Algae–bacteria interactions and their effects on aggregation and organic matter flux in the sea

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Summary
Aggregation of algae, mainly of diatoms, is an important process in marine pelagic systems, often terminating phytoplankton blooms and leading to the sinking of particulate organic matter in the form of marine snow. This process has been studied extensively, but the specific role of heterotrophic bacteria has largely been neglected, mainly because field studies and most experimental work were performed under non-axenic conditions. We tested the hypothesis that algae–bacteria interactions are instrumental in aggregate dynamics and organic matter flux. A series of aggregation experiments has been carried out in rolling tanks with two marine diatoms typical of temperate regions (Skeletonema costatum and Thalassiosira rotula) in an axenic treatment and one inoculated with marine bacteria. Exponentially growing S. costatum and T. rotula exhibited distinctly different aggregation behavior. This was reflected by their strikingly different release of dissolved organic matter (DOM), transparent exopolymer particles (TEP) and protein-containing particles (CSP), as well as their bacterial biodegradability and recalcitrance. Cells of S. costatum aggregated only little and their bacterial colonization remained low. Dissolved organic matter, TEP and CSP released by this alga were largely consumed by free-living bacteria. In contrast, T. rotula aggregated rapidly and DOM, TEP and CSP resisted bacterial consumption. Experiments conducted with T. rotula cultures in the stationary growth phase, however, showed rapid bacterial colonization and decomposition of algal cells.

Our study highlights the importance of heterotrophic bacteria to control the development and aggregation of phytoplankton in marine systems.

Introduction
There is ample evidence that phytoplankton blooms, in particular composed of diatoms, form aggregates (marine snow) which are one of the major components of sinking particulate organic carbon (POC) (Simon et al., 2002; Thornton, 2002). Transparent exopolymer particles (TEP) have been identified as an important agent for aggregation (Passow, 2002a). Various studies have shown that TEP is produced by planktonic algae, but also by bacteria and from dissolved precursor material (Zhou et al., 1998; Passow, 2002b; Engel et al., 2004). The role of another class of microparticles containing protein [Coomassie brilliant blue stainable particles (CSP); Long and Azam, 1996] in aggregation processes needs to be clarified. The specific role of heterotrophic bacteria in aggregation processes, i.e. the formation of TEP, other polymeric sticky material, and CSP, and the effect of bacteria on the adhesive properties and coagulation of this material is still unclear. This is because the great majority of studies have been carried out with non-axenic planktonic algae, thus masking the specific bacterial impact. Four scenarios are conceivable: (i) the organic material may be recalcitrant to bacterial decomposition; (ii) bacteria may consume it to varying extents, thus reducing the aggregation potential; (iii) bacteria may modify this material and change its adhesive properties; and (iv) the colonization of the algal cell surface by specific bacterial communities may directly affect the alga’s adhesive properties. These scenarios may be characteristic of an algal species, but may also vary with its physiological state, i.e. depend on the quality of the secreted material and the associated bacteria.

The role of heterotrophic bacteria in decomposing phytoplankton-derived POC and phytodetrital aggregates is much better understood. It has been shown that bacteria colonize planktonic algae, mainly when they become more senescent and aggregate (e.g. Smith et al., 1995), and that they solubilize phytodetrital aggregates (Smith et al., 1992; Grossart and Ploug, 2001), i.e. transforming particulate (POC) to dissolved organic carbon (DOC). In addition to POC solubilization, the high proteolytic activity of bacteria associated with diatoms greatly enhances sil-
ica dissolution and thus reduces the POC sinking flux (Bidle and Azam, 1999).

Hence, our present understanding of the role of heterotrophic bacteria in algae–bacteria interactions appears to be ambivalent. Whereas their role in phytodetrital POC decomposition appears to be rather well understood, we still lack a detailed understanding of the bacterial decomposition of specific phytoplankton-derived DOC compounds and how this affects aggregation processes. Further, we still know little about whether specific algal species attract distinct bacterial communities or populations, which may also have implications for phytoplankton–bacteria interactions (Schäfer et al., 2002; Bates et al., 2004; Rooney-Varga et al., 2005). The latter question has been addressed in an accompanying study using the same experimental set-up (Grossart et al., 2005).

