Composition analysis of organic matter released by cosmopolitan coral reef-associated green algae

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ABSTRACT: Coral reef-associated benthic algae can control important metabolic processes in reef ecosystems via organic matter release. However, information about the chemical composition of macroalgae exudates is very limited. We therefore quantified total and dissolved organic carbon (TOC, DOC) and total and dissolved organic nitrogen (TON, DON) of exudates released by 2 cosmopolitan coral reef-associated green algae species, Halimeda opuntia and Caulerpa serrulata. Glycosyl composition and content analyses, along with protein, lipid and chlorophyll a (chl a) quantifications of algae exudates were conducted. Findings revealed that organic matter released from both algae predominately consisted of carbohydrates (59 ± 5 %) and proteins (32 ± 7 %), which mainly (89 to 93%) dissolved in surrounding waters. Traces of fatty acids (C16:0; C18:0) and chl a were also found in algae incubation waters, but in a quantitatively negligible amount. Carbohydrate analysis further showed that glucose was the dominant glycosyl released by algae, accounting for 77 ± 8% of the carbohydrate fraction and 42 ± 8% of TOC. Galactose (9 ± 4% of carbohydrate fraction), mannose (6 ± 3%), xylose (4 ± 1%), rhamnose (3 ± 1%) and fucose (2 ± 1%) were also detected in all incubation water samples. High glucose and protein contents of algae exudates found in the present study confirm assumptions on the fast microbial degradability of these exudates, with ensuing potential negative effects on O₂ availability and on interactions between corals and algae in coral reef ecosystems.

KEY WORDS: Coral reef · Macroalgae · Organic matter · Chemical composition · Carbohydrates · Glucose

INTRODUCTION

Many coral reef ecosystems worldwide are facing shifts in benthic community composition from coral to macroalgae dominance (McCook et al. 1997, Bellwood et al. 2004, Work et al. 2008). These phase shifts (Done 1992) may lead to alterations in quantity and quality of organic matter released by the different primary producing organisms into the ecosystem (Wild et al. 2009). Previous studies showed that organic matter derived from hermatypic corals (Wild et al. 2004) and benthic algae (Hedges 2002) are key components of reef biogeochemical cycles. Algae have been shown to release 5 to 50% of their net primary production into their surroundings (Craigie & McLachlan 1964, Fogg 1966, Sieburth 1969, Hellebust 1974). Various processes may be responsible for the release of organic compounds, e.g. excretion, lysis, leaching and shedding of algal debris (Cole 1982, Wada et al. 2007).

Comparison of organic matter derived from various reef algae, hermatypic corals and seagrasses has, however, revealed significant differences in its quantity, quality and microbial degradability (Haas et al. 2010a, Naumann et al. in press, Wild et al. in press). The described differences may lead to alterations in nutrient cycles and O₂ availability in the reef ecosystem (Wild et al. 2009, Niggl et al. 2010).

Bio-labile organic matter derived from benthic algae has a highly stimulating effect on the activity of bacterioplankton in the ambient water column (Jonas 1997, Wild et al. 2009), with ensuing negative effects on O₂ availability (Haas et al. 2010a, Wild et al. in press). The resulting hypoxia or anoxia can be responsible for significant stress (Johannes 1975) or even mortality of...
corals (Simpson et al. 1993). Algae-derived organic matter may therefore negatively affect coral health, thus facilitating a negative feedback loop in reef degradation (Smith et al. 2006). This may potentially be explained by a high content of rapidly degradable organic carbon (Kline et al. 2006). In this context, the glycosyl composition is a key factor for microbial degradation (Arnosti 2000).

While organic matter derived from various hermatypic corals (Richards et al. 1983, Coffroth 1990, Wild et al. 2005) and phytoplankton (Hellebust 1965, 1974, Fogg 1966, Nguyen et al. 2005) has previously been subject to analyses, there is little data available on the chemical composition of macroalgae-released organic matter (Abdullah & Fredriksen 2004, Wada et al. 2007) and none that specifically address that released by coral reef-associated algae. However, organic matter composition of algae in the coral reef ecosystem may be essential to understand the underlying mechanisms of coral–alga interactions and a concomitant shift in benthic community composition.

