

Degradation of riverine dissolved organic matter by seawater bacteria

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ABSTRACT: The functional response of a seawater bacterial community transplanted into freshwater dissolved organic matter (DOM) was investigated together with the response of natural populations of bacteria to size-fractionated natural source water. Seawater bacteria were incubated over a period of 8 d in size-fractionated, freshwater DOM collected from Randers Fjord, Denmark, during spring (April) and summer (August) of 2001. Three fractions were used: 0.2 μm filtered (<0.2 μm -DOM), >1 kDa (high molecular weight, HMW-DOM) and <1 kDa (low molecular weight, LMW-DOM). The results were compared with parallel control incubations of freshwater bacteria in size-fractionated freshwater DOM and seawater bacteria in size-fractionated seawater DOM. There were few differences in bacterial abundance (BA) and production (BP) within each incubation type in spring, but this was not the case in summer. While the seawater bacteria transplanted into freshwater HMW-DOM performed similarly to those in seawater HMW-DOM, freshwater bacteria in freshwater HMW-DOM exhibited higher BA, BP and bacterial respiration (BR), indicating a difference in the physiological abilities of the seawater bacterial assemblage compared to that of the natural freshwater assemblage. Bacterial growth efficiency (BGE) varied between 11 and 41 % and the highest values were generally in the HMW-DOM size fraction. Comparison of the 'bioavailability' of the DOM predicted from BGE, the amino acid degradation index (DI), dissolved organic carbon degradation rates and the bioavailability index showed that net change in DI of dissolved combined amino acids over the course of an incubation can be a good predictor of most of the other indices. The separation of DOM into molecular weight size fractions resulted in different estimates of bioavailability than would have been predicted from the rates observed in the <0.2 μm -DOM fraction. These results further demonstrate the flexibility of bacteria in their ability to utilize different sources of DOM, and highlight the variability that can be observed when different indices are used to determine the bioavailability of organic matter to heterotrophic bacteria.

KEY WORDS: Bacterial growth efficiency · BGE · HMW · LMW · DOM · Randers Fjord Estuary · Amino acids · Degradation Index · DI

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INTRODUCTION

Estuaries are dynamic environments in which marine and fresh waters mix and present a unique ecosystem where organisms need to be able to adapt to the changes in the physical, chemical and biological parameters that occur on very short time scales (min-

utes to days). The distribution of chemical components such as dissolved organic matter (DOM) and inorganic nutrients along the estuary can be a primary determinant of the abundance of the biological components. The availability of nutrients and light can have a dramatic effect on phytoplankton abundance (Fisher et al. 1988).

Bacterial activity is the main pathway by which DOM and nutrients are remineralized, and as such bacteria play an important role in the functioning of ecosystems (del Giorgio & Cole 1998). Several studies have examined bacterial abundance (BA) and production (BP) along estuarine salinity gradients and the distribution of these parameters tends to vary with estuary (e.g. Kirchman & Hoch 1988, Revilla et al. 2000, del Giorgio & Bouvier 2002). For example, in Chesapeake Bay, a large coastal plain estuary, Smith & Kemp (2003) reported highest production rates in the mesohaline regions, where bacterial activity is often associated with the estuarine chlorophyll a maximum. This distribution was also observed by Crump et al. (2004) in the Plum Island Sound estuarine system. In contrast, in tidal and highly turbid estuaries, the distribution of BA and BP is often somewhat different. In these systems, BA and BP are generally highest in the lower salinity waters and decrease with increasing salinity (Heip et al. 1995, Iriarte et al. 1996, Goosen et al. 1997). Recently, del Giorgio & Bouvier (2002) remarked on the relationships between specific bacterial groups and the physico-chemical parameters in an estuary, and proposed that these parameters could control the distribution of the different bacterial groups and total BA. This has been further extended upon by Crump et al. (2004), who recently demonstrated the presence of 3 'types' of bacterial community along an estuarine salinity gradient. In the lower salinity waters, the bacterial community was dominated by phylotypes characteristic to freshwaters. This was mirrored in the coastal endmember, where the majority of the bacterial community was composed of typically marine bacterial populations. They also showed a unique estuarine bacterial community that existed at intermediate salinities during summer and fall.

As the bulk DOM pool crosses the estuarine salinity gradient it is subject to losses through biological utilization and to inputs from both allochthonous sources (e.g. adjacent marshes and small rivers) and autochthonous sources (e.g. autotrophic release, sedimentary release). Thus, changes in the DOM pool can occur not only from dilution along the salinity gradient, but also from biological and chemical processes, all of which affect the bulk composition, properties and, hence, bioavailability of the DOM, even if the bulk concentration remains unaltered (Peterson et al. 1994, Mannino & Harvey 1999, 2000).

Changes in BA, bacterial activity and bacterial growth efficiency (BGE) are also probably related to the concentration of organic and inorganic nutrients as well as to the bioavailability of DOM. Bioavailability itself is determined by the properties of the substrate as well as by the physiological abilities of the bacterial

communities present (del Giorgio & Davis 2003). Thus, bioavailability can be viewed as the product of the interaction of DOM with the diversity of microbial properties (Foreman & Covert 2003). Amon & Benner (1996) developed the concept of the size reactivity continuum and proposed that high molecular weight DOM (HMW-DOM) is generally of higher bioavailability than low molecular weight DOM (LMW-DOM). This is true for systems with low DOM concentrations and low proportions of HMW-DOM, such as open ocean waters, as well as for riverine and coastal waters with higher DOM concentrations and proportions of HMW-DOM. Raymond & Bauer (2000), examining the differences in DOM bioavailability along an estuarine salinity gradient, also reported that this paradigm seems to apply to the York River Estuary, a sub-estuary of Chesapeake Bay, with high freshwater DOC concentrations (430 $\mu\text{M C}$) and high proportions of HMW-DOM.

Although several studies have examined the bioavailability of size-fractionated DOM to bacteria and Gasol et al. (2002) have examined the differences in DOM bioavailability to bacteria at opposing ends of a lake, few have investigated the ability of bacteria to respond to DOM from another region of an estuarine salinity gradient (e.g. Stephanaukas et al. 1999). Here we present results from a study conducted in both spring and summer in a Danish estuary. The objectives of this experiment were 3-fold: (1) To determine if the bacterial response to different size classes was constant. (2) To examine the response of a natural seawater bacterial community grown in different size fractions of freshwater DOM. As bioavailability is determined by the interaction of the chemical properties of DOM with the physiological abilities of microbial communities it is probable that different measures of this parameter may yield different conclusions. The third objective was: (3) to compare a number of approaches to measure bioavailability, namely the decay constant of DOC degradation (k), BGE, the bioavailability index, enzyme activities, and the degradation index of dissolved free and combined amino acids (DFAA and DCAA, respectively) to determine if these approaches yield similar conclusions.

MATERIALS AND METHODS

Sample collection. Water samples were collected in spring (April 21 to 23, 2001) and summer (August 20 to 22, 2001) from 2 stations in Randers Fjord, Denmark. The first station (Stn F) was located at the freshwater end of the estuary in the town of Randers and the second station (Stn S) was located offshore of the mouth. These stations were chosen to represent as closely as

possible the freshwater and the seawater endmember. For each set of incubations, 65 l of water were collected from the surface mixed layer and stored in acid-washed (10% HCl) and Milli-Q water rinsed carboys and returned to the laboratory for processing.