The aim of the present study was to examine specific interactions between heterotrophic bacteria and two marine diatoms, *Skeletonema costatum* and *Thalassiosira rotula*, and their implications for phytoplankton aggregation and sinking flux. We chose these chain-forming species as they are common in the phytoplankton of temperate coastal marine regions. To test whether bacterial consumption and secretion of specific DOC compounds, such as dissolved amino acids and dissolved carbohydrates, affect the formation of TEP, CSP and aggregates, we conducted experiments with axenic and non-axenic cultures of these two diatoms.

**Results**

**Aggregation patterns**

The results exhibited distinct differences between the two diatoms with respect to aggregation processes. Whereas exponentially growing (exp) *S. costatum* aggregated only weakly, exponentially growing *T. rotula* readily formed aggregates (Table 1). The first aggregates >2 mm in size in cultures of *S. costatum* did not occur until 40 h of incubation, and even an increase of the initial cell concentration by one order of magnitude to $10^4$ cells ml$^{-1}$ only slightly reduced the time until aggregates formed (35 h). Image analysis revealed that the area of *S. costatum* aggregates remained small compared with *T. rotula*, although both types of aggregates were of similar length. Aggregation of *T. rotula* was faster in low light conditions than in high light conditions, yielding aggregates of >2 mm already within 4 h. However, in the former case aggregates remained smaller (Table 1). Under high light conditions, aggregates increased in size for about 60% of the incubation time and the algae and bacteria kept growing, except in the stationary-phase experiment. Aggrega-

![Image](image_url)

**Table 1.** Aggregation dynamics of *Skeletonema costatum* and *Thalassiosira rotula* cultures growing exponentially and in the stationary phase.

<table>
<thead>
<tr>
<th>Aggregation experiment</th>
<th>Skeletonema costatum</th>
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<th></th>
<th>Thalassiosira rotula</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1 Exponential</td>
<td>2 Exponential</td>
<td>3 Exponential</td>
<td>4 Exponential</td>
<td>5 Stationary</td>
<td>6 Exponential (axenic)</td>
<td>7 Exponential</td>
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<td>High light conditions</td>
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<tr>
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<td>10.5</td>
<td>14</td>
<td>15</td>
<td>9</td>
<td>12</td>
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<tr>
<td>Aggregate area (mm$^2$ ml$^{-1}$)</td>
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<td>nd</td>
<td>+/+/0</td>
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<td>+/+/0</td>
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<td>14</td>
<td>8</td>
<td>12</td>
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<tr>
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<td>nd</td>
<td>+</td>
<td>+++</td>
<td>o/−</td>
<td>o/−</td>
<td>o/+</td>
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<tr>
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<td>nd</td>
<td>++</td>
<td>+++</td>
<td>+/−</td>
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<tr>
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<td>−</td>
<td>+</td>
<td>+/−</td>
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<td>+/−</td>
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<tr>
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<td>nd</td>
<td>+/−</td>
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<td>o</td>
<td>o</td>
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<tr>
<td>TEP</td>
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<td>8–18</td>
<td>150–1200</td>
<td>25–95</td>
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<td>3</td>
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<tr>
<td>Aggregate area (mm$^2$ ml$^{-1}$)</td>
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<td>(+)+/−</td>
<td>o/−</td>
<td>(−)</td>
<td>o/+</td>
<td>(+)+/0</td>
<td>++/0</td>
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<tr>
<td>Maximum aggregate length (mm)</td>
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<td>nd</td>
<td>−/−</td>
<td>+/−</td>
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<tr>
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<tr>
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<td>5–17</td>
<td>7–42</td>
<td>2–20</td>
<td>2–20</td>
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</tbody>
</table>

nd, not determined; +, increase; o, no changes; −, decrease; ±, first increase then decrease; ( ), only slight changes.

*Skeletonema costatum* and *Thalassiosira rotula* cultures were inoculated with natural seawater bacteria and incubated under either high or low light conditions. The initial concentration of algal cells was approximately $10^4$ cells ml$^{-1}$, except in experiment 2, which started with approximately 10 000 cells ml$^{-1}$. Experiment 7 is a continuation of experiment 6 which was inoculated with seawater bacteria after 68 h.
tion of axenic *T. rotula* at high light was relatively fast compared with the non-axenic experiments but aggregate size remained smaller. In contrast, at low light conditions axenic *T. rotula* aggregated substantially slower than non-axenic ones. When bacteria were added to the axenic culture after 68 h aggregation was immediately enhanced, leading to strikingly larger aggregates within 10 h (Table 1, Fig. 1). Numbers and area of TEP and CSP did not exhibit clear-cut results with respect to the treatments and growth stages of *T. rotula* (Table 1).