The present study therefore aimed to provide the first data on the composition of organic matter derived from coral reef-associated green algae. For this purpose, total organic carbon (TOC) and nitrogen (TON) as well as dissolved organic carbon (DOC) and nitrogen (DON) released by 2 cosmopolitan coral reef-associated green algae species, Halimeda opuntia and Caulerpa serrulata, were used for this experiment. Two green algae of the order Bryopsidales (Chlorophyta, Bryopsidophyceae), namely Halimeda opuntia and Caulerpa serrulata, were used for this experiment. Three different specimens of each species (8 to 15 cm height) were carefully checked to exclude organisms infested by epibionts that could have affected experimental results by release or uptake of organic matter. For each of the 2 algae species, an independent beaker incubation experiment was conducted in triplicate after the method described in Herndl & Velimirov (1986) with some modifications. Autoclaved 5 l glass beakers were filled with 2000 ml sterile seawater. This sterile seawater was generated by dissolving a pure marine salt mixture (Tropic Marin® Sea Salt) in nanopure water. The beakers were provided with the respective algae subsequent to rinsing algae fragments in sterile seawater to minimize microbial epibionts. Beakers were then coated with transparent plastic foil in order to avoid external contamination, leaving small channels for air exchange. The beakers were left for 24 h in a water bath under identical conditions as described above. At the end of each incubation experiment, algae specimens were removed from the beakers using sterilized forceps to prevent contamination of the incubation water, and the remaining incubation water was sampled and analyzed as described below. Control incubations, containing sterile seawater only, were conducted using identical methodology.

Subsequently, algae surface area was determined by spreading each of the incubated algae 2-dimensionally on scaled paper. Photographs were then taken from directly above with a Sony Cybershot digital camera (5.1 megapixels resolution), and surface area was calculated with digital image processing software (ImageJ, version 1.37m, National Institutes of Health).

To allow for comparison with previous studies, where algal organic matter release was related to dry weight, standard measurements with different sized algae specimens were conducted to establish a conversion factor for surface area to dry weight. Algae surface areas were measured as described above (n = 10 for each species) and subsequently weighed after drying for at least 48 h at 40°C.

Organic carbon and nitrogen quantification. Subsamples of incubation water volumes were taken from each of the 6 algae and control treatments with a sterile syringe for subsequent organic carbon and nitrogen quantification. Samples for TOC and TON analysis (10 ml) were immediately transferred into precombusted (450°C, 4 h) glass vials. Samples for DOC and DON analysis (10 ml) were filtered through 0.2 µm sterile polyethersulfone membrane filters (Millepore, Millex®GP 0.22) before transferring them into the glass vials. Samples were then kept frozen at –20°C until analysis by high-temperature catalytic oxidation with a
Dimatec DIMA-TOC 100 TOC analyser equipped with a DIMA-N-Module for quantification of total bound nitrogen. Non-purgeable organic carbon was measured after sample acidification with hydrochloric acid to pH < 3, while sparging with O₂. For organic carbon quantification, potassium hydrogen phthalate was used as an elemental standard; for organic nitrogen, potassium nitrate and ammonium sulfate were used (SD < 3%). Particulate organic carbon (POC) and nitrogen (PON) concentrations were calculated by subtracting the dissolved fraction from the respective total content. Organic carbon release rates were then normalized to algae surface and duration of release.

**Glycosyl content and composition.** Algae incubation water subsamples (1000 ml from each incubation) were desalted prior to glycosyl composition analysis by dialysis. Dialysis was performed using Spectra/Por dialysis membranes with a molecular weight cut-off of 1000 Da. Samples were placed in 3 l deionized water and stirred for 8 h while the water was continuously refreshed. Samples were then transferred to 3 l of nanopure water and kept at 4°C for 48 h. Dialysis water was refreshed midway through the 48 h interval. After dialysis, each sample was lyophilized and glycosyl composition was performed on the resultant material.

Glycosyl composition analysis was performed by combined GC-MS of the per-O-trimethylsilyl (TMS) derivates of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis.