Preparation of DOM size fractions. Before preparing the ultrafiltrate, the water was prefiltered sequentially through pre-combusted GF/F filters (\varnothing 142 mm, Whatman) and a 0.2 μm (Durapore, Millipore) cartridge filter. Prefiltration through the GF/F filters was necessary to remove the particulates observed in the freshwater samples to prevent clogging of the 0.2 μm cartridge filters. Twenty liters of 0.2 μm filtrate were retained for the incubations; the other 30 l were ultrafiltered through a 1 kDa regenerated cellulose filter (Prep-Scale/TTF cartridge, Millipore). Before each use, the cartridges were cleaned as per the manufacturer's recommendations and were conditioned with a minimum of 10 l of 0.2 μm filtrate before collection of the ultrafiltrate. Although blanks were not performed during this filtration, previous analyses in the laboratory have shown that the protocol used minimized contamination. The ultrafiltration procedure resulted in 2 size fractions. The >1 kDa fraction (the retentate) is defined as the HMW fraction and the <1 kDa size fraction (the filtrate) is defined as the LMW fraction. In April, the concentration factor of the retentate for both the seawater and freshwater ultrafiltrations was 5. This resulted in a recovery of 105 and 84 % for the freshwater and seawater stations, respectively. In August, the concentration factors were slightly higher (8 for the Stn F and 7 for Stn S), which resulted in a recovery of 112 and 95 %, respectively. In April, a second ultrafiltration was performed to recuperate sufficient HMW-DOM for measurement of DFAA and DCAA; for this ultrafiltration concentration factors of 20 and 25 were used for the Stns F and S, respectively. In August, this second ultrafiltration was not performed and samples were collected from the same ultrafiltration as the incubation size fractions.

For each station 3 different water fractions were used for the incubations: (1) <0.2 μm -DOM; (2) low molecular weight (LMW-DOM); and (3) high molecular weight (HMW-DOM). The <0.2 μm -DOM fraction is considered to comprise of the entire DOM pool and the LWM-DOM fraction only the DOM with a molecular weight of less than 1 kDa. The HMW-DOM fraction was composed of 0.2 μm filtered water with an addition of HMW-DOM concentrate (Raymond & Bauer 2000). Thus, the HMW-DOM fraction is considered as a HMW-DOM enriched 0.2 μm -DOM pool. We chose this method to minimize the time needed for preparation of the size fractions. Our intention was that the addition of DOM in the HMW incubation would be 50 $\mu\text{mol l}^{-1}$ C; however, we did not always

achieve this goal. The lack of prior knowledge of the exact amounts of HMW-DOM in the water necessitated some guesswork that resulted in several of the incubations having a higher content of HMW-DOM than initially intended.

Preparation of bacterial inoculum. The natural bacterial inoculum was prepared from gravity filtered water (0.8 μm cellulose acetate filter, Osmonics) from each station and represented an addition of 5 % of the total volume of the incubation, e.g. 90 ml inoculum into 1800 ml of filtrate. Filtration through 0.8 μm was necessary to remove the phytoplankton, grazers and larger (>0.8 μm) non-living particulates from the inoculum. One of our goals was to examine the response of seawater bacteria to freshwater DOM. To address this, a bacterial inoculum from Stn S, the seawater site, was added to each of the prepared DOM fractions from Stn F, the freshwater site. This treatment is referred to as the transplanted treatment; the freshwater DOM with the freshwater bacterial inoculum is called the freshwater control treatment, and the seawater inoculum in seawater DOM is called the seawater control. In the transplantation treatment, the salinity of the freshwater filtrate was adjusted to 18 (salinity of Stn S) with an artificial seawater (ASW) mix (Guillard 1975) in order to minimize as much as possible the effects of the salinity change on the bacterial population. The ASW salts were all from Baker Insta-Analysed[®] and a previous experiment showed that the level of DOC contamination from this salt mix is minimal (~ 10 $\mu\text{M C}$). Duplicate 1.8 l samples were incubated for 8 d in the dark and at *in situ* temperature (6°C in April and 18°C in August; see Table 1). Throughout the incubation, 100 ml of sample were collected for dissolved organic carbon (DOC), BA and BP at Time 0, 18, 32, 90, 132 and 205 h.

Sample analyses. Samples for DOC were collected, after filtration through combusted (450°C, overnight) glass fiber filters (Whatman GF/F), in precombusted 10 ml glass ampoules, preserved with 12 μl 85 % phosphoric acid (H_3PO_4) and flame sealed. Samples were stored in the dark at 4°C until analysis. DOC concentration was measured on a Shimadzu 5000-TOC analyzer, using potassium phthalate calibration standards over the measurement range 0 to 800 $\mu\text{M C}$. Certified reference materials (Hansell Laboratory, University of Miami) were also used to assess the performance of the machine on and between measurement days. The machine blank, calculated as the difference between the measured concentration of the external standards and the 'real' concentration of the external values, was between 8 and 12 $\mu\text{M C}$ for the measurement days.

DFAA were measured by reverse-phase high-performance liquid chromatography (HPLC) using a

fluorescence detector and after precolumn derivatization with *o*-phthalaldehyde and *N*-isobutyrylcystein (Fitznar et al. 1999). DCAA were measured as for DFAA but after acid hydrolysis for 24 h at 110°C. After hydrolysis, the samples were evaporated to dryness and taken up in a borate buffer (Dauwe & Middelburg 1998). Urea was measured by colorimetry using the diacetyl-monoxime reagent.

Potential extracellular enzymatic activities were measured using methylumbelliferyl-labelled (MUF) or 7-amino-4-methylcoumarin (AMC)-labeled substrates. MUF-beta-glucose (beta-glucosidase), MUF-alfaglucoase (alpha-glucosidase), MUF-xylose (beta-xylosidase), MUF-butyrate (esterase), MUF-N-acetyl-D-glucosamine (chitinase) and AMC-leucine (leucine-aminopeptidase) were used at substrate-saturated concentrations. Briefly, substrate was added to 1 ml of unfiltered water, incubated at room temperature for 1 to 4 h and fluorescence increase measured using a Turner fluorometer.

BA was measured by a direct count method using epifluorescent microscopy and DAPI-stained samples (Porter & Feig 1980). Samples (10 ml) were preserved with 0.2 µm pore-size filtered borax-buffered formalin (2% final concentration) and stored at 4°C in the dark. Within 36 h, 10 ml samples were stained with 0.2 µm filtered DAPI solution for 10 min before filtering onto a black 0.2 µm pore size Osmonics filter. Filters were mounted on slides with non-fluorescent immersion oil and stored frozen until enumeration. For enumeration, at least 20 to 30 randomly selected microscope fields on each filter were counted with a Zeiss microscope at a magnification of 1250.

Bacterial biomass (BB) was calculated from the numbers of bacteria using a bacterial carbon content of 15 fg C cell⁻¹ (Caron et al. 1995). It has been shown elsewhere that bacterial cell size may increase over the incubation and to correct for this we multiplied the bacterial carbon biomass by 15% (Raymond and Bauer 2000).