Therefore, we re-examined the formation of TEP and CSP in relation to algal and bacterial abundance as well as concentration and quality of dissolved organic matter and bacterial activities in axenic versus non-axenic cultures of *T. rotula* and *S. costatum*.

**Phytoplankton growth dynamics**

Algal numbers of axenic and non-axenic cultures exhibited pronounced differences between algal species and growth stage (Fig. 2A and B). Numbers of axenic *S. costatum* were higher than of non-axenic *S. costatum* until the late exponential phase. The non-axenic treatment, however, reached higher algal cell numbers at the end of the exponential growth phase and enhanced numbers persisted throughout the stationary and declining phase until the end of the experiment. In contrast, numbers of axenic (exp) *T. rotula* were lower than those in the non-axenic treatment during the active growth phase from day 4 to 7 but remained higher thereafter. Numbers of axenic stationary (stat) *T. rotula* remained almost constant throughout the whole incubation period whereas those in the non-axenic treatment dramatically decreased already after 1 day, indicating a rapid bacterial degradation of the alga. In none of the axenic cultures were bacteria detectable by epifluorescence microscopy, demonstrating sterile conditions throughout the whole incubation.

**Dynamics of microparticles**

In both diatom cultures, concentrations of TEP continuously increased during exponential growth (Fig. 3A). The non-axenic (exp) *T. rotula* culture had higher numbers of TEP on days 7 and 9 than the axenic *T. rotula*. In contrast, the axenic (exp) *S. costatum* culture had substantially higher concentrations of TEP than the non-axenic culture after 2 days. In the (stat) *T. rotula* culture, TEP abundance increased over time in the axenic treatment but decreased in the non-axenic one (Fig. 3A). In axenic f/2 medium TEP
Fig. 2. Abundances of *Skeletonema costatum* (Skc, A), *Thalassiosira rotula* (Thr, B), free-living (C and D) and attached bacteria (E and F) and bacterial biomass production (G and H) in the course of experiments with the diatoms in exponential (exp) and stationary (stat) growth and in axenic (−) and non-axenic (+) cultures and in f/2 medium. Note the different scales of some corresponding y-axes. Error bars are given for replicates of the same experiment.
abundances were low and at times slightly higher in the non-axenic treatment. Concentrations of CSP in both (exp and stat) *T. rotula* cultures showed similar patterns as TEP (Fig. 3B). However, CSP abundance in the axenic (stat) *T. rotula* culture remained lower than TEP, whereas numbers of CSP decreased more strongly in the non-axenic culture than TEP. In contrast to TEP, numbers of CSP in both axenic and non-axenic (exp) *S. costatum* cultures increased until day 9. Thereafter, numbers further increased in the axenic treatment but decreased in the non-axenic one. In the f/2 medium, CSP abundance remained very low in both treatments except for days 4–7.

**Dynamic of bacterial numbers and activities**

Numbers of free-living and attached bacteria showed pronounced differences with respect to algal species and growth stage (Fig. 2C–F). Numbers of free-living bacteria in the (exp) *T. rotula* culture were similar to those in f/2 medium until day 7 with a peak on day 3. This suggests that growth was not due to algal-born substrates but due to DOC in the medium. When subtracting bacterial numbers in the f/2 medium (control) from those of the (exp) *T. rotula* culture, both numbers of free-living and attached bacteria remained low and only slightly increased towards the end. In contrast, free-living as well as attached bacteria in the (stat) *T. rotula* culture reached a pronounced maximum on day 3 and rapidly decreased thereafter. Numbers of free-living bacteria in the (exp) *S. costatum* culture strongly increased until day 7 and decreased thereafter, showing a similar but time-extended pattern as the (stat) *T. rotula* culture. In contrast, numbers of attached bacteria remained low and only slightly increased on days 9 and 11 when algal numbers declined, indicating enhanced bacterial colonization of senescent algae. Bacterial production (BP) co-varied with bacterial numbers both of (exp) *S. costatum* and of (stat) *T. rotula* with a pronounced peak on days 7 and 3 respectively (Fig. 2G–H). The initial increase of BP in (exp) *T. rotula* until day 3 co-varied with free-living bacterial numbers in
Dynamics of dissolved organic matter

