry~~, the most~~ rely on~~ wide spread approach involves 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 ~~immediately~~ submitted to analytical methods, such as GC-MS and LC-MS. Although useful, ~~these~~ dipping methods are rather problematic since solvent exposure can cause cell lysis and thereby contamination of surface extract with intracellular metabolites. Algae only tolerate exposure to rather unpolar solvents such as hexane for few seconds. However these solvents only cover a very limited range of unpolar metabolites and do not penetrate surface associated water. If~~, in contrast, methanol containing~~ solvent mixtures containing methanol are employed, massive damage of the algae can be observed, thereby questioning the validity ~~of the method~~of results. To overcome these limitations, we developed a new, non-destructive solvent free and universal method for extracting secondary metabolites from marine macroorganisms. The method is based on the adsorption of organic metabolites onto C18 extraction sorbent and has been optimized in terms of recovery, reproducibility and ease of use with the brown macro alga *Fucus vesiculosus* as 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 ~~investigation~~investigations of its chemical defense and anti-fouling capacity (Lachnit et al. 2013, Lachnit, et al. 2010, Saha et al. 2012, Saha et al. 2011). But also 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 on 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 algae were transported in plastic bags with pulp paper moistened with Baltic sea water at maximum 18°C to the University of Jena. Algae were immediately cleaned with deionized water to reduce epibionts. Then each individual was put into a 7 L aquarium filled with Instant Ocean Medium (Instant Ocean, Blacksburg, Virginia, USA), which was adjusted to the salinity of the Baltic Sea (14-16 PSU). The aquaria were kept in a temperature controlled (15 °C) climate chamber under a constant 14 h / 10 h light / dark regime (light intensity of 65 µmol m^{-2} s^{-1}) with aquarium pumps guaranteeing constant ventilation. In the first week, it is necessary to change water every two or three days in order to keep the algae clean, afterwards weekly change of water is required. Under these conditions algae survived in good shape for three weeks or up to a month. *Caulerpa taxifolia* was obtained by a tropical fish store (Aqua-Reptil-World, Jena, Germany) and transported to the lab in a plastic bag. Algae were washed carefully with deionized water and put into 7 L aquaria filled with Instant Ocean medium adjusted to Mediterranean salinity. 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 by 40 µmol m^{-2} s^{-1}. *Gracilaria vermiculophylla* was collected in the Kiel Fjord (54° 21'36.8" N, 10° 10' 44.0" E) during the last days of April / beginning of May 2014 and transported to Jena in plastic bags with pulp paper moistened with Baltic ~~sea~~ Sea water. Once in the laboratory, algae were washed carefully with medium and put into 7 L aquaria filled with Instant Ocean Medium (Instant Ocean, Blacksburg, Virginia, USA), which was adjusted to the salinity of the Baltic Sea (14-16 PSU). The aquaria were kept under comparable conditions as those of *C. taxifolia*.

Materials

All reagents used were of analytical grade or superior purity. The absorption material used was a fully ~~endcapped~~encapped silica Gel 90 C18 material (pore size 90 Å, particle's dimensions 40-63 µm, Sigma-Aldrich, Germany). For collection of absorption material, empty 6 mL polypropylene columns with PE frits (CHROMABOND, 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 algae (number of replicates n=5) were taken out of the tanks and hanged on clamps for ca. 2 minutes, in order to let most of the water drip off. This resulted in wet algal surfaces with comparable amounts of surface water. Algae should not be 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, weighted with a Kern ALJ 220-4 balance) was spread in 58 cm^2 Petri dishes. Then 36.5 ± 6.5cm^2 fragments of *F. vesiculosus* were put into the petri dishes and, after closing, the dishes were gently shaken for ca 10 seconds~~.~~, in order to obtain a full and uniform coverage of the algal surface with the absorption material (the entire procedure is illustrated in Figure 1). The extracted alga's surface was determined by taking photos of the algae 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 got into contact with it remained attached on the surface. The excess remaining material in the petri dish (ca. 0.4 g) did not contain any detectable surface metabolites (verified by UPLC/MS see below) and could be discarded. After covering with C18 material, the algae were left for 60 s in the Petri dishes without moving them. This incubation time was optimized for recovery of fucoxanthin in several experiments (20 to 300 s). Subsequently, the alga was rinsed with an excess of artificial sea water to wash of 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 vacuum was applied (ca. 550 Torr). The C18 absorption material settles at the bottom of the cartridge and attention must be paid not to dry the powder. The funnel and the C18 material were washed three times with 10 mL deionized water, in order to remove salts. Metabolites adsorbed on the C18 material were eluted with 3 x 0.5 mL of MeOH. The extracts were combined and splitted 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 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 can be stored at -20°C until further measurements.