BP was measured using ³H-leucine in accordance with the method of Smith & Azam (1992). Triplicate 1.5 ml aliquots of sample were incubated for 1 h with either 10 nM (10 nM ³H-leucine) or 90 nM leucine (10 nM ³H-leucine + 80 nM cold leucine) in April and 90 nM leucine (10 nM ³H-leucine + 80 nM cold leucine) in August. One TCA (100 µl 100% trichloroacetic acid)-killed control was ran in parallel with each group of replicates. At the end of the incubation period, all samples were killed with 100 µl 100% TCA and processed according to Smith & Azam (1992). The amount of radioactivity taken up in the particulate fraction was measured in a scintillation counter using Ultima Gold scintillation cocktail (Packard Instruments).

Bacterial respiration over the course of the experiment was also measured. For each incubation, six 60 ml BOD bottles were filled. Three bottles were fixed immediately with Winkler reagents. The other 3 bottles were incubated in the dark alongside the larger volume incubations until the end of the experiment. Oxygen concentration was titrated using an automated Winkler titration technique with potentiometric endpoint detection (Anderson et al. 1992). Reagents and standardizations were similar to those described by Knap et al. (1996).

The decay constant of DOC degradation (*k*) was calculated from the slope of the regression of ln(DOC) over the time of the incubation. Bacterial carbon demand (BCD) was calculated as the sum of the increase in BB and the decrease in oxygen expressed in carbon units using a respiratory quotient of 1:

$$\text{BCD} = (\Delta\text{BB} + |\Delta\text{O}_2|) \quad (1)$$

BGE was calculated as the ratio between bacterial biomass increase and BCD:

$$\text{BGE} = \frac{(\Delta\text{BB}) \times 100}{\text{BCD}} \quad (2)$$

BCD and BGE were calculated from the increase in BB rather than from BP rate measurements, because (1) there is a considerable difference in the time scales of the measurement of BP (1 h incubation) and bacterial respiration (duration of the experiment) and (2) the abundance of grazers was rather low in the incubations.

Bioavailability was calculated according to Amon & Benner (1996) using:

$$\text{Bioavailability index} = \frac{\text{BCD} \times 100}{(\text{DOC}_{\text{initial}})} \quad (3)$$

To avoid confusion between the use of the word 'bioavailability' as a general term to describe the utilization of organic matter by bacteria and that of the calculation of bioavailability proposed by Amon & Benner (1996; their *F*-value) we used the term 'bioavailability index' when referring to the above-calculated values.

Amino acid composition spectra were used to estimate the degradation index (DI) introduced by Dauwe & Middelburg (1998). The DI links amino acid composition to organic matter degradation state and varies from +1.5 for labile, fresh material to -3 for refractory, aged organic matter (Dauwe et al. 1999).

Statistical comparisons. Relationships between parameters were analyzed using ANOVA or Student's *t*-test and correlation analysis on untransformed data, when the assumptions were met, and on transformed data, when the assumptions of the tests were not met. *p*-values of more than 0.05 were deemed non-significant.

RESULTS

General conditions

Temperatures were low at both stations in April (6°C; Table 1) and had increased to 18°C by August. BA was higher in summer than in spring and was always higher in the low salinity waters, with Stn F exhibiting a BA approximately 1.5 to 2 times higher than at Stn S (Table 1). DOC concentrations also varied between seasons, with concentrations being slightly higher in April than in August for both stations (Table 1).

There were differences between both stations and seasons in the proportion of LMW-DOM. Stn F had a lower proportion of LMW-DOM for both seasons, with 67 and 42% of the total DOM being present in the LMW fraction for April and August, respectively (Table 2). The percentage of DOM in the LMW fraction was higher at Stn S and, unlike Stn F, the proportion remained relatively constant (82 and 84% for April and August, respectively). Although there was a large difference in both total DOM concentration and the percentage of DOM in the LMW fraction, the concentration of the LMW-DOM fractions was similar for Stns F and S in August (155 and 172 $\mu\text{M C}$ for Stn F and Stn S, respectively). This was not the case in April, when higher total DOM concentrations and relative percentages of LMW-DOM meant that Stn F had a higher concentration of LMW-DOM than Stn S (250 vs 184 $\mu\text{M C}$).

Amino acids and enzyme activities

Both free and combined amino acid concentrations were different between the 2 periods studied. In April, DFAA concentrations were higher at Stn F; however, the pattern was reversed in August, when highest concentrations were observed at Stn S (Table 1). DCAA did not exhibit a large change in concentration between April and August (1339 and 1272 nM, respectively) at Stn S; however, this was not the case at Stn F, where DCAA concentration was over 2 times higher in August compared to April (2705 vs 1298 nM, respectively).

Concentrations of DCAA in the HMW concentrate were 7 to 20 times higher than in the GF/F fraction ($[\text{HMW}] = [\text{GF/F}] - [\text{LMW}]$), indicating that they were concentrated during the ultrafiltration procedure (Table 1). However, the presence of DCAA in the LMW fraction and of, conversely, DFAA in the HMW fraction also highlights the imperfect separation of the components. DCAA composition spectra were used to estimate the DI of DOM. DIs were all lower than -1, except for HMW and the <0.2 μm -DOM fraction at Stn S during summer. The low DI values indicate that DOM in Randers Fjord has undergone significant degradation.

Potential rates of enzyme activities differed between stations and seasons, with rates being higher at Stn F and during August (Table 1). Rates of beta-glucosidase and beta-xylosidase (both involved in the degradation of macrophyte-derived detritus) were higher at Stn F.

Table 1. Conditions in surface waters at time of sampling. Amino acid concentrations in the dissolved combined amino acids (DCAA) and dissolved free amino acids (DFAA) (nmol AA l^{-1}) in the 2 molecular weight size fractions. Concentration factors from the ultrafiltration of the high molecular weight (HMW) size fraction were 25 and 20 for April at Stns F and S (freshwater and seawater stations, respectively), and 8 and 7 for August at Stns F and S, respectively. BA: bacterial abundance

	Stn F		Stn S	
	April	August	April	August
Salinity	0.2	0.2	21.4	21.8
Temperature (°C)	6.0	18.0	6.0	18.6
DOC ($\mu\text{M C}$)	370.2	353.4	224.1	205.6
BA (10^6 cells ml^{-1})	4.1	5.2	2.2	3.5
Urea ($\mu\text{M N}$)	0.83	1.88	1.12	2.09
Alpha-glucosidase ($\text{nmol l}^{-1} \text{h}^{-1}$)	28.1	29.5	3.0	40.7
Beta-glucosidase ($\text{nmol l}^{-1} \text{h}^{-1}$)	48.9	49.9	7.7	3.1
Beta-xylosidase ($\text{nmol l}^{-1} \text{h}^{-1}$)	9.7	44.5	1.1	12.8
Esterase ($\text{nmol l}^{-1} \text{h}^{-1}$)	819.1	1058.0	242.4	751.1
Chitinase ($\text{nmol l}^{-1} \text{h}^{-1}$)	36.5	35.6	6.7	12.9
Leucine aminopeptidase ($\text{nmol l}^{-1} \text{h}^{-1}$)	1310.7	1211.0	139.9	47.8
DCAA-GF/F (nmol AA l^{-1})	1339	1272	1298	2705
DCAA-HMW (nmol AA l^{-1})	23267	12508	11930	3084
DCAA-LMW (nmol AA l^{-1})	125	446	714	2258
DFAA-GF/F (nmol AA l^{-1})	3.7	944	13.9	268
DFAA-HMW (nmol AA l^{-1})	508	144	235	115
DFAA-LMW (nmol AA l^{-1})	680	172	80	109