A 200 µl aliquot was used from each sample and added to separate tubes with 20 µg of Inositol as internal standard. Methyl glycosides were then prepared from the dry sample following the mild acid treatment by methanolysis in 1 M HCl in methanol at 80°C for 16 h, followed by re-N-acetylation with pyridine and acetic anhydride in methanol for the detection of amino sugars. Samples were then per-O-trimethylsilylated by treatment with Tri-Sil (Pierce) at 80°C for 20 min. This analysis was carried out according to Merkle & Poppe (1994). GC-MS analysis of the TMS methyl glycosides were performed on an AT 6890N GC interfaced to a 5975B MSD, using a Supelco EC-1 fused silica capillary column (30 m × 0.25 mm ID). Electron-ionization MS was used for analysis with a scan range of 50 to 500 Da.

**Lipid content and composition.** For this analysis, a 100 ml subsample of each incubation water volume was used. Extraction of lipophilic components was achieved with a polystyrol-divinylbenzol-copolymer phase using a Varian Bond Elute™ Plexa™ cartridge (200 mg, 6 ml). Elution was conducted with methanol, and samples were then dried by sparging. The residue was dissolved in 990 µl methanol and 10 µl trimethylsulphoniumhydroxide, and lipid identification was carried out by GC-MS (Varian 3800 GC with Split-/Splitless-Injector 1177 interfaced to a 1200 MS with CTC CompiPAL for liquid-injection) with helium as the carrier gas.

**Protein content.** Protein content was determined by a protein assay described by Lowry et al. (1951) with BSA as the protein standard. One ml subsamples of undiluted incubation water were used for the procedure, and sterile seawater was used as a blank. The protein assay was conducted in triplicate for each of the 6 incubations, and the standard error between replicate measurements was <5% for each single treatment.

**Chl a content.** Chl a content was determined from a 30 ml incubation water subsample from all incubations. Chl a concentrations were analyzed with a GAT TD-700 fluorometer after the method described by Holm-Hansen et al. (1965).

**RESULTS**

**Organic carbon and nitrogen quantification**

Organic carbon quantification of algae incubation waters showed that *Halimeda opuntia* released 2.29 ± 0.11 mg TOC m⁻² surface area h⁻¹ (all values are given as means ± SE) during the incubation period. DOC was released in quantities of 2.08 ± 0.20 mg m⁻² h⁻¹ (91% of TOC), whereas POC release accounted for only 0.21 ± 0.13 mg m⁻² h⁻¹ (9% of TOC). By application of the calculated conversion factor for surface area to dry weight (DW) (1 g DW = 37.1 cm² surface area; R² = 0.90), *H. opuntia* showed release rates of 0.0095 ± 0.0004 mg g DW⁻¹ h⁻¹ TOC, composed of 0.0077 ± 0.0007 mg g DW⁻¹ h⁻¹ DOC and 0.0008 ± 0.0004 mg g DW⁻¹ h⁻¹ POC, respectively. Similar results were obtained for *Caulerpa serrulata* incubations, with release rates of 3.43 ± 0.54 mg TOC m⁻² h⁻¹, composed of 3.07 ± 0.44 mg DOC m⁻² h⁻¹ (90% of TOC) and 0.36 ± 0.10 mg POC m⁻² h⁻¹ (10% of TOC). The applied calculation factor (1 g DW = 223.3 cm² surface area; R² = 0.95) resulted in TOC release rates of 0.0766 ± 0.0120 mg g DW⁻¹ h⁻¹ consisting of 0.0686 ± 0.0099 mg g DW⁻¹ h⁻¹ DOC and 0.0080 ± 0.0021 mg g DW⁻¹ h⁻¹ POC. Incubation waters of both species therefore showed a high DOC:POC ratio of 13 ± 5. No organic carbon could be detected in the sterile seawater control incubations, indicating that organic matter release by the incubated algae was the exclusive source of organic carbon found in these treatments.

Total organic nitrogen was below the detection limit of 0.5 mg l⁻¹ in all incubation water samples, except one *Caulerpa serrulata* incubation sample. This single incubation indicated that nitrogen-containing com-
pounds were also released primarily in dissolved form (DON:PON ratio = 44) and that algae exudates exhibited high C:N ratios of 16. No significant (Mann-Whitney U-test) species-specific difference in quantity or quality of the released organic matter could be detected. Organic carbon and nitrogen contents of all incubation water samples are given in detail in Table 1.