After extraction algae were observed under a confocal light microscope (BX40, 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® (Massachusetts, USA) ACQUITY UPLC-MS system with a Micromass ® Q-ToF micro ESI-TOF mass spectrometer. 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, Biosolv) 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 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 wash step the polarity of the eluent was increased till 6 min (5 % B) and held till 6.5 min. Till 7.5 min the solvent was re-adjusted to 50 % B. The injection volume was of 10 µL, by means of an autosampler. 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 Thermo Scientific® (Massachussets, USA) Trace GC-ULTRA system coupled to a Thermo Scientific® ISQ EI-mass spectrometer, equipped with a quadrupole analyzer. The column used was an Agilent® 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 gas carrier 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).

Results and Discussion

Development of the extraction procedure

The optimum extraction method for surface metabolites is the one which maximizes 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 unipolar solvent allows only extraction of unpolar 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). We therefore developed an extraction method that covers a broad range of metabolites but causes no damage to the algae. The~~e extraction~~is method ~~developed in this work~~ relies on the absorption capabilities of solid-phase extraction material. By powdering the algal surface with this material, metabolites ~~that are found~~ in the boundary layer around the alga~~l~~ 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-encapped silica Gel 100 C18 reversed phase material (100 Å pore size, 40-63 µm particle's dimensions) 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~~ Fully encapped silica Gel 100 C18 reversed phase material (100 Å pore size, 15-25 µm particle's dimensions) was suitable for extraction of surface metabolites exhibiting low background but the very fine powdered material proved to be problematic in the handling (Supplemental information). The small particles attached poorly to the algal surface and could only be transferred incompletely into the extraction cartridges. ~~and fully endcapped~~encapped~~ silica Gel 90 C18 material (90 Å pore size, particle's dimensions 40-63 µm, all Sigma-Aldrich, Germany) were evaluated (data not shown).~~ Fully encapped silica Gel 90 C18 material (90 Å pore size, particle's dimensions 40-63 µm, Sigma-Aldrich, Germany) ~~Of these materials the end capped 90 Å material~~ proved to be superior with respect to the low background and the ease of handling (Supplemental Material, Figure 4) ~~above mentioned criteria~~.

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. Even if application using a sieve consumed less material, coverage was higher using the "Petri-dish method" that was further pursued. For *F. vesiculosus* a thallus piece of 36.5 ± 6.5 cm^2 proved to be sufficient for the generation of GC-MS and LC-MS samples. However, smaller sample sizes could be envisaged in cases of limitation of biological material since of the generated 200 µL UPLC and 100 µL GC samples only few microliters were required for analysis. The amount of

C18 material recovered in the cartridge, weighted at the end of the experiment after the complete evaporation of the remaining elution solvent, was 0.13 ± 0.01 g. Again, in case 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 gives the most uniform coverage of the alga and allows an easy handling. The incubation time of 60 s represents 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 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 seconds as indicated above (Figure 2a, Supplemental material 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 ~~duration of~~ incubation time with C18 material ~~incubation~~, visual inspection revealed that surface cells were not damaged or otherwise altered. In strong contrast, even after only 5 seconds of extraction with the "dipping" method, the colour of the *F. vesiculosus* surface changes from the typical yellow-brown to green, which is indicative for damage and pigment loss of the surface cells. Also fronds of *C. taxifolia* did not show any signs of damage after C18 treatment as examined by light microscopy (Supplemental material Figure S1). To visualize dead cells, they were stained with Evans Blue (Weinberger et al. 2005) and RGB (red, green, blue) colours of the recorded microscopic images were analysed. This test confirmed that already after 5 seconds of hexane / methanol dipping, cells were damaged as indicated by a substantial change in red/green ratio, while values did not differ significantly from controls when using the C18 method (Figure 2b). It has to be noted that, despite its broad application, the Evans Blue staining method is not unproblematic 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.