Table 2. DOC removal, rate constants and bacterial growth efficiency (BGE). Calculations used the data from the full 8 d of incubation. The slope (k) corresponds to the rate constant of the DOC removal. % LMW is the amount of LMW relative to $<0.2 \mu\text{m}$ -DOM filtered size fraction. Net decreases in DOC and O_2 are reported as the mean (\pm SE). Bioavailability is calculated according to Amon & Benner (1996): $(\text{BCD} \times 100)/(\text{DOC}_{\text{initial}})$, as in Eq. (3)

Water source	Bacteria source	Initial DOC ($\mu\text{M C}$)	LMW (%)	Slope (k) ($10^{-3} \mu\text{M C d}^{-1}$)	DOC removal ($\mu\text{M C}$)	Resp. ($\mu\text{M O}_2$)	BGE (%)	Bioavailability value (%)
April								
Stn F, $<0.2 \mu\text{m}$	Stn F	371		-5.2	17 (0.3)	9.7 (1.2)	18	3.2
Stn F, LMW	Stn F	249	67	-5.1	11 (0.1)	10.3 (1.0)	23	5.4
Stn F, HMW	Stn F	417		-4.7	17 (3.3)	8.4 (2.9)	28	2.8
Stn S, $<0.2 \mu\text{m}$	Stn S	224		-4.8	9 (0)	7.5 (0.8)	20	4.2
Stn S, LMW	Stn S	184	82	-10.3	16 (0.2)	5.2 (1.7)	36	4.4
Stn S, HMW	Stn S	292		-6.9	17 (0.3)	9.0 (3.2)	32	4.5
Stn F, $<0.2 \mu\text{m}$	Stn S	377		-8.0	26 (0.1)	11.6 (0.6)	16	3.7
Stn F, LMW	Stn S	251	67	-1.5	3 (2.3)	12.5 (0.6)	11	5.6
Stn F, HMW	Stn S	399		-2.4	9 (9.7)	9.9 (2.2)	21	3.1
August								
Stn F, $<0.2 \mu\text{m}$	Stn F	363		-13.7	53 (5.7)	24.5 (4.9)	25	9.0
Stn F, LMW	Stn F	146	46	-8.6	12 (5.8)	15.7 (1.5)	17	13.0
Stn F, HMW	Stn F	729		-18.2	73 (2.3)	33.6 (1.5)	41	7.8
Stn S, $<0.2 \mu\text{m}$	Stn S	206		-1.5	2 (1.3)	16.7 (2.8)	-0.1	8.0
Stn S, LMW	Stn S	172	84	-4.7	11 (2.9)	9.4 (2.8)	23	6.9
Stn S, HMW	Stn S	301		-6.1	19 (1.3)	11.5 (2.6)	-0.1	3.8
Stn F, $<0.2 \mu\text{m}$	Stn S	346		-3.6	12 (0.1)	13.2 (0.5)	30	5.5
Stn F, LMW	Stn S	165	42	-9.2	13 (0.1)	13.7 (0.6)	28	11.5
Stn F, HMW	Stn S	677		-14.3	80 (0.1)	18.8 (3.8)	29	4.0

Potential activities of leucine-aminopeptidase (degradation of proteinaceous material) and chitinase were also significantly higher at Stn F than at Stn S. These differences in enzyme activities likely reflect the combined effect of substrate quantity and quality, and the composition of the microbial community.

Incubations

Bacterial abundance and production

In April, BA was initially low in all the incubations and increased very slowly over the first 48 h. Bacteria in the control incubations (Fig. 1A,C), regardless of the molecular weight and quantity of DOM, generally continued to increase until the end of the experiment. In the transplanted incubation (Fig. 1B), BA leveled off towards the end of the experiment; however, BA was not statistically significantly different between the DOM size fractions from each station (AVOVA, $p > 0.05$) and there was no evidence that the transplantation of the seawater bacterial community into freshwater-DOM inhibited the bacteria as the change in BA was similar to the freshwater and seawater controls (ANOVA, $p > 0.05$).

In August, although DOC concentrations were lower in most incubations than in April (Table 2), BA was

higher in the incubations (Fig. 1D–F, Student's t -test, $p < 0.01$). As in April, BA was initially low (0 and 18 h) but began to increase in all incubations from 24 h onwards, sooner than was observed for April. The only exceptions were the $<0.2 \mu\text{m}$ -DOM and the HMW-DOM incubations for the seawater control, for which there was a rapid crash in BA between 132 h and the end of the experiment at 205 h (Fig. 1D). Unlike in April, there were differences between the size fractions in both freshwater control and the transplanted incubation. In both of these incubations, the increases in BA were always higher in the HMW-DOM fraction relative to the LMW-DOM incubation (Student's t -test, $p < 0.05$). This was particularly evident in the freshwater HMW-DOM control incubation, where a rapid increase in BA was observed towards the end of the incubation ($>17 \times 10^6 \text{ cells ml}^{-1}$, Fig. 1F).

In April, there were no large differences in cell-specific BP between the size fractions within a treatment or between transplanted and control incubations as a whole (AVOVA, $p > 0.05$; Fig. 2A–C). In August, cell-specific BP was similar in the transplanted and freshwater control incubations (Fig. 2D,E), with maximum rates of cell-specific BP 3 to 10 times higher than the seawater control incubation after 132 h. High cell-specific BP rates were also observed in the HMW-DOM treatment of the seawater control at the end of the incubation (Fig. 2D).

In summary, there appeared to be little difference in terms of BA and BP between different size fractions within each control or transplanted incubation (ANOVA, $p > 0.05$ in all comparisons). Comparing cell-specific BP between the control and transplanted incubations, although there were some differences in the patterns of production between the incubations in April, this was not as evident in August. The only exception was the HMW-DOM freshwater incubation, for which very high BA was. These results indicate that the bacterial community composition within the incubations was either very plastic in its ability to use a wide range of substrates or that it was able to adapt to changes in substrate supply in very short periods of time (hours).