Concentrations of DOC were almost similar in the axenic and the non-axenic cultures of (exp) *T. rotula* (Fig. 4). They increased towards the end of the experiment, co-varying only with enhanced AMP activities. In contrast, DOC concentrations in non-axenic cultures of (stat) *T. rotula* and (exp) *S. costatum* were much lower compared with the respective axenic treatments, indicating a rapid bacterial solubilization and decomposition of the algal-born organic matter. Dissolved organic carbon concentration in the axenic (exp) *S. costatum* culture constantly increased until the end of the experiment. In the (exp) *T. rotula* culture β-glucosidase activity remained low (<0.3 µmol l⁻¹ h⁻¹), except towards the end of the incubation period.

Concentrations of dissolved free (DFAA) and dissolved combined amino acids (DCAA) were generally lower in all non-axenic treatments (Fig. 5A and B). In contrast to DOC, amino acid concentrations remained low at the end of the experiment in the non-axenic treatment of the (exp) *T. rotula* culture. Organic carbon bound in dissolved amino acids constituted between 1% and 10% of DOC with highest proportions in the axenic (stat) *T. rotula* culture. Dissolved free amino acids released by both axenic algae were dominated by aspartic and glutamic acid, constituting 20–29 and 24–39 mol% respectively. In the (exp) *T. rotula* culture, bacteria preferentially consumed the acidic DFAA, as indicated by greatly reduced mol% of these amino acids to <13 mol% in the non-axenic treatments towards the end of the incubations. Mol% composition of DCAA was similar for both axenic and non-axenic algal cultures.

Dynamics of dissolved free neutral (DFCHO) and dissolved combined neutral monosaccharides (DCCHO) were rather similar to those of DFAA and DCAA with generally lower concentrations in the non-axenic algal cultures (Fig. 6A and B). Organic carbon bound in dissolved carbohydrates constituted between 1% and 13% of DOC with highest values in both axenic (stat) *T. rotula* and axenic (exp) *S. costatum* cultures. Glucose dominated the DFCHO and DCCHO released by both (exp) axenic algae (50–78 and 48–54 mol%) but constituted only 20 mol% in the (stat) axenic *T. rotula* culture. Other monosaccharides occurring in both pools included fucose, ribose, rhamnose, arabinose, galactose and mannose. Glucose was always consumed preferentially, as indicated by the strongly reduced mol% of glucose in the DFCHO pool of the non-axenic relative to the axenic treatments towards
the end of the incubation. In the DCCHO of the (exp) S. costatum culture, glucose and galactose were preferentially consumed as indicated by their reduced mol% in the non-axenic relative to the axenic treatments towards the incubation end. In the (stat) T. rotula culture, however, all neutral DCCHO were consumed in rather equal proportions.

Discussion

The two investigated diatoms represent two completely different lifestyles and types of interactions with bacteria. Whereas in S. costatum bacterial degradation of exudates prevents aggregation until a late growth stage, cells of T. rotula aggregate much more rapidly and irrespective of their bacterial colonization, but the presence of bacteria does enhance aggregation. Hence, bacteria prevent aggregation and subsequent sedimentation in the first case but favour these processes in the second one. These experiments with axenic and non-axenic diatom cultures clearly demonstrate that specific interactions of bacteria with the algae are instrumental for growth dynamics, aggregation, and sinking of these or other diatoms with similar properties during the course of a bloom. Specific algae–bacteria interactions have been largely neglected so far, but are presumably as important as inorganic nutrient supply and grazing in controlling the development and fate of diatom blooms (Kiørboe et al., 1994; Passow and Allardregde, 1995; Smith et al., 1995; Tiselius and Kuylenstierna, 1996). These interactions may vary to a certain extent, depending on the light conditions, the changing composition of the bacterial community during the various growth stages (Grossart et al., 2005) and varying temperatures. They may explain the variability of aggregation properties of diatoms observed in several studies (e.g. Kiørboe and Hansen, 1993; Passow and Allardregde, 1995).