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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 step. In initial experiments we monitored for the presence of the carotenoid fucoxanthin, a dominant pigment of brown algae such as *Fucus*. This metabolite is ideally suitable for method development and comparison since earlier studies using the hexane / methanol dipping method as well as Raman-imaging already indicated that this compound is released into the surface environment of *Fucus* (Grosser, et al. 2012, Saha, et al. 2011). 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

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For quantitative determination, canthaxanthin, another carotenoid pigment that is not found in *F. vesiculosus,* was used as 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 5 specimens, with an average extracted surface of 36.5 ± 6.4 cm^2. Since extracts were split into two equal parts for GC-MS and LC-MS determination the values determined correspond to a 18.25 cm^2 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 ~~entire~~ extraction protocol was performed this indicates a very good reproducibility of the extraction procedure~~, since only~~. Only minor additional variability in comparison to sample drying, re-dissolution and measurement is introduced by the C18 extraction procedure. ~~As with all available surface extraction protocols, the quantitative determination of metabolites is impossible since proper recovery rates cannot be determined. To overcome this limitation we~~ We quantitatively compared the C18 method to the established hexane / MeOH dipping. ~~From Figure 3b it can be concluded that, given the fact that the relative amount of added canthaxanthin standard is similar, the absolute recovered~~ Recovered fucoxanthin in dipping experiments is significantly higher ~~in dipping~~ compared to C18 experiments (Figure 3b). This can be due to overall better extraction success, but most likely also to contributions of fucoxanthin released by the lysed cells in the hexane / MeOH treatment. An external calibration ~~by~~based on the evaluati~~ng~~on of the peak areas of the standard canthaxanthin in relation to areas resulting from different amounts of fucoxanthin (Supplemental Figure S3) allowed estimating the extracted fucoxanthin. Even if quantification is problematic since no reference method for the determination of absolute amounts of surface chemicals is available the procedure allows relating extraction success of this study to studies in the literature. C18 extraction gives ca. 1.2 µg fucoxanthin cm^{-2} while the hexane / methanol dipping recovers absolute amounts of ca. 14 µg fucoxanthin cm^{-2}. Previous studies using the dipping method gave similar values (0.7-9 µg fucoxanthin cm^{-2}) and it can be concluded that algae in our study and the analytical work-flow are matching those in the literature within the margins of natural and experimental variability (Saha et al. 2011). It can however not be finally answered if the C18 method quantitatively extracts surface metabolites and if hexane / methanol overestimates the content due to cell lysis or if the C18 method underestimates surface metabolites due to non-quantitative extraction. ~~It is also evident from~~ The reproducibility of the C18 method however suggests a highly reliable measurement. ~~the~~ The standard deviation of signals in the C18 extract is ~~that the dipping method introduces a~~ substantially lower than that in hexane / methanol extracts. ~~higher amount of variation compared to the C18 protocol.~~ Given the facts that the C18-method is by far better reproducible and that it does not introduce cell damage this method has to be considered as superior. Lower recovery ~~has not be considered as~~is not problematic, since ~~with~~ even small thallus fragments provide sufficient extract for~~,~~ the entire analytical process. ~~can~~ Extraction and measurement can be easily ~~performed~~ carried out without additional concentration steps using routine instrumentation ~~without additional concentration steps~~. Experiments using hexane as extraction solvent did not result in detectable amounts of extracted fucoxanthin (data not shown). This solvent that causes minimum damage of algal surfaces is thus not suitable for the extraction of the metabolite using a sample size sufficient for the C18-method and hexane / methanol dipping.

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To test the universal applicability of the C18-method we also investigated two macroalgae with hitherto unknown surface chemistry. No adaptation in the protocol was required for surface extraction of the green alga *Caulerpa taxifolia* and the red alga *Gracilaria vermiculophylla*. In the case of *C. taxifolia* 45.8±4.0 cm^2 (n = 3) algal surface was extracted without causing damage (Supplemental material Figure S1) and LC-MS revealed the presence of caulerpenyne (identified by comparison to authentic material (Jung and Pohnert 2001)). Again, elevated amounts were detected using the hexane / methanol dipping protocol. Since caulerpenyne is a very dominant intracellular metabolite, this elevated value can 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) of the alga. ~~It~~ This is the first report demonstrating that caulerpenyne is also present at the surface of the alga (Figure 3c) ~~and motivates~~motivatin further investigation of its potential role as surface defense compound or natural anti-fouling metabolite.