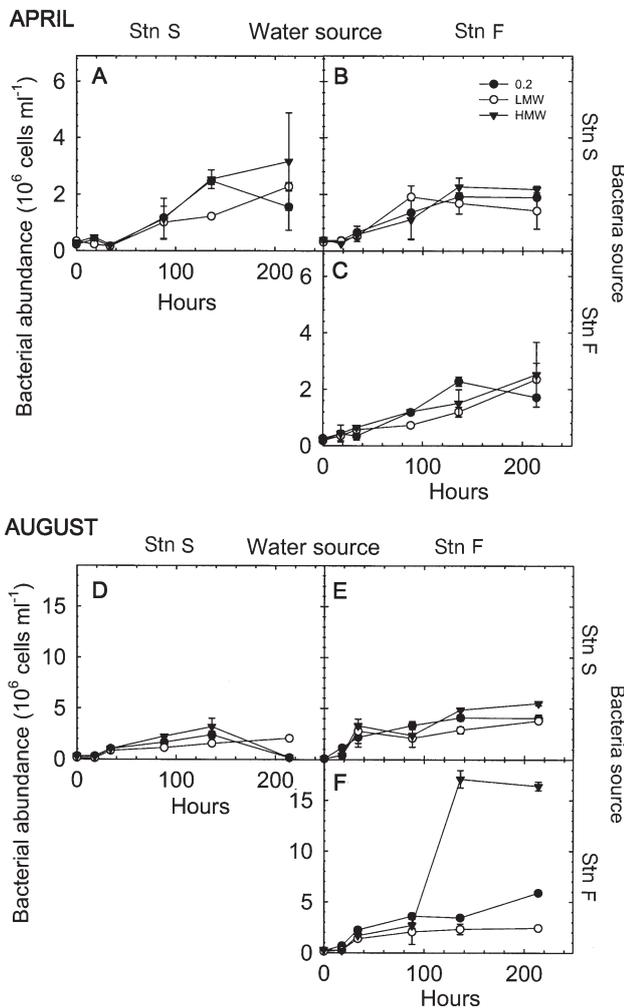


Fig. 1. Changes in bacterial abundance (BA) during seawater control incubations in (A) April and (D) August, transplanted incubations (seawater bacteria in freshwater DOM) in (B) April and (E) August and freshwater control incubations in (C) April and (F) August. Note that the y-axis scales in April and August differ

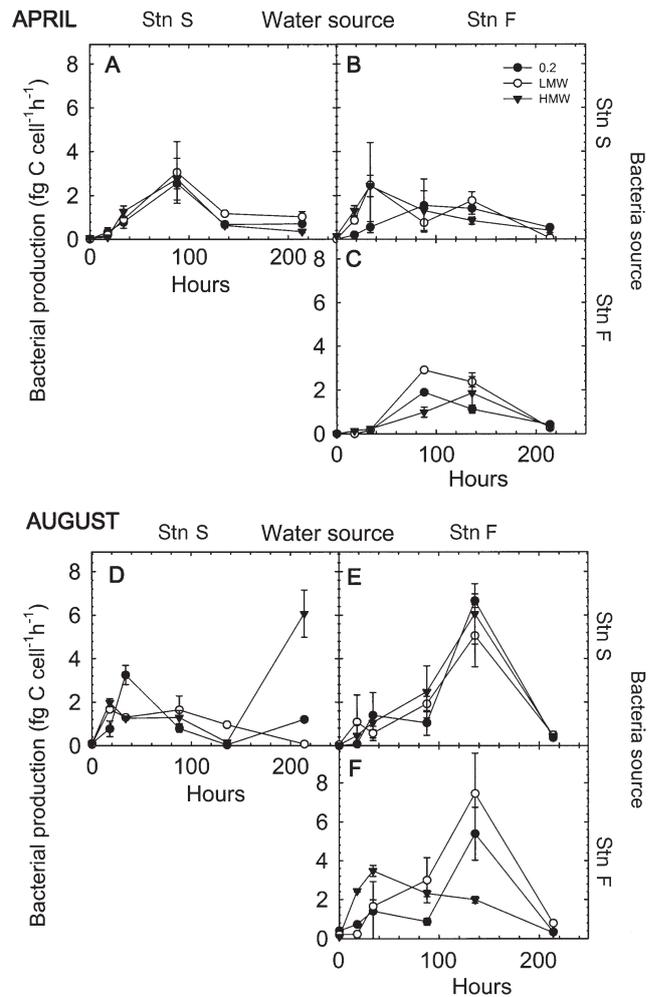


Fig. 2. As for Fig. 1, except for cell-specific bacterial production (BP)

O₂ and DOC

The concentrations of oxygen decreased during the 205 h of incubation. Although there were no differences in the changes of dissolved oxygen between the size fractions in spring within each incubation (1-way ANOVA, $p > 0.05$), significant differences in oxygen removal were observed between the 2 controls and the transplanted incubations (1-way ANOVA, $p < 0.01$). Respiration was significantly higher in the transplanted incubation compared to the seawater control incubation (Student's t -test, $p < 0.05$), although, interestingly, there was no significant difference between the respiration rates between the seawater and freshwater control incubations (t -test, $p > 0.05$).

In August, respiration was significantly higher than in April (t -test, $p < 0.01$) and, as observed in April,

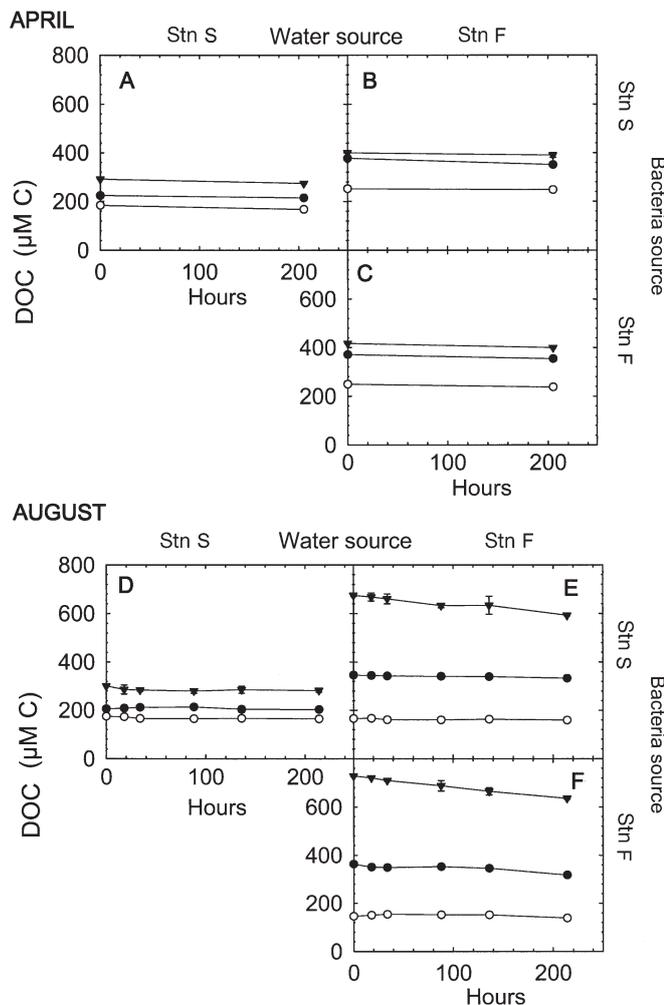


Fig. 3. As for Fig. 1, except for dissolved organic carbon concentration (DOC)

there were significant differences between the control and transplanted incubations (ANOVA, $p < 0.01$). Oxygen removal was significantly higher in the freshwater control incubation compared to both the seawater control incubation and the transplanted incubation (t -tests, $p < 0.01$). In both the seawater and freshwater control incubations, oxygen consumption in LMW-DOM was less than that of HMW-DOM (t -test, $p < 0.01$). This decrease was most noticeable in the freshwater control incubation, where decreases of $33.6 \mu\text{M O}_2$ were observed in the HMW-DOM incubation compared to only $15.7 \mu\text{M O}_2$ in the LMW-DOM incubations (Table 2).

In summary, oxygen consumption was up to 2–3 times higher in August than in April. Furthermore, in summer, significant differences in oxygen consumption were evident between the different size fractions within each control or transplanted incubation, with LMW-DOM having lower oxygen consumption than

the HMW-DOM treatments. There were no significant differences in oxygen removal between the size fractions in spring.