So far, growth and aggregation experiments have been carried out with non-axenic cultures (Allardregde et al., 1993; Passow et al., 1994). Hence, it has been impossible to elucidate the role of heterotrophic bacteria in the aggregation process. Kiørboe and colleagues (1994), for example, found that a diatom bloom in a Danish fjord was...
dominated by S. costatum, and five other diatoms, including T. rotula, constituted minor proportions. The adhesive properties of the former diatom were much lower than those of most of the other species, but interactions with other diatoms enhanced the adhesive properties of S. costatum. The critical concentration of S. costatum for aggregation, however, was much higher than that of the other algae, explaining its numerical dominance until the termination of this bloom by aggregation and sedimentation. According to our experiments we assume that free-living bacteria consumed the DOC released by S. costatum, whereas the other diatoms were colonized faster by bacteria, thus enhancing aggregation and presumably their subsequent sedimentation. Because in early spring the water temperature in the fjord was much lower than in our experiments (2–4°C versus 15°C) and bacterial growth and physiological activity are low at this temperature, the situation in this field study resembled the situation in our axenic cultures.

Our observations complement previous studies which address the specific role of bacteria in the decomposition of diatoms and their silicate frustules as well as in the formation of diatom aggregates (Smith et al., 1992; 1995; Bidle and Azam, 1999). Our results, however, show that bacteria are also important in the initial phase of diatom blooms and can contribute to controlling the development of these blooms by more subtle and specific interactions with the diatoms present. Further, bacteria may also determine whether diatoms are subject to zooplankton grazing as single cells or colonies, or whether they aggregate and sink and thus escape zooplankton grazing (Jackson, 2001).

**Experimental procedures**

**Experimental design**

Two axenic marine diatoms (T. rotula, CCMP 1647 and S. costatum, CCMP 1332) were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, ME, USA). The algae were incubated in batch cultures in Guillard’s f/2 medium at 15°C in 2.5 l rolling tanks (5 r.p.m.) and illuminated in a 12:12 h light:dark cycle. For further experiments, one replicate of each a 12-day-old (exponentially growing) and a > 8-week-old (stationary
equivalent spherical diameter (ESD) and the equivalent images were used to assess the aggregate abundance, the light all aggregates within each window. The transformed chosen and the images were grey-scale transformed to high-

The total aggregate abundance in the experimental tanks (analySIS V 3.0, Soft Imaging System, Muenster, Germany). Amino acids

Axenic cultures of S. costatum and T. rotula both at exponential (exp) and stationary (stat) growth were incubated at concentrations of approximately 1000 cells ml$^{-1}$ in sterile polyvinyl tanks (1.2 l) rotating horizontally at 6 r.p.m. The tanks were illuminated for 24 h with either full light (100 µmol m$^{-2}$ s$^{-1}$) or low light (4.0 µmol m$^{-2}$ s$^{-1}$). All algal cultures were inoculated with a natural bacterial community (Wadden Sea samples) and incubated for at least 65 h at 15–20°C. Numbers and size classes of formed aggregates were determined by video and image analysis and growth of diatoms and bacteria as well as concentrations of TEP and CSP by microscopy (see below).

In an additional experiment, we incubated an axenic exponentially growing T. rotula culture in full light under sterile conditions for 68 h. Thereafter, the culture was inoculated with natural seawater bacteria and incubated for another 69 h.

Image analysis

Rolling tanks were illuminated with side-arm focus lamps to capture sample images with a Sony 900CCD handy cam (680K gross pixels) in a volume of 45 µm$^3$. In order to analyse the images, a 250 × 250-pixel window (1 pixel = 60 µm) was chosen and the images were grey-scale transformed to highlight all aggregates within each window. The transformed images were used to assess the aggregate abundance, the equivalent spherical diameter (ESD) and the equivalent spherical area (mm$^2$) by computer-assisted image analysis (analySIS V 3.0, Soft Imaging System, Muenster, Germany). The total aggregate abundance in the experimental tanks was corrected for the aggregate abundance in the corresponding control tanks.

Sampling

Samples for algal and bacterial abundance, dissolved substrates, bacterial activities, TEP and CSP were collected under sterile conditions (laminar flow) at regular intervals and were immediately processed for further tests and analyses. The volume withdrawn was replaced by an equal volume of sterile medium to avoid air bubbles inside the rolling tanks.