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As with *F. vesiculosus* extracts, hexane / ~~MeOH~~ methanol dipping resulted in overall higher caulerpenyne recovery, but also in substantial cell damage (data not shown) and a very high standard deviation (Figure 3d). Initial experiments with *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 to extract a broad range of ~~apolar~~ non-polar and medium polar compounds, we tested its capability to extract structurally diverse surface metabolites. Since LC-MS techniques do not allow for ~~simple~~ easy compound identification we used the exploratory power of GC-MS supported by library identification of metabolites to test for additional compound classes picked up by the C18 method. The major metabolites that were extracted from *F. vesiculosus* surfaces were fatty acids that were transformed by the solvent MeOH to ~~their~~ the corresponding methyl esters during elution. This transformation was verified by control measurements where EtOH ~~was used~~ 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* ($[C_6H_7]^+$ indicative for polyunsaturated fatty acids) and 74 *m/z* (a McLafferty ion indicative for saturated fatty acids) were detected and structure elucidation ~~of the algal metabolites~~ was performed ~~with~~ by comparison with authentic standards (FAME, Sigma- Aldrich©, Germany). A representative chromatogram and the assigned metabolites can be found in Figure 4. ~~The rather polar f~~Fatty acids are common in brown algae (Pereira et al. 2012, Schmid and Stengel 2015), but were previously never detected as surface metabolites. Their presence in surface extracts can be explained with an active release mechanism of free fatty acids or alternatively with a partial hydrolysis of ~~surface~~ lipids on the surface of the alga. The fact that fatty acids as surface metabolites were overlooked till now might be due to limitations of previous experimental approaches. In accordance, samples that were generated with the hexane / ~~MeOH~~ methanol dipping method contained only few fatty acids and only in trace quantities (GC/MS after derivatisation, data not shown). In addition to free fatty acids we could identify substantial amounts of phytol and a not further identified steroid, confirming the broad extraction potential of the C18-method.

Conclusions

Investigation of the algal surface chemistry is central for the understanding of ecological interactions on and around these 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 in a very limited polarity range and can cause substantial damage to the algal tissue. Alternatively, ~~elaborate~~ expensive instrumentation ~~as it is~~ required for desorption electrospray MS (Lane et al. 2009) or Raman techniques (Grosser, et al. 2012) is needed for surface investigations. The introduced method that is based on covering the algal surface with C18 extraction sorbent and collecting the material for subsequent extraction does not cause ~~stress~~ surface damage ~~to~~ in 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 reliable results reflected by low standard deviations make 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.

Acknowledgments

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

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Figure 1 Schematic work flow of the C18 method. 1) Algae are removed from the water and left for 2 min to remove excess water by dripping; 2) algae are transferred to Petri dishes and covered with absorption material; 3) the C18 material is washed off with excess sea water 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.

Figure 2 Evaluation of surface damage by different extraction methods. A) Photographs of *F. vesiculosus* surfaces (Scale bars 100 µm). Top row control after removal from water for 5, 60 and 600 seconds. Middle row algae after C18 extraction and lowest row after hexane / methanol dipping for the same time spans. B) Evaluation of cell damage after Eavns blue staining by red / green ratio analysis at 5, 30, 60, 120, 300, 600 seconds exposure to C18 material (grey), hexane / methanol dipping (white) and control (black), (n = 5 ± SD)

Figure 3 a,b) Average chromatographic areas of fucoxanthin extracted from *Fucus vesiculosus* surfaces in relation to cantaxanthin as internal standard (Std.): a) C18 method, n = 5, surface extracted = 36.5 ± 6.4 cm^2 ; b) hexane / methanol dipping method, n=5, surface extracted = 29.3 ± 3.0 cm^2. c, d) Average chromatographic areas of caulerpenyne extracted from *Caulerpa taxifolia* surfaces and cantaxanthin as internal standard. c) C18 method, n=3, mean surface extracted= 45.8 ± 4.0 cm^2 ; d) hexane / methanol dipping method, n=3, mean surface extracted= 36.0 ± 7.3 cm^2.

Figure 4 GC-MS run of a C18 extract of *F. vesiculosus* performed with 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 (α-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.