DOC concentrations also decreased throughout the experiment in both months (Fig. 3, Table 2). In April, net DOC removal was significantly lower than in August (t -test, $p < 0.05$) and there were significant differences between the size fractions within each incubation as well as between the control and transplanted incubations. In April, net DOC removal was significantly higher in the freshwater control incubation compared to the transplanted incubation (t -test, $p < 0.05$); however, this was not the case when the seawater control was compared with either the freshwater control (t -test, $p > 0.05$) or the transplanted incubation (t -test, $p > 0.05$). Nevertheless, within each incubation, there were differences between the size fractions, particularly between the LMW-DOM fraction and the 2 other fractions. In both the freshwater control and the transplanted incubation, DOC removal was significantly lower in LMW-DOM (t -tests, $p < 0.01$), whereas in the seawater control incubation, DOC removal in the LMW-DOM fraction was significantly greater than in the other 2 fractions (t -test, $p < 0.05$).

In August, DOC removal was higher in the freshwater control incubation than in the seawater control (t -test, $p < 0.05$). However, there was no difference between either of the control incubations and the transplanted incubation (t -test, $p > 0.05$). In terms of size fractions, as in April, there were differences between the incubations. In the seawater control, the fractionation of DOM did not appear to have any effect on DOC removal (t -test, $p > 0.05$); however, in both the

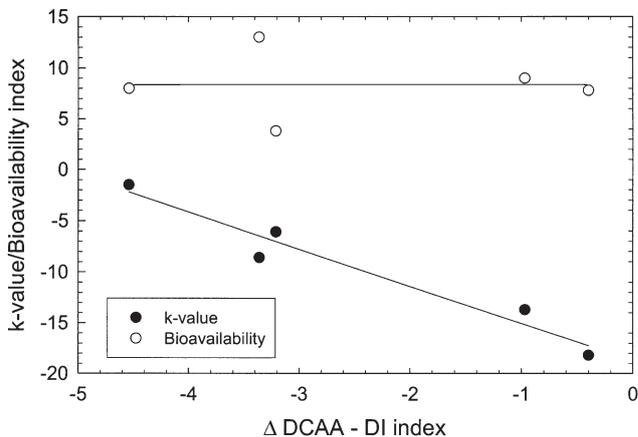


Fig. 4. Net change in degradation index (DI) for dissolved combined amino acids (DCAA) between the initial and final time points and decay constant of DOC degradation (k), the bioavailability index. The outlier values from the seawater < 1 kDa fraction (low molecular weight, LMW-DOM) incubation are not included in the figure or in the statistical analyses

freshwater control and the transplanted incubation, fractioning of the DOM did effect DOC removal. In both of these incubations, the LMW-DOM treatment always had lower net removal of DOC than either the HMW or 0.2 μm fractions (*t*-test, $p < 0.05$).

The decay constant of DOC degradation (k), calculated from DOC measurements at each time point, varied between -1.5 and $-10.3 \times 10^{-3} \mu\text{M C d}^{-1}$ for April and -1.5 and $-18.2 \times 10^{-3} \mu\text{M C d}^{-1}$ for August, with no significant difference between the months (*t*-test, $p > 0.05$). In April, there were no significant differences between removal rates, although values tended to be highest in the seawater control incubations (LMW and HMW fractions) and lowest in the transplanted incubation (LMW and HMW fractions). In August, removal rates were significantly higher (*t*-test, $p < 0.05$) in the freshwater control incubations than in the other incubations. The lowest rates were observed in the seawater control incubations.

Amino acids and DI

In August, samples were collected from the 12 (6 duplicated treatments) control incubations at the initial and final time points for measurement of DCAA and DFAA. Both DFAA and DCAA decreased in all incubations, regardless of the source or fraction of the water. At the beginning of the experiment, the average (\pm SE) DFAA concentration was 113 (30) nmol l^{-1} ; this decreased to an average of 39 (17) nmol l^{-1} by the end of the experiment (Table 3). The average decrease for DCAA in the incubations was 332 (248) nmol l^{-1} .

In general, decreases in concentration of amino acids were mirrored by a decrease in the DI, reflecting the increasing degree of degradation of the DCAA and DFAA in the incubations (Table 3). For the DCAA, the only exception was the Stn S LMW incubation, for which a decrease in DCAA was reflected in an increase in DI. The DI of the DFAA also increased in

this size fraction over the course of the incubation, potentially indicating a source of contamination during the experiment. Therefore, this incubation was not included in the comparison of the different indices of bioavailability.

Bacterial growth efficiency and bioavailability index

In April, BGE ranged from 11 to 36% for the incubations, and was highest in the control incubations (Student's *t*-test, $p < 0.05$; Table 2). Within the transplanted treatment, the HMW-DOM fraction exhibited the highest BGE (21%). The BGE in the LMW-DOM size fraction of the transplanted incubation was 36% lower than that in the LMW-DOM of the freshwater control and was 70% lower than in the seawater control incubation (Table 2).

Although in August BGE covered a wider range than in April, there was no significant difference between August and April in terms of BGE (*t*-test, $p > 0.05$). In the seawater control incubation, we obtained negative BGE for the 2 incubations in which the BA crashed (HMW-DOM and $<0.2 \mu\text{m}$ -DOM; Table 2). In the freshwater control incubation, BGE was significantly higher in the HMW-DOM fraction (41%; *t*-test, $p < 0.05$) than in the other 2 fractions. There was little difference between the size fractions in the transplanted incubations, with all BGE falling in a very close range (28 to 30%; ANOVA, $p > 0.05$).

In April, within both the control and transplanted incubations, the DOM bioavailability index was highest in the LMW-DOM incubations (*t*-test, $p < 0.01$). However, there were no differences between the control and transplant incubations as a whole (ANOVA, $p > 0.05$); indeed, the values were very similar in both the freshwater control incubations and the transplanted incubations, with values for each size fraction being within 1% of each other (Table 2). Although the index was higher in August than in April (*t*-test, $p <$

Table 3. DCAA and DFAA concentrations in August incubations. Samples were only measured for the Stn F control incubation and the Stn S control incubation and for the initial and final time point. DI; degradation index (Dauwe & Middelburg 1999); Diff.: final value subtracted from the initial value

	DCAA (nmol l^{-1})			DI			DFAA (nmol l^{-1})			DI		
	Initial	Final	Diff.	Initial	Final	Diff.	Initial	Final	Diff.	Initial	Final	Diff.
Stn F												
<0.2 μm	1739	1547	-192	-1.14	-2.11	-0.97	112	66	-46	-0.93	-2.10	-1.17
LMW	498	381	-117	0.67	-2.69	-3.36	172	23	-149	-0.39	-1.07	-0.68
HMW	4701	4539	-162	-1.57	-1.94	-0.40	103	36	-67	-0.71	-1.06	-0.35
Stn S												
<0.2 μm	1570	1098	-317	2.76	-1.78	-4.54	101	25	-76	-2.08	-2.9	-0.82
LMW	867	442	-425	-1.58	-0.40	+1.18	109	30	-79	-2.26	-0.88	+1.38
HMW	1634	836	-784	2.48	-0.73	-3.21	83	55	-28	-1.93	-1.37	+0.56

0.01), the increase was uniform across the incubations and, hence, the differences between the control and transplanted incubations were not significant. In terms of size fractions within each incubation type, in general, as in April, the LMW-DOM fraction had a higher bioavailability than the HMW-DOM and $<0.2 \mu\text{m}$ -DOM fractions (Table 2; *t*-test, $p < 0.05$).