Microparticles

Duplicate subsamples (2 ml each) were filtered onto polycarbonate membranes (0.2 µm pore size) under vacuum (< 10 mbar pressure) to enumerate TEP and CSP. Transparent exopolymer particle samples were stained with 0.22 µm pre-filtered 0.02% Alcian Blue prepared in 0.06% glacial acetic acid (pH 2.5; Aldredge et al., 1993). Similarly, CSP samples were stained with 1 ml of 0.04% Coomassie brilliant blue (G-250) according to Long and Azam (1996). Samples were filtered dry, placed over a drop of oil on a frosted slide (Cyto-clear TM, Poretics, USA), and enumerated by using a Zeiss Axioplan microscope under bright field illumination and at a magnification of 100–200×. The abundance of TEP and CSP in the experimental tanks was corrected for that in the control tanks.

Algal and bacterial abundance

After staining with DAPI (4′,6′-diamidino-2-phenylindole) free-living and attached bacteria from 1 to 5 ml subsamples were counted on 0.2 and 5.0 µm Nuclepore membranes, respectively, by epifluorescence microscopy (Axioplan, Zeiss, Germany) at 1000× magnification (Porter and Feig, 1980). Algae were counted by simultaneously using light and epifluorescence microscopy at 400–1000× magnification. A minimum of 10 replicates was counted for each sample.

Dissolved organic carbon

Samples (10 ml) were collected in glass ampoules after filtration through 0.2 µm polycarbonate membranes (Nuclepore, USA). Samples were acidified with 100 µl of 85% H$_2$PO$_4$, flame sealed, and stored until analysis at 4°C in the dark. Dissolved organic carbon was analysed by high temperature combustion (Shimadzu TOC-5000). The standard deviation between three injections per sample was usually < 1%. The instrument blank (6–12 µM C) was measured using UV-irradiated Milli-Q water and was subtracted from each sample.

Neutral carbohydrates

Samples (10 ml) were filtered using 0.2 µm pore size polycarbonate filters (Nuclepore) and stored frozen at −20°C until analysis. Concentrations of DFCHO were analysed by HPLC using a Carbopac PA 10 column (Dionex, USA) and pulsed amperometric detection (Mopper et al., 1992). NaOH (20 mM) was used as eluent. Before analysis, samples were desalted by ion-exchange chromatography according to Borch and Kirchman (1997). DCCHO were analysed by HPLC as DFCHO after 20 h of hydrolysis with 0.09 N HCl at 100°C.

Amino acids

Samples (10 ml) were filtered through 0.22 µm pore size low protein binding acrodisc filters (Pall Corporation) and stored frozen at −20°C until analysis. Concentrations of DFAA were
analysed by HPLC after ortho-phthalaldehyde derivatization (Lindroth and Mopper, 1979). Dissolved combined amino acids were hydrolysed with 6 N HCl at 155 °C for 1 h and analysed as DFAA.

Bacterial production and hydrolytic enzyme activities

Bacterial production was determined by incorporation of [14C]-leucine (14C-Leu; Simon and Azam, 1989). Triplicates and a formalin-killed control were incubated with [14C]-Leu (292 mCi mmol−1), Hartmann Analytik, Braunschweig, Germany) at a final concentration of 50 nM which ensured saturation of leucine uptake systems. Incubation was performed in the dark at 15 °C for 1 h. Further processing, including fixation, filtration and radioassay followed the procedure outlined in Grossart and colleagues (2004). BP was calculated according to Simon and Azam (1989).

Aminopeptidase and β-glucosidase activities were measured using L-leucine-methyl coumarinyl amide (Leu-MCA) and methyl-umbelliferyl-β-D-glucoside (β-D-Gluc-MUF) as substrate analogues according to Hoppe (1993). For each substrate, triplicates and a formalin-killed control were incubated at 15 °C in the dark for 1 h. Final concentrations of substrate analogues were 100 µM which ensured maximum hydrolysis as determined by saturation kinetics. Fluorescence of both fluorochromes was measured in a TD 700 fluorometer (Turner Design, USA) at 300–400 nm (excitation) and 410–610 nm (emission).

Acknowledgements

We thank Birgit Kürzel and Rolf Weinert for neutral monosaccharide analysis and Lars Borchers, Isabel Schmalenbach and Florian Levold for sample collection. This work was supported by the Deutsche Forschungsgemeinschaft within the Research Group BioGeoChemistry of the Wadden Sea (FG 432-TP5).

References