**Composition analyses**

Composition analysis of algae-derived organic matter revealed that the main component of algae exudates were carbohydrates (*Halimeda opuntia*: 53 ± 7%; *Caulerpa serrulata*: 64 ± 7% of TOC) followed by proteins (*H. opuntia*: 37 ± 11%; *C. serrulata*: 27 ± 9% of TOC). Traces of chl a (<0.1%) and fatty acids (<1%) were also found in both algae treatments (Table 1).

Glycosyl composition analysis showed that the dominant carbohydrate component in all algae exudates was glucose (77 ± 8% of all carbohydrates) followed by galactose (9 ± 4%), mannose (6 ± 3%), xylose (4 ± 1%), rhamnose (3 ± 1%) and fucose (2 ± 1%). Traces (<1%) of arabinose and N-acetyl glucosamine were detected in *Halimeda opuntia*, but not for *Caulerpa serrulata* exudates (Fig. 1). Palmitic acid (C16:0) and stearic acid (C18:0) were found in 2 of 3 incubation water samples of each algae genus, but not in a quantitatively determinable concentration (<10 µg l⁻¹).

**DISCUSSION**

Organic matter quantification conducted in the present study revealed that both investigated coral reef-associated green algae, *Halimeda opuntia* and *Caulerpa serrulata*, released organic matter primarily in dissolved form into their surroundings. This is in line with previous studies (Khailov & Burlakova 1969, Sieburth 1969, Abdullah & Fredriksen 2004, Haas et al. 2010b), which congruently suggested that algae-derived organic matter may primarily dissolve in ambient waters. Quantities of organic matter released during incubation experiments were comparable to a previous study by Brylinsky (1977), who found release rates of 0.0059 to 0.0533 mg m⁻² h⁻¹ for coral reef-associated algae. Preliminary studies in the northern Red Sea additionally showed organic matter release rates for *C. serrulata* in comparable quantities (4.9 ± 0.9 mg m⁻² h⁻¹) when normalized by surface area (Haas et al. 2010b).

C:N ratios of algae exudates in the present study considerably exceed those of marine organic matter usually found in coral reef environments (Redfield

Table 1. Analytical contents of algae incubation waters (mg m⁻² h⁻¹; for Chl a, µg m⁻² h⁻¹). DOC: dissolved organic carbon; POC: particulate organic carbon; TOC: total organic carbon; DON: dissolved organic nitrogen; PON: particulate organic nitrogen; TON: total organic nitrogen; Chl a: chlorophyll a; nd: not detectable.
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1934). This is also confirmed by previous studies (Biddanda & Benner 1997, Haas et al. 2010b) and may be explained by a high fraction of carbohydrates in algae-derived organic matter. Total carbohydrate fractions of the analysed algae incubation water samples were 59 ± 5% of TOC, which is similar to carbohydrate contents described for exudates of various phytoplankton cultures (52 ± 2% of TOC; Biersmith & Benner 1998). These carbohydrate contents may even be considered a conservative approximation, as carbohydrates with a molecular weight <1 kDa were not assessed by the composition analysis and therefore contributed to the other organic carbon fraction (Fig. 1). Carbohydrate fractions of exudates from the temperate brown algae *Ecklonia cava* analyzed by Wada et al. (2007) were considerably lower (5.3 to 14.0%). This might be attributed to a lower photosynthetic performance owing to the more complex morphology of the kelp, as it requires higher proportions of structural tissue (Littler & Littler 1984), and a lower surface area to volume ratio. Lower temperature and light availability in temperate surroundings may further lead to reduced photosynthetic performance (Verity 1981). This may also explain the lower contents of glucose (7 to 14%), the primary product of photosynthesis, in organic matter released by these temperate algae.

Glucose was found to be the main component of the bulk carbohydrate fraction, accounting for 42 ± 8% of the TOC released by algae. Next to glucose, other C6 sugars (mannose, galactose) followed by smaller amounts of 6-deoxysugars (rhamnose, fucose) represented the principal carbohydrate components of algae-derived organic matter. The only C5 sugar found in noticeable amounts in incubation waters was xylose. In a number of marine algae of the order Bryopsidales, including *Halimeda* and *Caulerpa*, cellulose, consisting of β(1→4) linked D-glucose units, has been found to be completely absent (Lewin 1974). Instead, cell walls contained β(1→4) linked xylan as the structural polysaccharide (Frei & Preston 1964). Xylose glycosides therefore likely derived from structural cell components of the siphonous algae, which were shed during the incubation period.