DISCUSSION

Response of bacterial community

One of the goals of the present study was to examine how bacterial communities respond to a change in substrate quality on short time scales (hours to days). More specifically, the objective was to determine its functional response (production and respiration) to sudden changes in the composition of DOM. Our results demonstrate that bacteria possess a plasticity which allows them to use different DOM sources. There was little difference in functional response of the bacteria to a change in the DOM source (freshwater or seawater). Transplanted bacteria did not exhibit a large suppression of activity compared to the control incubations, in fact there was a clear stimulation of growth in some incubations, for example in the transplanted LMW-DOM incubation in August.

The incubating of size-fractionated water did result in clear differences between different size fractions in August, but not in April. For example, freshwater LMW-DOM always exhibited a bioavailability index considerably higher than that of the other fractions in August, or of the same fractions from April. Furthermore, in the later stages of the summer incubations, there was a large increase in BA and BP in the HMW-DOM freshwater control that was not observed in the transplanted incubation or in the $<0.2 \mu\text{m}$ -DOM fraction freshwater control. The rapid increase in bacterial biomass may indicate bacterial succession in the incubation resulting in the dominance of 1 species or group of species as a response to the elevated levels of HMW-DOM in that incubation. It has been shown that enclosure incubations can result in rapid changes in bacterial community composition (Gasol et al. 2002) and different bacterial phylogenetic groups can exhibit differences in the assimilation of proteins and amino acids (Cottrell & Kirchman 2000). Indeed, comparing the freshwater HMW-DOM incubation to the HMW-DOM transplanted incubation, it is clear that the succession was not just a response to the HMW-DOM addition, but also that a seed population was necessary. Bacterial community composition changes along estuarine salinity gradients (del Giorgio & Bouvier 2002, Troussellier et al. 2002, Crump et al. 2004);

hence, it is probable that there were differences in the seed populations between the freshwater control and transplanted incubations. Nevertheless, our results demonstrate that separating the size fractions can result in quite different dynamics in the incubations.

In August, BA, BP and enzyme activities were generally higher (up to a factor of 10 in some cases). However, there were no large differences in BGE between the 2 study periods, indicating that bacterial respiration and BP increased at a relatively constant ratio. The large decrease in BA in the seawater control incubation carried out in August makes it difficult to make any firm conclusions on how the bacteria performed in the transplanted incubation relative to the seawater control. However, comparing the results from the freshwater control, it appears that at least in terms of BGE, there was little difference between the size fractions, and some stimulus of BGE was even detected in the LMW-DOM incubation. The pattern was somewhat different in April, with lower BGE in all transplanted incubations, whether the values were compared to the freshwater control (same initial DOM) or to the seawater control (same initial bacteria). This suggests a lesser ability of the transplanted bacterial community to adapt to the DOM in April than in August. The reasons for this remain unclear but are related to the bioavailability of the DOM and nutrients, or even to a change in the seed population of bacteria in the inoculum. The increase in bacterial activity may also result from increased abundance and/or from the 12°C increase in temperature as bacteria may be able to adapt quicker to new environmental conditions at warmer temperatures. In their study of DOM bioavailability in the York River Estuary, Raymond & Bauer (2000) also concluded that temperature was the main control on bacterial activity.

The similarity in magnitude of the response of the bacterial populations to changes in DOM, both in terms of size fraction and in terms of source, indicates that the bacterial populations in these incubations were very plastic. In their study of the bioavailability of wetland-derived organic nitrogen to marine bacteria, Stephanaukas et al. (1999) found that marine bacteria could assimilate a substantially larger fraction of wetland-derived dissolved organic nitrogen DON than could freshwater bacteria. Our results are in contrast to these and show rather that there was little difference in the assimilation of DOM when freshwater and seawater bacteria were compared. As discussed below, the index used to calculate bioavailability of DOM can lead to differences and this, combined with fact that in this research we looked at utilization of carbon and not nitrogen, may also explain the differences between the results of our work and theirs. Nevertheless, this work

supports the conclusion of Stephanauskas et al. (1999) that freshwater DOM is bioavailable to marine bacteria and further underlines the plasticity of bacteria in using different DOM sources.

Bioavailability

Bioavailability is the result of the interactivity of DOM and the bacterial community (Sinsabaugh & Findlay 2003) and it can be estimated by several methods, each of which measures either the properties of the substrate, the community, or a mix of the two. These include increases in enzyme activities, DOC degradation rates, BB, BP, bacterial total carbon demand, oxygen respiration rate, and the amino acid DI. For example, the amino acid DI is a measure of the properties of the substrate, whereas BGE combines a measure of the properties of the substrate with those of community. Thus, the ability of bacteria to rapidly adapt, either through succession or physiological acclimation, can also determine the degree of bioavailability of the DOM. In the present study, we used a series of methods to estimate the bioavailability of DOM and the variability in the results obtained is discussed in the following section.

Enzyme activities

Enzyme activities represent the response of the microbial community to the composition and availability of polymeric substrates in POM and DOM. Activities at Stn F are almost the same for both months, suggesting that the composition of the DOM was relatively constant. The only exception was beta-xylosidase, which showed a 4-fold increase. The relatively high activities of beta-glucosidase and beta-xylosidase, enzymes involved in the degradation of cellulose and hemicelluloses, also suggests that material derived from higher plants is an important source of organic matter at this site. However, the data for Stn S are very different as they show major shifts between April and August, despite relatively constant bulk DOC concentrations. alpha-glucosidase, beta-xylosidase, esterase and chitinase all increased in activity, sometimes by a factor of 10, whereas activity of beta-glucosidase and aminopeptidase decreased. This indicates that enzyme activities were induced or down-regulated in response to a shift in the composition of the available substrates and also that the bacterial community was degrading different compounds at the 2 sites and during the 2 field trips. This supports the suggestion that the microbial community is very flexible and capable of quickly adapting to changes in substrate composition.

DOC degradation rates

The DOC degradation rates (k) varied by a factor of 10 between seasons, size fractions and treatments. In spring, k was highest in the LMW fraction, indicating that removal rates of DOC were faster in this fraction. This implies that the LMW-DOM was more bioavailable to bacterial heterotrophs than in the other incubations, which is also reflected in the higher BGE. In contrast in August, degradation rates were higher in the HMW fractions of the freshwater control and transplanted incubations. Thus, it appears that degradability of the DOC was higher in the LMW fraction in spring at both the freshwater and seawater sites and that this then switched to a higher degradability of HMW relative to LMW in summer at both sites. The transplantation of bacteria from the seawater DOM to freshwater DOM altered this pattern of degradation in spring, suggesting an inhibition of DOC consumption in both molecular weight size fraction incubations. In summer, the differences between the rates in the freshwater control incubations and the transplanted incubation were less, although there was some evidence of a repression of DOC consumption in the transplanted <0.2 μm -DOM fraction. Comparing the different indices of bioavailability between the LMW and HMW fractions and the <0.2 μm -DOM fraction it is evident that the degradability of DOM was changed considerably by separating DOM into size fractions. Indeed, this is reflected in all the indices used to measure degradability or bioavailability and is potentially related to changes in bacterial community structure within the incubations (Gasol et al. 2002, Gattuso et al. 2002).