Glycosyl composition analysis, together with DOC and TOC quantification, revealed that the main part of algae-derived organic matter was released as dissolved C6 sugars and primarily as glucose. Glucose is used as universal energy source in most organisms (Vollhardt & Schore 2000) and, along with mannose and galactose (Aluwihare & Repeta 1999), is the most substantial component for heterotrophic bacterial production in marine environments (Rich et al. 1996, Hama et al. 2004). Glucose and, to a lesser extent, mannose, have together been shown to fuel more than 40% of the total bacterial respiration in aquatic ecosystems (Rich et al. 1996). Hama et al. (2004) further proved a high bioreactivity of the low (<10 kDa) as well as the high molecular weight fraction (>10 kDa) of algae-derived dissolved glucose. Other dissolved monosaccharides, especially the more refractory deoxysugars, play a rather subordinate role in heterotrophic bacterial production (Amon et al. 2001, Ogawa et al. 2001).

Proteins, the second largest fraction in algae exudates besides carbohydrates, can significantly contribute to the dissolved combined amino acid (DCAA) pool in marine ecosystems (Billen 1991). DCAAs also serve as rapidly degradable substrate for bacterial growth (Hagström et al. 1984, Keil & Kirchman 1993) and contribute 12 to 50% of the heterotrophic bacterial N requirements (Tupas & Koike 1990) in the generally
nutrient-poor coral reef ecosystem (Lapointe 1997). Other components of algae exudates (lipids, chl a, N-acetyl glucosamine) were only found in negligible amounts (<1%) during the present study. Yoshimura et al. (2009) already indicated that the release of lipid material (0.7%) is not an essential metabolism of algae. This indicates a rather homogeneous structure of algae exudates, with 2 substance groups (carbohydrates and proteins) accounting for 91 ± 2% of the total released organic carbon.

Ecological implications

Comparison with glycosyl composition analyses of mucus released by various hermatypic corals, i.e. Acropora (n = 8), Fungia (n = 4), Ctenactis (n = 1), Pocillopora (n = 1), Stylophora (n = 1), Pachyseris (n = 1) (Meikle et al. 1988, Wild et al. 2010) showed that corals release a more heterogeneous carbohydrate mix than algae (glucose 19 ± 14% of all carbohydrates, galactose 1 ± 1%, mannose 20 ± 6%, fucose 39 ± 14%, arabinose 11 ± 11%, N-acetyl glucosamine 10 ± 8%). Contrary to the 2 tested algae genera, coral mucus carbohydrate content and composition was rather genus-specific (Wild et al. 2005, Wild et al. in press) and contained significantly lower proportions of glucose (1-way ANOVA, df = 12, p = 0.006). This coral mucus generally provided a good substratum for microbes in the pelagic and benthic environment of coral reef habitats with ensuing positive effects on microbial activity (Wild et al. 2004, 2005).

However, coral reef-associated algae exudates may provide an even more rapidly degradable substratum for the ambient heterotrophic microbial community (Wild et al. 2009, in press, Haas et al. 2010a). This is supported by the present study, as high concentrations of glucose and its polymers (Hama et al. 2004) and DCAAs released by algae represent a matrix readily available for heterotrophic microbial utilisation.

The present study therefore strengthens the hypothesis of alterations in cycles of matter and O2 availability following changes in benthic community structure from coral to algae dominance (Niggl et al. 2010, Wild et al. in press). It also provides essential chemical composition information to link findings documenting deleterious effects of dissolved glucose on corals (Kuntz et al. 2005, Kline et al. 2006) and on interactions between corals and with algae (Haas et al. 2009), with those recent findings hypothetically assigning algae exudates a key role in microbi-induced coral mortality (Smith et al. 2006). Finally, it supports the assumption that algae-released organic matter facilitates a positive feedback loop created during phase shifts from coral to algal dominance.

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