Indices of bioavailability

Bioavailability of organic matter refers to the extent and rate at which organic matter is consumed by bacteria and other heterotrophs. In the present study, several proxies for DOM availability to heterotrophic bacteria were used: a first-order decay constant of DOC degradation (k), an amino acid based compositional DI, BGE and the bioavailability index. It is clear from the results in Tables 2 & 3 and the previous paragraphs that different conclusions can be drawn depending on the method used. Three of the applied bioavailability measures (bioavailability index, DI and k) relate to the entire DOM pool and their absolute values, and magnitudes of change depend somewhat on the relative importance of refractory background DOM components. BGE only includes measurement of BB accumulation and bacterial respiration and does not depend on absolute DOM concentrations. The bioavailability

index and BGE both take into account the accumulation of BB and bacterial respiration and are thus based on the implicit assumption that BB accumulation is only limited by carbon (i.e. energy) availability and not by nutrients or viral infections. The DI of amino acids is a chemically based index of the degradation state of organic matter based on compositional changes in amino acid patterns (Dauwe & Middelburg 1998, Dauwe et al. 1999). These various proxies for bioavailability vary in their reliance on substrate properties and bacterial community properties, with DI reflecting mainly substrate properties, BGE depending most on bacterial community properties and the other two incorporating both substrate and bacterial community characteristics. Since DI values as introduced by Dauwe & Middelburg (1998) and Dauwe et al. (1999) do not depend on the time scale, it might be more appropriate to compare the net change in DI with k , BGE and bioavailability index in experimental incubations.

Although there is a clear correlation between the net change in DI of DCAA in the August incubations and k ($r^2 = 0.95$, $p < 0.01$), there is no correlation between DCAA and the bioavailability index ($r^2 = 0.08$, $p > 0.05$). Similarly, the relationships between the DI of DFAA and either k ($r^2 = 0.08$, $p > 0.01$) or the bioavailability index ($r^2 = 0.49$, $p > 0.05$; data not shown) are non-significant. The paucity of points ($n = 3$) excluded a rigorous statistical comparison between BGE and the net change of either the DI of DCAA or of DFAA. However, there appears to be a trend of lower BGE in the incubations exhibiting large decreases in DI. Of the indices that apply to the entire data set (BGE, k and bioavailability index), only k and BGE are significantly correlated ($r^2 = 0.45$, $p < 0.05$), suggesting that high BGE was found in the incubations with the highest DOC removal rates. There is no correlation between either the bioavailability index and k ($r^2 = 0.10$, $p > 0.05$) or BGE ($r^2 = 0.01$, $p > 0.05$). It is therefore clear from this comparison of the different indices of DOM that the index used can lead to very different conclusions regarding the bioavailability of the substrate.

DOM and the size reactivity continuum

In a recent paper examining the changes in DOM size in a Bayou river system, Engelhaupt & Bianchi (2001) have shown that both bulk DOC concentration and the contribution of the HMW fraction to total DOC changes throughout the system and over an annual cycle. This is also the case in Randers Fjord, where the contribution of the HMW fraction at the freshwater site varies greatly despite small changes in the bulk DOC. The amount of HMW-DOM was considerably lower at Stn S during both field trips (34 to 40 $\mu\text{M C}$ vs 124 to

199 $\mu\text{M C}$ at Stn F) and neither the proportions of low and high molecular weight DOM nor the concentrations changed between the 2 cruises. Despite this relative constancy in bulk concentration and the proportions of HMW and LMW fractions, the large changes in enzyme activities between spring and summer at Stn S suggest that the DOM composition was not constant.

The variability in DOM proportions at the Stn F can be due to a change in either the rate of supply, the degree of processing of the DOM, the mechanisms of physical removal or a combination of all three. The first hypothesis is unlikely because riverine inflow is generally higher in April than in August, and total DOM concentration is remarkably similar between the 2 seasons with only the proportions of HMW-DOM differing. A more active processing, and hence removal, of HMW-DOM throughout the estuary in August is potentially due, in part, to increased temperatures and to the increased residence time in the estuary in summer as a consequence of the reduced streamflow. The increased residence times, combined with increased removal rates, as calculated from the rate constants of DOC removal, may explain the lower contribution of HMW-DOM downstream. In August, the rate constants were 3 times higher than in April in the freshwater HMW-DOM control incubation (Table 2) and correspond to an average removal of 13% of the HMW-DOM over the incubation. The freshwater residence time (estimated as the time required to replace 50% of water) varies between 3 and 8 d (Nielsen et al. 2001). Assuming that the residence time is 8 d in August (the period of low freshwater flow) and 3 d in April (a time of higher freshwater flow), one can estimate the average bacterial consumption of HMW-DOM throughout the estuary. Multiplying the daily DOC removal rates by the residence time provides values of 92 $\mu\text{M C}$ in August compared to 6.4 $\mu\text{M C}$ in April. These values represent a potential removal of HMW-DOM by bacterial consumption of 13 and 4% for August and April, respectively.

Although concentrations of LMW-DOM in April were lower at Stn S than at Stn F, they were approximately the same in August, indicating a change in the uptake and release processes between the 2 seasons. The apparent lack of change in concentration indicates that there is a source of LMW-DOM throughout the estuary in August. This portion of the DOM pool can be used by bacteria (Figs. 1 & 2) and removal therefore cannot be ruled out. Consequently, there has to be another source of LMW-DOM in the estuary. Photolysis of HMW-DOM could be one such source (Moran & Zepp 1997, and references therein), as photolysis is known to produce LMW carbon moieties.

In their paper proposing the size reactivity continuum, Amon & Benner (1996) showed that HWM-DOM

has a higher bioavailability index than LMW-DOM. Our results seem to be contrary to their's, particularly in August when the bioavailability index was consistently higher in the LMW-DOM fractions, regardless of the source of the water and bacteria, with the only exceptions being in April in the seawater control incubation, when it was equal to that of the other 2 fractions. There are several reasons as to why our data might yield a different pattern to that of the size reactivity continuum, the most obvious of which is that the DOM was different between this environment and the environments studied by Amon & Benner (1996). The area surrounding Randers Fjord is characterized by large-scale agricultural land with both arable and animal husbandry taking place and the littoral area is dominated by the common reed. It is likely that these features represent a large source of DOM to the estuary, which is supported by the activities of betaglucosidase and beta-xylosidase enzymes. A second reason may be that there are different bacterial species or groups present in these incubations, although the apparent bacterial plasticity observed in these incubations and the proposed ubiquity of bacterial species does not support this second hypothesis (Findlay et al. 1997).

The results of this experiment show that freshwater DOM is utilizable by marine bacteria and that the bioavailability of the DOM is not constant, although the degree of variability is dependent upon the index used to estimate bioavailability. Furthermore, it is clear from these results that the separation of molecular weight size fractions resulted in very different estimates of bioavailability. In other words, the sum of the fractions was not equal to the total as rates in the unfractionated DOM incubations were generally less than the sum of the rates in the LMW and HMW size fractions. Finally, these results also demonstrate that care must be taken when comparing different estimates of bioavailability.

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